Differential Regulatory Mechanism of Ca^{2+}/Calmodulin-Dependent Protein Kinase Kinase Isoforms

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ABSTRACT: We have previously demonstrated that the α isoform of Ca^{2+}/calmodulin-dependent protein kinase kinase (CaM-KKα) is strictly regulated by an autoinhibitory mechanism and activated by the binding of Ca^{2+}/CaM [Tokumitsu, H., Muramatsu, M., Ikura, M., and Kobayashi, R. (2000) *J. Biol. Chem. 275*, 20090−20095]. In this study, we find that rat brain extract contains Ca^{2+}/CaM-independent CaM-KK activity. This result is consistent with an enhanced Ca^{2+}/CaM-independent activity (60−70% of total activity) observed with the recombinant CaM-KK*β* isoform. By using various truncation mutants of CaM-KK*β*, we have identified a region of 23 amino acids (residues 129−151) located at the N-terminus of the catalytic domain as an important regulatory element of the autonomous activity. A CaM-KK*β* deletion mutant of this domain shows a significant increase of Ca^{2+}/CaM dependency for the CaM-KK activity as well as for the autophosphorylation activity. The activities of CaM-KKα and CaM-KK*β* chimera, in which autoinhibitory sequences were replaced by each other, were completely dependent on Ca^{2+}/CaM, suggesting that the autoinhibitory regions of CaM-KKα and CaM-KK*β* are functional. These results establish for the first time that residues 129−151 of CaM-KK*β* participate in the release of the autoinhibitory domain from its catalytic core, resulting in generation of autonomous activity.

Ca^{2+}/calmodulin-dependent protein kinase kinase (CaM-KK)[1] has been identified and cloned as an activating protein kinase for two multifunctional CaM kinases, CaM-KI and CaM-KIV. Phosphorylation by CaM-KK of a Thr, located in the T-loop of the catalytic domain of CaM-KI and CaM-KIV, results in a large increase in the catalytic efficiency of each CaM kinase (reviewed in refs *1* and *2*). In mammals, two CaM-KK genes (α and *β*) have been cloned (*21*−*23*). The highest expression of the α and *β* isoforms of CaM-KK is observed in brain, although the α isoform is also expressed in various peripheral tissues such as thymus and spleen (*21*, *22*).

Previous studies demonstrated that the CaM-KK/CaM-KIV cascade is present and/or functional in various cell types such as Jurkat cells (*3*), cultured hippocampal neurons (*4*), and transfected COS-7 cells (*5*). An important role for the CaM-KK/CaM-KIV cascade in the regulation of Ca^{2+}-dependent gene expression through phosphorylation of transcription factors such as CREB (cAMP response element binding protein) has been demonstrated (*4*, *6*−*9*). Recent studies using mice deficient in CaM-KIV revealed that the CaM-KIV mediated pathway plays important roles in the function and development of the cerebellum and is critical for male and female fertility (*10*−*12*). The CaM-KK/CaM-KI cascade is activated in PC12 cells upon membrane depolarization although its physiological role remains to be determined in this model (*13*). The CaM kinase cascade has been shown to cross-talk with other signaling pathways. Activation of the CaM kinase cascade activates MAP kinases while direct phosphorylation of CaM-KK by PKA downregulates the cascade (*14*−*16*). Recently, a kinase homologous to mammalian CaM-KI (*Ce*CaM-KI) has been cloned in *Caenorhabditis elegans*. An amino-terminal nuclear localization signal localizes *Ce*CaM-KI predominantly in the nucleus of transfected cells (*17*). In *C. elegans*, CaM-KK and CaM-KI reconstitute a signaling pathway which is able to mediate Ca^{2+}-dependent phosphorylation of CREB and CRE-dependent transcription in transfected cells similarly to the mammalian CaM-KK/CaM-KIV pathway. The physiological significance of the CaM kinase cascade in nematode remains, however, to be investigated. Two CaM kinases (CMKB and CMKC), respectively homologous to the mammalian CaM kinase cascade members, have also been cloned in *Aspergillus nidulans*, suggesting that a CaM kinase cascade also exists in this organism (*18*). In addition to CaM-KI and CaM-IV, protein kinase B (PKB) has been identified as a target for CaM-KK in transfected cells and in vitro (*19*, *20*). The phosphorylation and activation of PKB by CaM-KK are responsible for the antiapoptotic effect upon a limited elevation of intracellular Ca^{2+} in NG108 cells (*19*).

CaM-KK, like other CaM kinases, contains an amino-terminal catalytic domain and a regulatory domain (autoinhibitory and CaM-binding segments) at its carboxyl terminus (*5*, *24*, *25*). The catalytic domain of CaM-KK contains a unique Arg-Pro-rich 22 residue insert, which plays an important role for the recognition of downstream CaM kinases (*26*). Using site-directed mutagenesis and synthetic peptides, the CaM-binding region of αCaM-KK, spanning

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[1] Abbreviations: CaM-KK, Ca^{2+}/CaM-dependent protein kinase kinase; CaM, calmodulin; PKA, cAMP-dependent protein kinase; MLCK, myosin light chain kinase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; GST, glutathione *S*-transferase; MBP, maltose binding protein; CREB, cAMP response element binding protein; PBS, phosphate-buffered saline.

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residues 438−463, was identified (*24*). The three-dimensional structure of the Ca^{2+}/CaM complex with the CaM-KKα peptide (residues 438−463) resolved by NMR spectroscopy revealed that the orientation of the CaM-KK peptide with respect to the two CaM domains is opposite to that of the MLCKs and CaM-KII peptides (*27*). The characteristic feature of the CaM-binding domain in CaM-KK was also observed with the corresponding peptide (residues 331−356) in *C. elegans* CaM-KK by X-ray crystallography (*36*). A recent study has shown that the binding orientation of CaM to its binding site in CaM-KKα is not essential for the release of the autoinhibitory domain from the catalytic core; however, the unique regulatory sequence is critical for the function of CaM-KK autoinhibition (*28*).

Most of the information about the regulatory mechanisms of CaM-KK has been obtained from experiments using CaM-KKα, and very few information is available regarding the regulation of the *β* isoform. The binding of Ca^{2+}/CaM to CaM-KKα is absolutely required for its activation and efficient phosphorylation of target protein kinases (*5*, *28*−*30*), while CaM-KK*β* activity is enhanced by but not completely dependent on Ca^{2+}/CaM, in vitro as well as in transfected cells (*23*). In this report, using mutants and chimeras of CaM-KKα and CaM-KK*β*, we characterize the different regulatory mechanisms of each isoform of CaM-KK.

EXPERIMENTAL PROCEDURES

Materials. CaM-KKα cDNA (GenBank accession number L42810) was obtained from a rat brain cDNA library (*21*). CaM-KK*β* cDNA was obtained from rat brain cDNA (Clonetech) by PCR amplification using LATaq polymerase (Takara). PCR was performed with a sense oligonucleotide (5′-ATGTCATCATGTGTCTCTAGC-3′) and an antisense oligonucleotide (5′-CTACTCCGGCTCCATGGCCTC-3′) from the published sequence (*22*, *23*) using the following condition: 94 °C for 2 min and then 30 cycles at 98 °C for 20 s and at 55 °C for 10 min. The amplified product was purified and cloned into the TA vector (Invitrogen). Mammalian expression plasmid carrying the cDNA of CaM-KK*β* (pME-CaM-KK*β*) was constructed by introduction of the *Sma*I−*Xba*I digest from CaM-KK*β* in the TA vector using blunt-ended *Eco*RI−*Xba*I digested of pME18s. CaM-KK*β* cDNA and all other deletion or substitution constructs were sequenced by automated sequencing using an Applied Biosystems 377 DNA sequencer. GST-CaM-KI (1−293, K49E) and GST-CaM-KI (1−374, K49E) were constructed and purified as previously reported (*28*). Recombinant CaM-KKα wild type was expressed in *Escherichia coli* BL-21 (DE3) using the pET16b expression plasmid lacking the His-tag sequence. CaM-KKα was inserted in this plasmid at the *Nco*I site and purified by CaM−Sepharose chromatography (Amersham Pharmacia Biotech). Recombinant rat CaM was expressed in *E. coli* BL-21 (DE3) using pET-CaM (*31*) (kindly provided from Dr. Nobuhiro Hayashi, Fujita Health University, Toyoake, Japan) and purified by phenyl-Sepharose column chromatography. Rat brain extract was prepared as previously described (*32*). A 26-residue synthetic peptide corresponding to the CaM-binding domain of rat CaM-KKα (residues 438−463) (*24*) was synthesized by Peptide Institute Inc. (Osaka, Japan). All other chemicals were from standard commercial sources.

*Construction of CaM-KK*β *Mutants.* The expression vector (*pGEX-KG-PreS*) was constructed to introduce the PreScission protease (Amersham Phaermacia Biotech) cleavage site (Leu-Glu-Val-Leu-Phe-Gln-Pro) between the GST moiety and the fused protein as follows: pGEX-KG plasmid was digested with *Bam*HI and *Xba*I and then ligated with preannealed double-stranded oligonucleotides (5′-GATCCCTGGAAGTTCTGTTCCAG GGGCCCATT-3′ and 5′-CTAGAATGGGCCCCTGGAACAGAACTTCCAGG-3′) coding the PreScission protease cleavage site. For construction of truncation and deletion mutants of CaM-KK*β* (2−587, 2−499, 129−499, 139−499, 144−499, 152−499, 162−499), each cDNA was amplified by PCR using Ex Taq (Takara) and the corresponding sense primers and antisense primers containing the *Xba*I site as follows. Sense primers: 2−587 and 2−499, 5′-GGTCTAGAGTCATCATGTGTCTCTAG-3′; 129−499, 5′-GCTCTAGAGCCAGCCAGCTCCCCACAG-3′; 139−499, 5′-GCTCTAGAGATGCCCCGGCGGCCCAC-3′; 144−499, 5′-GCTCTAGAGACAGTGGAGTCGCACCAC-3′; 152−499, 5′-GCTCTAGAGATCACGGGTTTGCAGGAC-3′; 162−499, 5′-GCTCTAGAGAATCAGTACACGCTGAAG-3′. Antisense primers: 2−499, 5′-CCGTCTAGATCAAAATGGGTTCCCAAAAG-3′; 2−587, 5′-CCTCTAGACTACTCCGGCTCCATGG-3′. Each PCR product was digested with *Xba*I and ligated into the *Xba*I digested *pGEX-KG-PreS* vector. CaM-KK*β* 129−151 deletion mutant (Δ129−151) and Ala substitution mutant (SA mutant, S131, S132, S135, S136) were produced by site-directed mutagenesis using the GeneEditor in vitro site-directed mutagenesis system (Promega Co.) and *pGEX-KG-PreS*-CaM-KK*β* 2−587 plasmid as a template. Mutagenic oligonucleotides are described as follows: 129−151 deletion mutant, 5′-TCCCTGTCCTACTCAATCACGGGTTTGCAG-3′; SA mutant, 5′-CTACTCACCAGCCGCCGCCCCACAG-GCCGCTCCCCGGATGCCCC-3′. Construction of *pGEX-KG-PreS*-CaM-KK*β* 2−470 and *pGEX-KG-PreS*-CaM-KK*β* 2−470 lacking residues 129−151 (Δ129−151) was also performed by site-directed mutagenesis to introduce a stop codon at residue 471 using a mutagenic oligonucleotide (5′-GAAGAGGAGGTCTAGAATTCAGTCAAA-3′) and *pGEX-KG-PreS*-CaM-KK*β* 2−587 and *pGEX-KG-PreS*-CaM-KK*β* 2−587 (Δ129−151) as templates.

Construction of CaM-KK α/β *Chimera Mutants.* Construction of CaM-KKα 126−463 was performed by PCR using pME-CaM-KK (wild-type) plasmid as a template and sense and antisense oligonucleotides as follows: sense primer, 5′-TCTCTAGAGAACCAGTACAAGCTG-3′; antisense primer, 5′-GCTCTAGATCAAAATGGGTTTCCAAA-3′ followed by ligation into the *Xba*I digested *pGEX-KG-PreS* vector (*pGEX-KG-PreS*-CaM-KKα 126−463). For CaM-KKα/*β* chimera mutants, CaM-KK*β*162−499 was mutated at position 1338 (G by C) to create a *Bst*EII site and at position 1398 (C by T) to remove an endogenous *Bst*EII site without changing any amino acid. The two mutagenic oligonucleotids used were 5′-CCCTTGGGTCACCAGGCACGGGGCC-3′ and 5′-GGTCGAGGTGACTGAAGAGGAGGTC-3′. After digestion of the plasmid with *Bst*EII, the fragment containing the regulatory domain of CaM-KK*β* (residue 446−499) was replaced by the *Bst*EII digested fragment of *pGEX-KG-PreS*-CaM-KKα 126−463 containing the regulatory domain of CaM-KKα (residue 410−463). This CaM-KK chimera mutant (CaM-KK α/*β*) contains the CaM-KKα catalytic

domain fused to the regulatory domain of CaM-KKβ at residue 409. The reverse chimera (CaM-KKβ/α) encoding the CaM-KKβ catalytic domain fused with the regulatory domain of CaM-KKα at residue 445 was also constructed by replacement of the *Bst*EII fragment of each isoform of *pGEX-KG-PreS*-CaM-KK plasmids.

Expression and Purification of Recombinant CaM-KKs. GST fusion proteins of CaM-KK mutants including wild-type, truncation, deletion, and chimera mutants were expressed in *E. coli* JM109 as follows: transformed *E. coli* JM109 with the different *pGEX-KG-PreS*-CaM-KK plasmids were cultured overnight in LB medium containing 100 μg/mL ampicillin at 37 °C, and the 10 mL culture was inoculated into 100 mL of LB medium. Further growth of the culture was performed at 25 °C with vigorous shaking until $OD_{600} = 1.2$. IPTG was added into the *E. coli* culture at a final concentration of 1.0 mM. After 18 h *E. coli* expressing GST-CaM-KKs were collected by centrifugation and washed with cold 30 mM Tris-HCl (pH 7.5), lysed on ice by sonication in 20 mL of PBS containing 0.2 mM PMSF. After centrifugation at 12000*g* for 15 min, the supernatant was applied to a glutathione−Sepharose column (0.5 mL) and washed with 20 mL of PBS containing 0.2 mM PMSF. Elution was carried out by using a solution containing 50 mM Tris-HCl (pH 7.5), 10 mM glutathione, and 0.2 mM PMSF. $CaCl_2$ (2 mM final concentration) was immediately added to the eluate, which was then applied onto a CaM−Sepharose column (Amersham Pharmacia Biotech, 0.5 mL gel volume) preequilibrated with 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 0.2 mM $CaCl_2$, 1 mM DTT, and 0.2 mM PMSF (buffer A). After the column was washed with 10 mL of buffer A, elution was carried out by buffer A containing 2 mM EGTA and 1 mM EDTA. The fraction containing the GST fusion protein was digested with 1 unit of PreScission protease (Amersham Phaermacia Biotech) for 15 h at 5 °C. The digested sample was applied onto a glutathione−Sepharose column (0.5 mL gel volume), and the pass-through fraction was reapplied onto the column three times to completely remove digested GST, undigested GST fusion protein, and PreScission protease (also a GST fusion protein). The pass-through fraction was collected and stored at −30 °C after addition of an equal volume of a solution containing 80% glycerol and 20% ethylene glycol.

Transient Expression and Partial Purification of CaM-KKs. COS-7 cells were maintained in Dulbeco's modified Eagle's medium containing 10% fetal bovine serum. Cells were subcultured in 10 cm dishes for 12 h before transfection. The cells were then transferred to serum-free medium and treated with a mixture of either 10 μg of pME18s plasmid DNA (DNAX Research Institute, Inc.), CaM-KKα, or CaM-KKβ cDNA containing plasmid DNAs and 60 μg of LipofectAMINE Reagent (Life Technologies, Inc.) in 6.4 mL of medium. After 32−48 h incubation, the cells (two plates) were collected and homogenized with 1.5 mL of lysis buffer [150 mM NaCl, 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1% NP-40, 10% glycerol, 0.2 mM PMSF, 10 mg/L leupeptin, 10 mg/L pepstatin A, 10 mg/L trypsin inhibitor] at 4 °C. After centrifugation at 15000*g* for 15 min, the supernatant was diluted by addition of 2 volumes of 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 0.2 mM PMSF, 10 mg/L leupeptin, 10 mg/L pepstatin A, and 10 mg/L trypsin inhibitor (buffer B) and applied to Q-Sepahrose column (0.5 mL bed volume) which had been preequilibrated with 50 mM NaCl containing buffer A. After the column was washed with 10 mL of the equilibration buffer, CaM-KK was eluted by the addition of 0.5 mL of buffer A containing 0.6 M NaCl and stored at −80 °C.

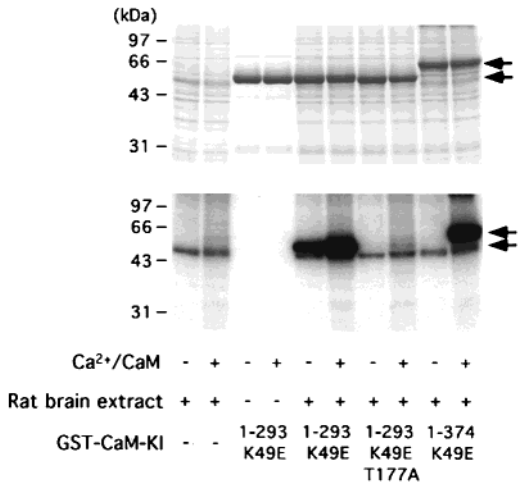

FIGURE 1: Ca^{2+}/CaM requirement of CaM-KK activity in rat brain extract. Rat brain extract (0.28 mg/mL) was incubated at 30 °C for 20 min with either buffer (−), GST-CaM-KI 1−293 K49E mutant (10 μg), GST-CaM-KI 1−293 K49E, T177A mutant (10 μg), or GST-CaM-KI 1−374 K49E mutant (10 μg) in a solution (25 μL) containing 50 mM HEPES, pH 7.5, 10 mM $Mg(OAc)_2$, 1 mM DTT, 100 μM [γ-^{32}P]ATP, and 1 μM mycrocistin LR in the presence of either 1 mM $CaCl_2$/3 μM CaM (+) or 1 mM EGTA (−). After termination of the reaction by addition of SDS−PAGE sample buffer, $^1/_3$ volume of the sample was applied to SDS−10% PAGE followed by autoradiography (lower panel). The upper panel shows Coomassie Brilliant Blue R-250 staining of the gel. Arrows indicate GST-CaM-KI mutants.

In Vitro Assay for CaM-KK Activity. Either partially purified CaM-KKs from transfected COS-7 cells or *E. coli* expressed CaM-KKs were incubated with 10 μg of GST-CaM-KI (1−293, K49E) at 30 °C for the indicated times in a solution (25 μL) containing 50 mM HEPES (pH 7.5), 10 mM $Mg(OAc)_2$, 1 mM DTT, and 100 μM [γ-^{32}P]ATP (1000−2000 cpm/pmol) in the presence of either 1 mM EGTA or 1 mM $CaCl_2$/3 μM CaM. The reaction was initiated by the addition of [γ-^{32}P]ATP and terminated by spotting aliquots (15 μL) onto phosphocellulose paper (Whatman P-81) followed by several washes with 75 mM phosphoric acid (*33*). A 5 min reaction was chosen to determine CaM-KK activity (Figure 2). Phosphate incorporation into GST-CaM-KI (1−293, K49E) was quantitated by liquid scintillation counting of the filters. In other experiments, the ^{32}P incorporation into either GST-CaM-KI (1−293, K49E), GST-CaM-KI (1−293, K49E, T177A), or GST-CaM-KI (1−374, K49E), reflecting CaM-KK activity in rat brain extract, was revealed by autoradiography of SDS−PAGE gels. A similar assay was used to determine CaM-KK autophosphorylation activity in the same reaction mixture as described above without exogenous substrate.

Other Methods. Western blotting was performed using anti-CaM-KK antibody (Transduction Laboratory) at a dilution of 1:1000 followed by using horseradish peroxidase conjugated anti-mouse IgG (Amersham) as a secondary antibody. Biotinylated CaM overlay in the presence of either 1 mM $CaCl_2$ or 2 mM EGTA was done as previously described (*24*). Detection was performed by using chemi-

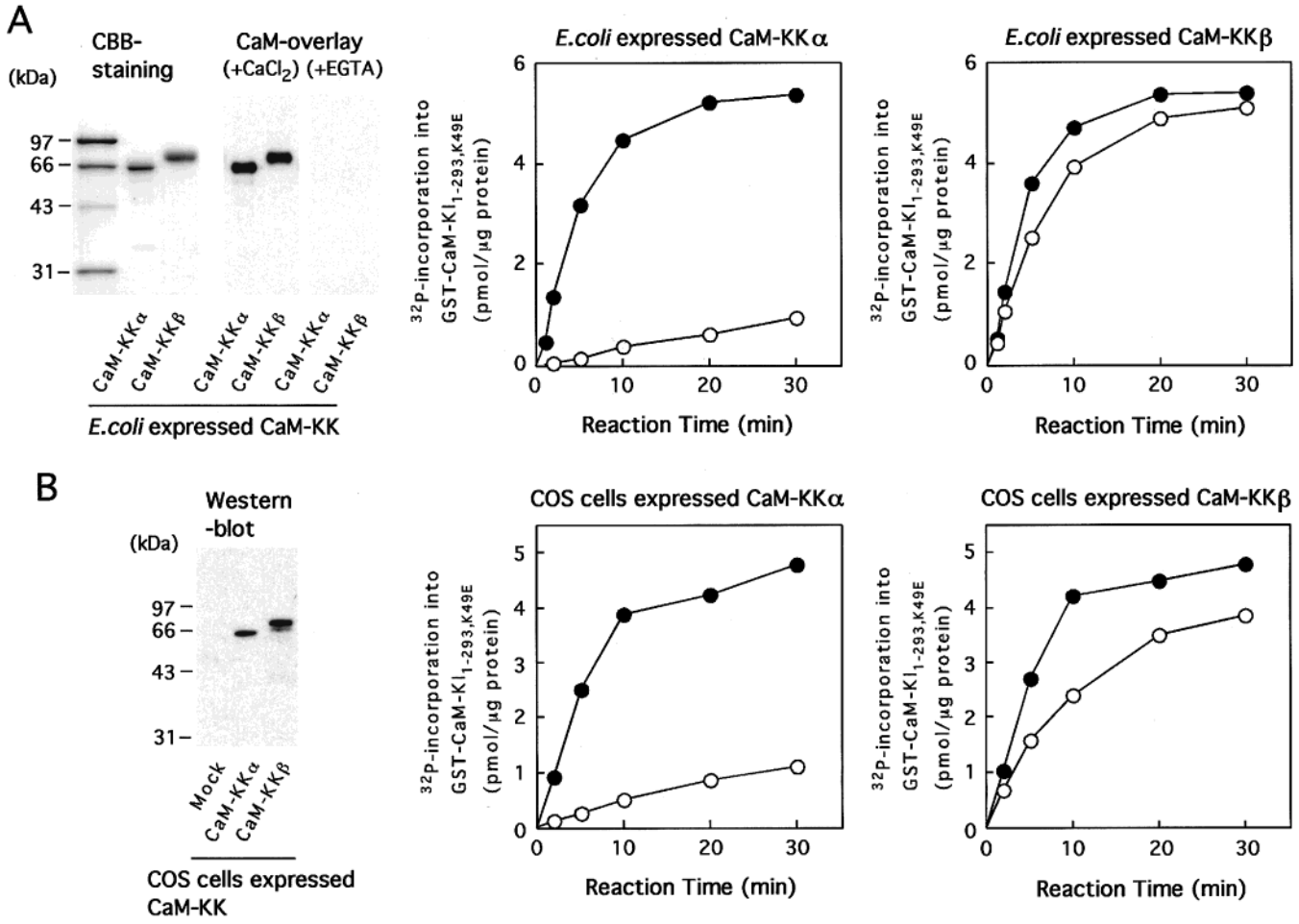

FIGURE 2: Characterization of recombinant rat CaM-KK isoforms. (A) α and β isoforms of rat CaM-KK expressed in *E. coli* and purified as described in Experimental Procedures were subjected to SDS−10% PAGE followed by either protein staining (left panel, 0.6 μg of each enzyme with molecular weight marker) or CaM overlay (60 ng of each enzyme) in the presence of either 1 mM $CaCl_2$ (+$CaCl_2$) or 2 mM EGTA (+EGTA). CaM-KK activities of α (0.3 μg/mL) and β (0.5 μg/mL) isoforms of rat CaM-KK were measured at 30 °C for the indicated times using GST-CaM-KI 1−293 K49E mutant (0.4 mg/mL) as a substrate in the presence of either 1 mM $CaCl_2$/3 μM CaM (closed circles) or 1 mM EGTA (open circles) as described in Experimental Procedures. (B) Partially purified α and β isoforms of rat CaM-KK (∼1 μg of protein) from transfected COS-7 cells including empty vector transfected cells (Mock) as described in Experimental Procedures were subjected to SDS−10% PAGE followed by Western blotting using anti-CaM-KK antibody (left panel). CaM-KK activity of α and β isoforms of rat CaM-KK (∼ 50 μg of protein/mL) were measured at 30 °C for the indicated times using GST-CaM-KI 1−293 K49E mutant (0.4 mg/mL) as a substrate in the presence of either 1 mM $CaCl_2$/3 μM CaM (closed circles) or 1 mM EGTA (open circles) as described above.

luminecsence reagent (DuPont NEN). Protein concentration was estimated by Coomassie dye binding (Bio-Rad) using bovine serum albumin as a standard (*34*).

RESULTS AND DISCUSSION

Recent studies have demonstrated that the CaM kinase cascade, a Ca^{2+}-triggered signal transduction pathway which requires phosphorylation and activation of CaM-KI and CaM-KIV by CaM-KK, is initiated by the binding of Ca^{2+}/CaM to both upstream and downstream CaM kinases (*5*, *21*, *25*, *29*, *30*). Investigation of the signaling cascade was mainly performed by using the CaM-KKα isoform as the activating enzyme. To clarify the requirement of Ca^{2+}/CaM for the CaM kinase cascade, especially for the CaM-KK, we examined the activity of the endogenous CaM-KK in rat brain. A GST fusion protein of an inactive form of rat CaM-KI (1−293, K49E) lacking the autoinhibitory and CaM-binding domains was used as a substrate. This assay has already been used to determine the requirement of Ca^{2+}/CaM for CaM-KK activity (*28*). As shown in Figure 1, rat brain extract increased the CaM-KI phosphorylating activity when the assay was performed in the presence of Ca^{2+}/CaM. The mutation of Thr177 to Ala in the T-loop of CaM-KI abolished the effect of the rat brain extract. In the absence of Ca^{2+}/CaM, a significant Thr177 phosphorylating activity (CaM-KK activity) was still observed in the extract, indicating that rat brain contained both Ca^{2+}/CaM-dependent and -independent CaM-KK activities. When we used an inactive form of full-length CaM-KI as a substrate, CaM-KK activity from the rat brain extract was only detected in the presence of Ca^{2+}/CaM. These results demonstrate the requirement for the Ca^{2+}/CaM binding to CaM-KI in order to expose Thr177 to the activating kinase (*25*, *30*).

Since two CaM-KK genes (α and β) have been cloned from rat brain (*21*−*23*), both CaM-KKs were expressed in *E. coli* to examine their activities. Both CaM-KKs were purified to near homogeneity by CaM−Sepharose (Figure 2A, left panel). Ca^{2+}-dependent binding of CaM to both enzymes was also demonstrated by CaM overlay in the presence of either 1 mM $CaCl_2$ or 2 mM EGTA. Next, we

measured the activity of both the α and *β* isoforms of CaM-KK in the absence or presence of Ca^{2+}/CaM, using GST-CaM-KI (1−293, K49E) as a substrate. As shown in Figure 2A, CaM-KKα exhibited a high Ca^{2+}/CaM-dependent activity (936 nmol min^{-1} mg^{-1}) with a weak autonomous activity (44 nmol min^{-1} mg^{-1}). This result is consistent with other reports indicating that CaM-KKα is tightly regulated by a Ca^{2+}/CaM-dependent autoinhibitory mechanism (*5*, *25*, *28*). On the other hand, bacterially expressed CaM-KK*β* was able to phosphorylate GST-CaM-KI (1−293, K49E) in a Ca^{2+}/CaM-independent manner (60−70% of total activity, 430 nmol min^{-1} mg^{-1}) and could be further activated by Ca^{2+}/CaM (616 nmol min^{-1} mg^{-1}). This result is correlated with a previous report demonstrating that the bacterially expressed MBP fusion protein of CaM-KK*β* activates a truncated version of CaM-KIV (residues 1−317) in a Ca^{2+}/CaM-independent manner and that the addition of Ca^{2+}/CaM further enhanced CaM-KK*β*-mediated $CaM\text{-}KIV_{1-317}$ activity (*23*). We have further confirmed the distinct Ca^{2+}/CaM requirement of CaM-KK isoforms by using partially purified enzymes from transfected COS-7 cells. Both enzymes were detected by Western blotting, and their activities were measured as in Figure 2A (Figure 2B). As a result, the Ca^{2+}/CaM dependencies of CaM-KK isoforms expressed in COS-7 cells were similar to that observed with the enzymes expressed in *E. coli*. These results indicate that the Ca^{2+}/CaM-independent CaM-KK activity detected in rat brain extract probably reflects on the autonomous activity of CaM-KK*β*.

We attempted next to identify the region of CaM-KK*β* involved in the generation of its autonomous activity. Various truncation mutants were expressed as GST fusion proteins and purified by glutathione−Sepharose followed by CaM−Sepharose. The GST moiety of the fusion proteins was removed by PreScission protease digestion at 5 °C followed by a glutathione−Sepharose column chromatography. A 5 min kinase reaction assay (based on the results obtained in Figure 2A) was chosen to measure the activities of various CaM-KK*β* mutants. As shown in Figure 3B, all of the recombinant CaM-KK*β* enzymes were purified to homogeneity. The ability of each mutant to bind Ca^{2+}/CaM was detected by a Ca^{2+}/CaM overlay method since they all contain the putative regulatory region (residues 474−499) which includes the autoinhibitory and CaM-binding domains (similar to the ones previously characterized in the α isoform) (*24*, Figure 6B). The C-terminal truncation mutant (residues 2−499) exhibited significant Ca^{2+}/CaM-independent activity similar to that observed with the wild-type enzyme. We, therefore, constructed a series of N-terminal truncation mutants of the C-terminal truncated mutant (residues 2−499). Deletion of the first N-terminal 128 amino acid residues had little or no effect on the Ca^{2+}/CaM-independent activity compared to the wild-type or C-terminal truncated enzyme (Figure 3C). Further truncation from residue 129 resulted in a gradual increase of the Ca^{2+}/CaM dependency, and both CaM-KK*β* 152−499 and 162−499 mutants were completely dependent on Ca^{2+}/CaM. These results suggest that the region spanning residues 129−151 of CaM-KK*β*, located at the N-terminus of the catalytic domain, is involved in the generation of autonomous activity. The total activities of those mutants were comparable to that of the wild-type enzyme, indicating that the series of truncation did not affect

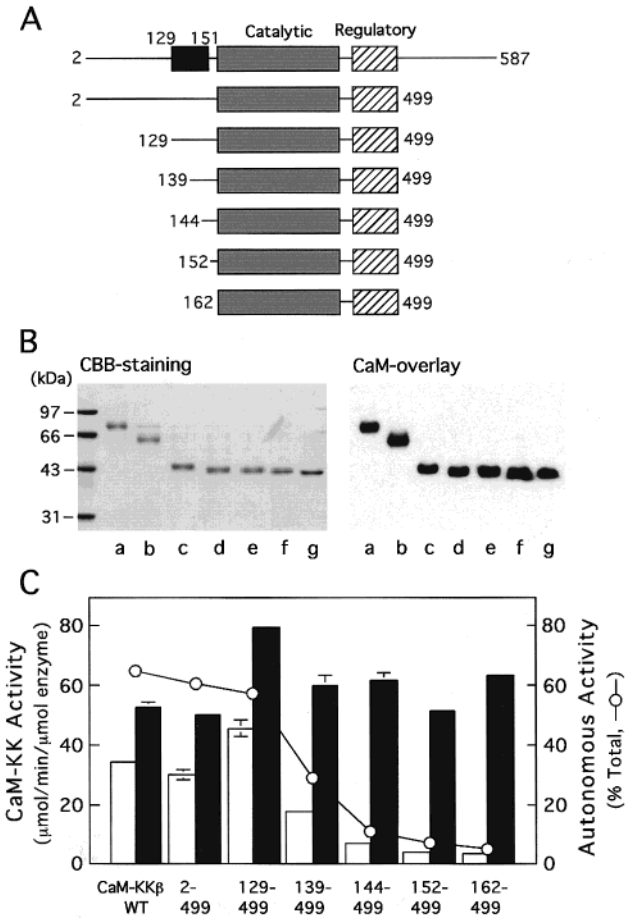

FIGURE 3: Characterization of truncation mutants of rat CaM-KK*β*. (A) Schematic representation of wild-type and truncated mutants of CaM-KK*β*. A series of truncation mutants of CaM-KK*β* were constructed and expressed in *E. coli* JM109 as a GST fusion protein followed by digestion with PreScission protease (Amersham Phaermacia Biotech) to remove GST and purified as described in Experimental Procedures. (B) Purified CaM-KK*β* mutants were subjected to SDS−10% PAGE followed by either protein staining (∼0.5 *μ*g of enzymes, left panel) or CaM overlay (∼50 ng of enzymes, right panel) in the presence of 1 mM $CaCl_2$. Lanes: a, CaM-KK*β* wild type (2−587); b, 2−499; c, 129−499; d, 139−499; e, 144−499; f, 152−499; g, 162−499. (C) Purified CaM-KK*β* mutants (0.2−0.4 *μ*g/mL) were incubated with GST-CaM-KI 1−293 K49E mutant (0.4 mg/mL) at 30 °C for 5 min in the presence of either 1 mM $CaCl_2$/3 *μ*M CaM (closed bars) or 1 mM EGTA (open bars) as described in Experimental Procedures. Autonomous activity of each recombinant enzyme (% of total, open circle) is plotted. The experiment was performed in triplicate for each mutant, and the results are presented as the mean and SE of three experiments. CaM-KK*β* WT, wild type (2−587).

CaM-KK catalytic activity. These results also suggest that, as for CaM-KKα, the autoinhibitory segment of CaM-KK*β* is functional. This was confirmed by the fact that the activities of the purified CaM-KK*β* (162−499) as well as of the chimera mutants containing the catalytic domain of either CaM-KKα (residues 126−409) or CaM-KK*β* (residues 162−445) fused with the regulatory domain of either CaM-KK*β* (residues 446−499) or CaM-KKα (residues 410−463) are fully dependent on Ca^{2+}/CaM (Figure 4).

To examine the mechanism involving residues 129−151 in the generation of autonomous activity of CaM-KK*β*, we constructed and expressed other mutants including a mutant lacking residues 129−151 (Δ129−151). The residues in CaM-KKα corresponding to residues 129−151 of CaM-KK*β*

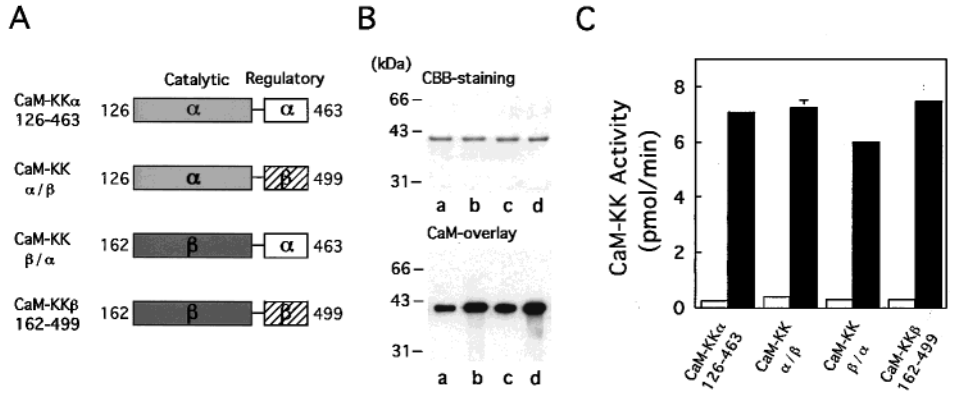

FIGURE 4: Catalytic and regulatory domains of CaM-KK isoforms are interchangeable. (A) Schematic representation of chimera mutants of CaM-KK isoforms. Chimera mutants of CaM-KK isoforms were constructed and expressed in *E. coli* JM109 as a GST fusion protein followed by digestion with PreScission protease (Amersham Phaermacia Biotech) to remove GST and purified as described in Experimental Procedures. (B) Purified CaM-KK chimera mutants were subjected to SDS−10% PAGE followed by either protein staining (1 μg of enzymes, left panel) or CaM overlay (200 ng of enzymes, right panel). Lanes: a, CaM-KKα (126−463); b, CaM-KKα/β; c, CaM-KKβ/α; d; CaM-KKβ (162−499). (C) Purified CaM-KK chimera mutants (0.4 μg/mL) were incubated with GST-CaM-KI 1−293 K49E mutant (0.4 mg/mL) at 30 °C for 5 min in the presence of either 1 mM $CaCl_2$/3 μM CaM (closed bars) or 1 mM EGTA (open bars) as described in Experimental Procedures. The experiment was performed in triplicate for each mutant, and the results are presented as the mean and SE of three experiments.

are very homologous in the C-terminal part of this region while the N-terminal sequence is highly divergent. Most notably, the sequence of CaM-KKβ shows four serine residues (Figure 5A). It has been shown that the recombinant CaM-KKβ fused with MBP exhibited significantly high Ca^{2+}/CaM-independent autophosphorylation activity (*23*). Therefore, we constructed a Ser to Ala mutant of CaM-KKβ to determine if these residues are important for the autonomous activity of the enzyme. As expected, a CaM-KKβ mutant lacking residues 129−151, CaM-KKβ (Δ129−151), exhibited significantly low autonomous CaM-KK activity (10% of total activity) compared to the wild-type enzyme (Figure 5B) while there was no differences regarding Ca^{2+}/CaM-dependent activity. The substitution of the four Ser residues by Ala did not altered the Ca^{2+}/CaM-independent activity which was indistinguishable from the wild-type enzyme. The autophosphorylation activity of CaM-KKβ is highly independent of Ca^{2+}/CaM. This result is consistent with the CaM-KI phosphorylating activity of CaM-KK isoforms (Figure 2) reported here and in a previous report (*23*). However, CaM-KKα exhibited complete Ca^{2+}/CaM-dependent autophosphorylation activity (Figure 5C). Deletion of the residues 129−151 significantly decreased the Ca^{2+}/CaM-independent autophosphorylation as well as the CaM-KK activity (Figure 5B). The SA mutation of CaM-KKβ did not affect the ability of the enzyme to autophosphorylate in the absence of Ca^{2+}/CaM. These results suggest that residues 129−151 contribute to prevent the autoinhibition of CaM-KKβ resulting in an increased Ca^{2+}/CaM-independent CaM-KK activity and autophosphorylation activity. The observed increased autophosphorylation activity does not target the serine residues located in this region.

We have previously identified and characterized the regulatory region of rat CaM-KKα (residues 438−463) as a functional autoinhibitory peptide (*28*). This region is highly homologous between the α and β isoforms (Figure 6B). Residues such as Ile (at position 477 in CaM-KKβ and position 441 in CaM-KKα) located at the −3 position from the CaM anchoring region are important for the autoinhibitory function (*28*). The regulatory region was interchangeable between the two CaM-KK isoforms as demonstrated in Figure 4. To examine the inhibitory effect of the CaM-KKα peptide against the catalytic domain of CaM-KKβ, we constructed and expressed two constitutively active mutants lacking the putative autoinhibitory domain (residues 471−499) with either the presence or absence of residues 129−151 (Figure 6A). As shown in Figure 6C, both mutants were inhibited by the CaM-KKα peptide (residues 438−463) in the absence of Ca^{2+}/CaM similarly (IC_{50} of 10−20 μM) to the observed inhibition of a constitutively active CaM-KKα (IC_{50} of ~15 μM) (*28*). This result indicates that residues 129−151 did not directly block the interaction between the catalytic and autoinhibitory segments.

In summary, we have demonstrated that, unlike CaM-KKα, CaM-KKβ exhibits a Ca^{2+}/CaM-independent activity. This activity is caused by the region spanning residues 129−151, located at N-terminus of the catalytic domain, which interferes with the autoinhibitory mechanism of the enzyme. The Ca^{2+}/CaM-independent activity of CaM-KKβ is probably the autonomous CaM-KK activity detected in rat brain. The effect of the residue 129−151 deletion or truncation on Ca^{2+}/CaM dependency of CaM-KKβ activity is not likely due to decreased enzymatic stability in the absence of Ca^{2+}/CaM resulting in creating an inactive enzyme, since Ca^{2+}/CaM-independent activity of the 129−151 deletion mutant lacking the regulatory domain (CaM-KKβ 2−470, Δ129−151) is comparable to that of the nondeletion mutant (CaM-KKβ 2−470) (Figure 6). These results support the previous observation that CaM-KKβ could enhance CREB-dependent transcriptional activation independently from Ca^{2+}/CaM in transfected Jurkat cells (*23*). On the basis of the results observed with the CaM-KK chimera mutants, the region from residue 471 to residue 499 of CaM-KKβ is a functional autoinhibitory domain similar to the one characterized in the α isoform. Taken together, this is the first demonstration for CaM kinases that an intramolecular interference of the autoinhibition generates autonomous activity. Other exceptions are the autophosphorylation of CaM kinase II on Thr286 and the phosphorylation of CaM kinase IV at Thr196 by CaM-KK, both inducing their autonomous activity

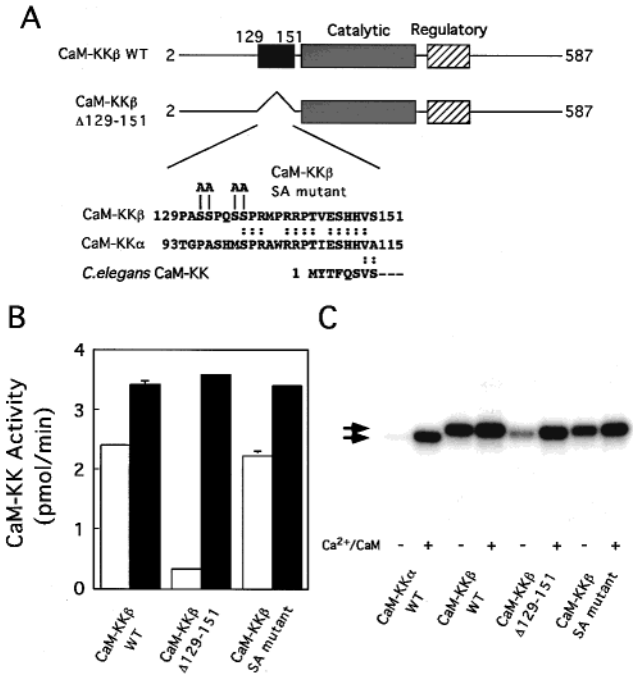

FIGURE 5: Involvement of residues 129−151 in the autonomous activity of CaM-KKβ. **(**A) Schematic representation of CaM-KKβ mutants and amino acid sequence comparison of the regions in various CaM-KKs (CaM-KKβ, CaM-KKα, and *C. elegans* CaM-KK) corresponding to the residues 129−151 of CaM-KKβ. CaM-KKβ mutant lacking the residues 129−151 (Δ129−151) and Ala substitution mutant at Ser131, 132, 135, 136 (SA mutant) were constructed and expressed in *E. coli* JM109 as a GST fusion protein followed by digestion with PreScission protease (Amersham Phaermacia Biotech) to remove GST and purified as described in Experimental Procedures. (B) Purified CaM-KKβ mutants (0.25 μg/mL) including wild type were incubated with GST-CaM-KI 1−293 K49E mutant (0.4 mg/mL) at 30 °C for 5 min in the presence of either 1 mM $CaCl_2$/3 μM CaM (closed bars) or 1 mM EGTA (open bars) as described in Experimental Procedures. The experiment was performed in triplicate for each mutant, and the results are presented as the mean and SE of three experiments. CaM-KKβ WT, wild type (2−587). (C) Purified CaM-KKβ mutants including wild type and CaM-KKα wild type (0.6 μg) were subjected to the autophosphorylation reaction at 30 °C for 10 min in the presence of either 1 mM $CaCl_2$/3 μM CaM (+) or 1 mM EGTA (−) followed by SDS−10% PAGE and autoradiography as described in Experimental Procedures. Arrows indicate autophosphrylated CaM-KKs.

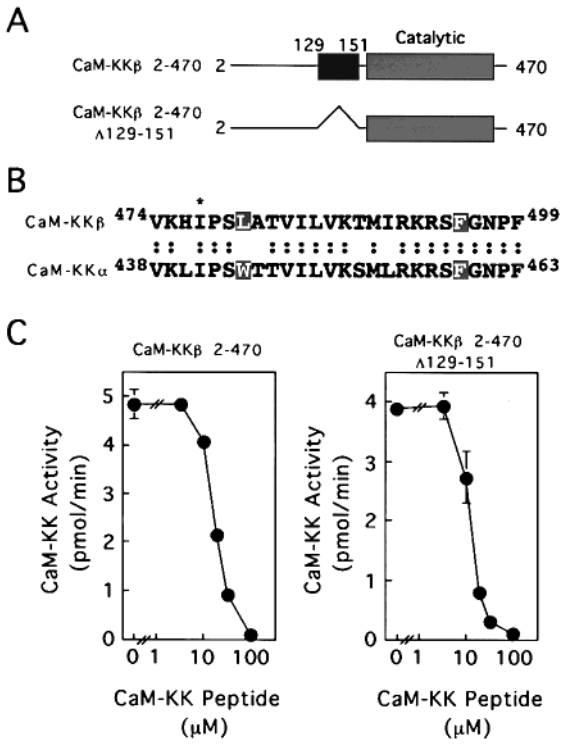

FIGURE 6: Effect of CaM-KKα autoinhibitory peptide on the activities of CaM-KKβ mutants lacking the regulatory domain. (A) Schematic representation of CaM-KKβ mutants lacking the regulatory domain. CaM-KKβ mutant lacking the regulatory domain (CaM-KK 2−470) in either the presence or absence of residues 129−151 (Δ129−151) was expressed in *E. coli* JM109 and purified as a GST fusion protein as described in Experimental Procedures. (B) Amino acid sequence comparison of the regulatory domain of CaM-KK isoforms (residues 474−499 in CaM-KKβ and residues 438−463 in CaM-KKα) based on the previous identification of the regulatory domain of CaM-KKα (*24*). The key hydrophobic residues binding to the hydrophobic pockets of Ca^{2+}/CaM are shaded (*27*). The Ile residue (Ile441 in CaM-KKα and Ile477 in CaM-KKβ) which is critical for autoinhibitory function is indicated by an asterisk (*28*). (C) The activities of purified CaM-KKβ mutants lacking the regulatory domain (CaM-KKβ 2−470) in either the presence or absence of residues 129−151 (Δ129−151) were measured at 30 °C for 5 min in the presence of 1 mM EGTA with various concentrations of CaM-KKα autoinhibitory peptide (residues 438−463, 0−100 μM) as shown in Figure 5. The experiment was performed in triplicate for each mutant, and the results are presented as the mean and SE of three experiments.

(reviewed in ref *35*). However, our results suggest that no phosphorylation within residues 129−151 is involved in the enhanced autonomous activity. Residues 129−151 do not directly prevent the inhibition of CaM-KKβ activity by the autoinhibitory peptide (Figure 6C). The suppression of the autoinhibitory function of CaM-KKβ in the absence of Ca^{2+}/CaM could be due to the distortion of the proper alignment of the autoinhibitory domain toward the catalytic core through direct interaction with residues located N-terminally from the regulatory domain. Further studies remain to be performed to characterize the intramolecular mechanism through which residues 129−151 of CaM-KKβ prevent the expected autoinhibitory function of this enzyme. It is noteworthy that *C. elegans* CaM-KK has been shown to activate constitutively active mouse CaM-KIV in a complete Ca^{2+}/CaM-dependent manner. *C. elegans* CaM-KK lacks the residues corresponding to amino acids 129−143 of CaM-KKβ, and the other residues corresponding to amino acids 144−151 of CaM-KKβ are highly divergent (*26*, Figure 5A).

The phosphorylation of the activating Thr within the T-loop of CaM-KI and CaM-KIV is tightly regulated by the autoinhibitory domain of each kinase. In the case of the CaM kinase cascade initiated by CaM-KKα, the Ca^{2+}/CaM complex regulates the activation of both CaM-KK and downstream CaM kinases. Our result suggests that, in the case of signaling triggered by CaM-KKβ, the Ca^{2+}/CaM complex only regulates the activation of the downstream CaM kinases (Figure 1). Depending on the CaM-KK isoforms expressed in different tissues or cell types, Ca^{2+} can regulate the signaling cascade at different steps. Previous studies revealed that CaM-KKβ is predominantly expressed in brain but is not detected in peripheral tissues (*22*, *23*) while CaM-KKα was detected in brain and also in other tissues such as spleen and thymus (*3*, *21*).

In conclusion, on the basis of our biochemical characterization of CaM-KK isoforms and other studies, CaM-KKα and CaM-KKβ also share a common signaling cascade such

as the intracellular Ca^{2+}-mediated CaM kinase cascade but may contribute to a distinct pathway with regard to the requirement of intracellular Ca^{2+}.

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