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Regulatory Mechanism of Ca^{2+}/Calmodulin-dependent Protein Kinase Kinase*

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Ca^{2+}/calmodulin-dependent protein kinase kinase (CaM-KK) is a novel member of the CaM kinase family, which specifically phosphorylates and activates CaM kinase I and IV. In this study, we characterized the CaM-binding peptide of αCaM-KK (residues 438–463), which suppressed the activity of constitutively active CaM-KK (84–434) in the absence of Ca^{2+}/CaM but competitively with ATP. Truncation and site-directed mutagenesis of the CaM-binding region in CaM-KK reveal that Ile^{441} is essential for autoinhibition of CaM-KK. Furthermore, CaM-KK chimera mutants containing the CaM-binding sequence of either myosin light chain kinases or CaM kinase II located C-terminal of Leu^{440}, exhibited enhanced Ca^{2+}/CaM-independent activity (60% of total activity). Although the CaM-binding domains of myosin light chain kinases and CaM kinase II bind to the N- and C-terminal domains of CaM in the opposite orientation to CaM-KK (Osawa, M., Tokumitsu, H., Swindells, M. B., Kurihara, H., Orita, M., Shibanuma, T., Furuya, T., and Ikura, M. (1999) *Nat. Struct. Biol.* 6, 819–824), the chimeric CaM-KKs containing Ile^{441} remained Ca^{2+}/CaM-dependent. This result demonstrates that the orientation of the CaM binding is not critical for relief of CaM-KK autoinhibition. However, the requirement of Ile^{441} for autoinhibition, which is located at the −3 position from the N-terminal anchoring residue (Trp^{444}) to CaM, accounts for the opposite orientation of CaM binding of CaM-KK compared with other CaM kinases.

Ca^{2+}/calmodulin-dependent protein kinase kinase (CaM-KK)[1] has been identified and cloned as an activator for two multifunctional CaM kinases, CaM-KI and IV. The identification of this new CaM kinase regulating other CaM kinases revealed a novel intracellular Ca^{2+}-mediated signal transduction pathway, the CaM kinase cascade (1–6). Phosphorylation by CaM-KK of Thr, located in the activation loop of the catalytic domain of CaM-KI and CaM-KIV, results in a large increase in the catalytic efficiency of each CaM kinase (5–7). Two CaM-KK genes (α and β) have been cloned in mammal (4, 8, 9), whereas only one gene was characterized in *Caenorhabditis elegans* (10, 11). In mammals, the highest expression of the α and β isoforms of CaM-KK is observed in the brain, although the α isoform is also expressed in various peripheral tissues such as thymus and spleen (4).

Numerous studies demonstrated that the CaM-KK/CaM-KIV cascade is present and functional in various cell types such as Jurkat cells (12), cultured hippocampal neurons (13), and transfected COS-7 cells (7). 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 cAMP-response element-binding protein has been demonstrated (13–17). The CaM-KK/CaM-KI cascade is activated in PC12 cells upon membrane depolarization, although its physiological role remains to be determined (18). Recently a kinase homologous to mammalian CaM-KI (*Ce*CaM-KI) has been cloned in *C. elegans* (19). Unlike mammalian CaM-KI, overexpressed *Ce*CaM-KI is predominantly localized in the nucleus of transfected cells because of the presence of an N-terminal nuclear localization signal. In transfected cells, *Ce*CaM-KK and *Ce*CaM-KI reconstitute a signaling pathway that mediates Ca^{2+}-dependent phosphorylation of cAMP-response element-binding protein and cAMP-response element-dependent transcription, similarly to the mammalian CaM-KK/CaM-KIV pathway. In addition to CaM-KI and IV, protein kinase B has been identified as a target for CaM-KK. The phosphorylation and activation of protein kinase B by CaM-KK is responsible for the anti-apoptotic effect upon modest elevation of intracellular Ca^{2+} in NG108 cells (20).

CaM-KK has an N-terminal catalytic domain and a regulatory domain containing autoinhibitory and CaM-binding segments at its C terminus in a similar manner to other CaM kinases. The catalytic domain of CaM-KK contains a unique Arg-Pro-rich 22 residues insert, which plays an important role for recognition of downstream CaM kinases (11). The binding of Ca^{2+}/CaM to CaM-KK is absolutely required for its activation and efficient phosphorylation of target protein kinases (7, 27). Previous studies using site-directed mutagenesis and synthetic peptides identified the CaM-binding region of αCaM-KK as spanning residues 438–463 (21). The three-dimensional structure of the Ca^{2+}-CaM complex with the αCaM-KK peptide (residues 438–463) has been resolved by NMR spectroscopy. The CaM-KK peptide forms a fold comprising an α-helix (residues 444–454) and a hairpin-like loop (residues 455–459) whose C terminus folds back onto the helix (22). This unique loop structure in CaM-KK peptide is stabilized by intramolecular hydrophobic interaction between Met^{453} and Phe^{459}, which anchor to the C-terminal domain of CaM, as well as interactions involving Phe^{463}, Ile^{448}, and Leu^{449}. This was confirmed by mutation of either Phe^{459} or Phe^{463} to Asp, which resulted in a significant reduction of CaM-binding ability of

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[1] The abbreviations used are: CaM-KK, Ca^{2+}/CaM-dependent protein kinase kinase; CaM, calmodulin; MLCK, myosin light chain kinase; GST, glutathione *S*-transferase; sk, skeletal muscle; sm, smooth muscle.

CaM-KK. Trp[444] was also identified as another anchoring residue to the N-terminal domain of CaM. Therefore, 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 (35–37). These observations raised the question why CaM-KK requires an opposite orientation of CaM binding to that of other CaM kinases for the regulation of its protein kinase activity. In this report, we present evidences using CaM-KK mutants and chimeras to assume the critical role of the unique regulatory domain of CaM-KK and the orientation of CaM binding for the kinase autoinhibition.

EXPERIMENTAL PROCEDURES

Materials—αCaM-KK cDNA (GenBank™ accession number L42810) was from a rat brain cDNA library (4). GST-CaM-KK-(84–434) was constructed by the insertion of a *Xba*I-digested fragment from pME-CaM-KK-(1–434, 21) into pGEX-KG vector, and the recombinant enzyme was expressed in *Escherichia coli* JM109 and purified on glutathione-Sepharose. GST-CaM-KI-(1–293, K49E) into pGEX-4T3 was constructed by polymerase chain reaction followed by mutagenesis using a mutagenic oligonucleotide (5′-AAACTGGTGGCCATCGAATG-CATTGCCAAGAAG-3′) and a GeneEditor *in vitro* site-directed mutagenesis system (Promega Co.). Expressed GST-CaM-KI-(1–293, K49E) in *E. coli* JM109 was purified on glutathione-Sepharose. Recombinant rat CaM was expressed in *E. coli* BL-21 (DE3) using pET-CaM (23), which was kindly provided from Dr. Nobuhiro Hayashi (Fujita Health University, Toyoake, Japan), and purified by phenyl-Sepharose column chromatography. Nucleotide sequences of those constructs were confirmed. Twenty six-residue synthetic peptides corresponding to the calmodulin-binding domain of both rat αCaM-KK (residues 438–463) (21) and *C. elegans* CaM-KK (residues 331–356) (11) were synthesized by the Peptide Institute Inc. (Osaka, Japan). Purity of either peptide was estimated at higher than 95% judging by high pressure liquid chromatography. All other chemicals were from standard commercial sources.

Construction of CaM-KK Mutants—Mutagenesis of CaM-KK using pME-CaM-KK (wild-type, 4) plasmid as a template was carried out by a GeneEditor *in vitro* site-directed mutagenesis system (Promega Co.) and mutagenic oligonucleotides as follows; I441A, 5′-AACTCAGTCAAGCTTGCCCCCAGCTGGACC-3′; I441R, 5′-AACTCAGTCAAGCTTCGCCCCAGCTGGACC-3′; I441D, 5′-AACTCAGTCAAGCTTGACCCCAGCTGGACC-3′.

For CaM-KK truncation and chimera mutants, pME-CaM-KK-(1–463, 21) was mutated at Ile[441] by a mutagenic oligonucleotide (5′-AAGAACTCAGTCAAGCTTAAGCCCAGCTGGACCACT-3′) to introduce the *Afl*II site and then digested with *Afl*II followed by treating with mung bean nuclease to create a blunt end at the first codon (C) of Leu[440]. After digestion with *Not*I, the linearized plasmid ligated with pre-annealed double-stranded oligonucleotides containing the *Not*I site as follow: pME-CaM-KK-(1–440), 5′-TCTGACATATGCGCTCTAGAACTAGTGC-3′ and 5′-GGCCGCACTAGTTCTAGAGCGCATATGTCAGA-3′; pME-CaM-KK-(1–441), 5′-TCATCTGACATATGCGCTCTAGAACTAGTGC-3′ and 5′-GGCCGCACTAGTTCTAGAGCGCATATGTCAGATGA-3′; pME-CaM-KK-(1–443), 5′-TCATCCCCAGCTGACATATGCGCTCTAGAACTAGTGC-3′ and 5′-GGCCGCACTAGTTCTAGAGCGCATATGTCACCAGCTGGGGATGA-3′; pME-CaM-KK-(1–444), 5′-TCATCCCCAGCTGGTGACATATGCGCTCTAGAACTAGTGC-3′ and 5′-GGCCGCACTAGTTCTAGAGCGCATATGTCACCAGCTGGGGATGA-3′; pME-CaM-KK-440/smMLCK (chicken gizzard, residues 797–816), 5′-TCAGAAGAAAATGGCAGAAAACAGGCCATGCTGTCCGAGCAATAGGAAGACTGTCATCCATGTGAGC-3′ and 5′-GGCCGCTCACATGGATGACAGTCTTCCTATTGCTCGGACAGCATGGCCTGTTTTCTGCCATTTTTCTTCTGA-3′; pME-CaM-KK-440/skMLCK (rabbit skeletal muscle, residues 577–596), 5′-TCAAGAGGCGCTGGAAGAAAAACTTCATTGCCGTCAGCGCTGCCAACCGCTTCAAGAAGATCTGAGC-3′ and 5′-GGCCGCTCAGATCTTCTTGAAGCGGTTGGCAGCGCTGACGGCAATGAAGTTTTTCTTCCAGCGCCTCTTGA-3′; pME-CaM-KK-440/CaM-KII (rat, residues 296–313), 5′-TCCGACGGAAATTGAAGGGTGCCATCTTGACAACTATGCTGGCTACGAGAAATTTTTGAGC-3′ and 5′-GGCCGCTCAAAAATTTCTCGTAGCCAGCATAGTTGTCAAGATGGCACCCTTCAATTTCCGTCGGA-3′. pME-CaMKK-(1–448) and (1–434) were constructed as described previously (21).

For construction of Ile[441]-inserted CaM-KK chimera mutants, each chimeric mutant described above was digested with *Eco*RI and *Not*I and re-inserted into a newly digested pME18s plasmid, which was used as a template for mutagenesis. Mutagenic oligonucleotides are described as follows: pME-CaM-KK-441/skMLCK, 5′-AAGAACTCAGTCAAGCTTATCAAGAGGCGCTGGAAGAA; pME-CaM-KK-441/smMLCK, 5′-AAGAACTCAGTCAAGCTTATCAGAAGAAAATGGCAGAA-3′; pME-CaM-KK-441/CaM-KII, 5′-AAGAACTCAGTCAAGCTTATCCGACGGAAATTGAAGGG. The nucleotide sequence of each mutant was confirmed by automated sequencing using an Applied Biosystems 377 DNA sequencer.

Transient Expression and Partial Purification of CaM-KK Mutants—COS-7 cells were maintained in Dulbeco's modified Eagle's medium containing 10% fetal calf serum. Cells were subcultured in 10-cm dishes 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.) or CaM-KK cDNA containing plasmid DNAs and 60 μg of LipofectAMINE Reagent (Life Technologies, Inc.) in 6.4 ml of medium. After a 32–48-h incubation, the cells (3 plates) were collected and homogenized with 1 ml of lysis buffer (150 mM NaCl, 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1% Nonidet P-40, 10% glycerol, 0.2 mM phenylmethylsulfonyl fluoride, 10 mg/liter leupeptin, 10 mg/liter pepstatin A, 10 mg/liter trypsin inhibitor) at 4 °C. After centrifugation at 15,000 × *g* for 15 min, the supernatant was diluted by the addition of twice the volume of 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 0.2 mM phenylmethylsulfonyl fluoride, 10 mg/liter leupeptin, 10 mg/liter pepstatin A, and 10 mg/liter trypsin inhibitor (buffer A) and then applied to a Q-Sepahrose column (0.5-ml bed volume), which had been pre-equilibrated with 50 mM NaCl containing buffer A. After washing the column with 10 ml of the equilibration buffer, CaM-KK was eluted by the addition of 0.5 ml of 0.6 M NaCl containing buffer A and stored at −80 °C.

In Vitro Assay of CaM-KK Mutants—Either partially purified CaM-KK (1 μl) from transfected COS-7 cells or *E. coli* expressed GST-CaM-KK-(84–434, 0.7 μg/ml) was incubated with 10 μg of GST-CaM-KI-(1–293, K49E) at 30 °C for 5 min in a solution (25 μl) containing 50 mM HEPES (pH 7.5), 10 mM $Mg(Ac)_2$, 1 mM dithiothreitol, 100 μM [γ-^{32}P]ATP (1000–2000 cpm/pmol) in the presence of either 2 mM EGTA or 2 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 washing in 75 mM phosphoric acid (24). Phosphate incorporation into GST-CaM-KI-(1–293, K49E) was quantitated by liquid scintillation counting of the filters. For each experiment, ~10-fold volume of partially purified CaM-KKs used in the assay was analyzed by Western blotting to check the amount of the enzymes. CaM-KK activity was measured for 5 min, which was under the linear condition.

Others—Western blotting was carried out using antiserum (1/1000 dilution) against a peptide corresponding to a conserved protein kinase motif (residues 132–146 of CaM-KII), and the biotinylated-CaM overlay was done as described previously (4). Detection was performed by using chemiluminescence reagent (NEN Life Science Products). Protein concentration was estimated by Coomassie Blue dye binding (Bio-Rad) using bovine serum albumin as a standard (25).

RESULTS AND DISCUSSION

We have previously identified the CaM-binding domain at the C-terminal of rat αCaM-KK (residues 438–463) by using mutagenesis and a synthetic peptide (Fig. 1*A*) (21). Recently, a study using NMR spectroscopy has shown that the CaM-binding peptide (residues 438–463) of αCaM-KK bound to the N- and C-terminal domains of CaM in an opposite direction to all other known CaM kinases such as MLCKs and CaM-KII (22). This result raised the possibility that CaM-KK is regulated by a mechanism distinct from that controlling other CaM kinases. Indeed, we have found that the CaM-binding peptide (residues 438–463) of αCaM-KK is a weak inhibitor of the enzyme, when CaM-KIV activation assay was used to measure CaM-KK activity (21). In this study, we performed a direct phosphorylation assay toward the GST-CaM-KI-(1–293, K49E) mutant as a substrate to determine CaM-KK activity. The use of this CaM-KI mutant has numerous advantages. First, CaM-KI lacking a regulatory domain (including an autoinhibitory and CaM-binding domains) is more suitable for measuring the Ca^{2+}/CaM sensitivity of CaM-KK. Second, substitution of Lys[49] by Glu abolishes the binding of ATP and allows us to rule out the possibility of a feedback phosphorylation of CaM-KK by activated CaM-KI, which would indirectly affect CaM-KK ac-

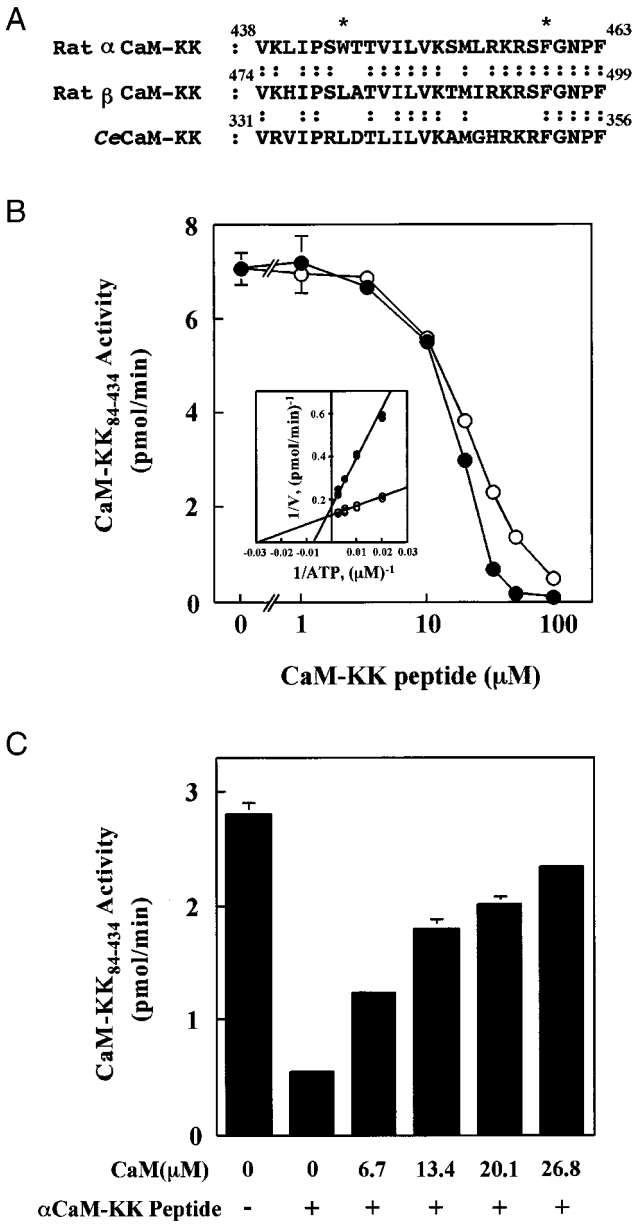

FIG. 1. **Inhibition of CaM-KK (84–434) activity by αCaM-KK peptide (residues 438–463).** *A*, amino acid sequence alignment of the Ca^{2+}/CaM-binding region of various CaM-KKs (rat α and β isoforms and *C. elegans*) (4, 8–11). *CeCaM-KK*, *C. elegans* CaM-KK. *B*, GST-CaM-KK-(84–434, 0.7 μg/ml) was incubated with GST-CaM-KI-(1–293, K49E, 0.4 mg/ml) without Ca^{2+}/CaM in the absence or presence of either αCaM-KK peptide (residue 438–463, 1–100 μM, *closed circle*) or *C. elegans* CaM-KK peptide (residue 331–356, 1–100 μM, *open circle*) for 5 min at 30 °C as described under "Experimental Procedures." *Inset*, GST-CaM-KK-(84–434) activity was measured as described in *A* in the either absence (*open circle*) or presence of 20 μM αCaM-KK peptide (residue 438–463, *closed circle*) using 50–400 μM [γ-^{32}P]ATP. The results are presented as double reciprocal plots (Lineweaver-Burk). *C*, GST-CaM-KK-(84–434) activity was measured as described in *A* using 20 μM of [γ-^{32}P]ATP in the either absence (−) or presence of 24 μM αCaM-KK peptide (residue 438–463, +) with various concentrations of calmodulin (0–26.8 μM) as indicated. The experiments were performed in triplicate for each point and the results are presented as the mean and S.E. of three experiments.

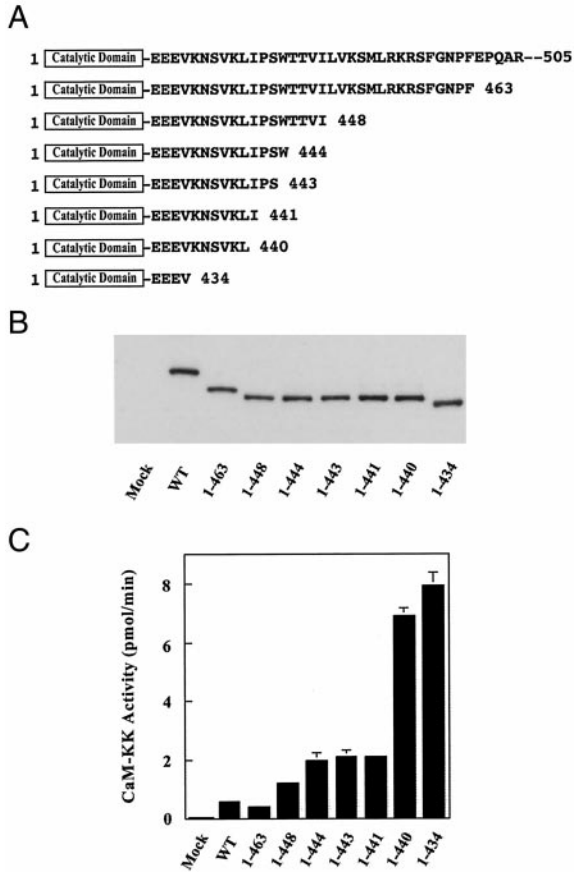

FIG. 2. **Ca^{2+}/CaM-independent activity of truncated αCaM-KK mutants.** *A*, schematic representation of wild-type and truncated mutants of αCaM-KK. A series of truncation mutant of CaM-KK were constructed and expressed in COS-7 cells followed by partial purification as described under "Experimental Procedures." *B*, partially purified truncation mutants of CaM-KK described in *A* (~1 μg of protein) including Mock were subjected to SDS/10% polyacrylamide gel electrophoresis followed by Western blotting using antiserum (1/1000 dilution) against a peptide corresponding to a conserved protein kinase motif (residues 132–146 of CaM-KII). *C*, partially purified truncated CaM-KKs (~0.1 μg of protein) were incubated with GST-CaM-KI-(1–293, K49E, 0.4 mg/ml) in the absence of Ca^{2+}/CaM for 5 min at 30 °C as described under "Experimental Procedures." The experiment was performed in triplicate for each point, and the results are presented as the mean and S.E. of three experiments. *WT*, wild type.

tivity (26). Finally, a direct phosphorylation assay allows us to perform kinetic analysis of CaM-KK activity. Thus we examined the inhibitory effect of the αCaM-KK peptide (residues 438–463) on the constitutively active mutant (84–434) of CaM-KK lacking both an autoinhibitory and CaM-binding domains (7). As shown in Fig. 1*B*, the peptide inhibits CaM-KK 84–434 activity with an IC_{50} of ~15 μM in the absence of Ca^{2+}/CaM. This inhibition is comparable to that obtained with the corresponding peptide from *C. elegans* CaM-KK (residues 331–356). Kinetic analysis indicated that the peptide inhibition of CaM-KK 84–434 was competitive with respect to ATP (Fig. 1*B, inset*) but not to its protein substrate (data not shown). When Ca^{2+}/CaM was added into the inhibitory reaction using 24 μM CaM-KK peptide, the activity of CaM-KK 84–434 was restored up to 80–90% of its original activity in a dose-dependent manner (Fig. 1*C*). These results indicated that the CaM-binding peptide could also interact with the catalytic domain of CaM-KK and function as an autoinhibitory peptide. Previous mutagenesis study revealed that block mutation of either Lys^{435}-Asn^{436}-Ser^{437} or Val^{438}-Lys^{439}-Leu^{440} by triple Asp resulted in increased Ca^{2+}/CaM-independent activity (10–20% of total activity), suggesting that these residues are involved in autoinhibitory function either directly or indirectly (21). Because constitutive activity of those mutants is low compared with that of fully constitutive active mutant (1–434), we tested the possibility that other regions of CaM-KK, further C-terminal from residue 440, could be important for autoinhibition.

When we truncated at residue 448 in CaM-KK, the mutant was inactive (21). A recent study demonstrated that the truncation mutant of CaM-KK at Trp^{444}, which turns out to be the anchoring residue to N-terminal domain of CaM (22), was also inactive (27). Based on these results, we postulated that the important residue(s) for the autoinhibition are located between residue 438 and 444.

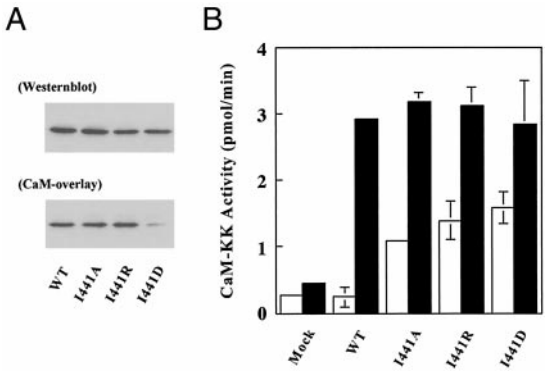

FIG. 3. **Point mutation of Ile^{441} in αCaM-KK.** *A*, wild-type CaM-KK was mutated at Ile^{441} by Ala (I441A), Arg (I441R), or Asp (I441D) and expressed in COS-7 cells followed by partial purification as described under "Experimental Procedures." Each mutant (~2 μg of protein) was analyzed by either Western blotting (*upper panel*) as shown in Fig. 2*B* or the CaM overlay method (*lower panel*). *B*, each CaM-KK mutant (~0.3 μg of protein) including Mock was incubated with GST-CaM-KI-(1–293, K49E, 0.4 mg/ml) in the either absence (*open bar*) or presence (*closed bar*) of Ca^{2+}/CaM for 5 min at 30 °C as described under "Experimental Procedures." The experiment was performed in triplicate for each point, and the results are presented as the mean and S.E. of three experiments. *WT*, wild type.

We, therefore, constructed other truncated CaM-KK mutants (Fig. 2 *A*) and overexpressed them in COS-7. The expression level of each mutant was analyzed by immunoblotting after partial purification on Q-Sepharose (Fig. 2*B*). Direct phosphorylation assay toward GST-CaM-KI-(1–293, K49E) by each mutant was performed in the absence of Ca^{2+}/CaM (Fig. 2*C*). Consistent with previous observation (21), a truncated version of CaM-KK at Phe^{463} did not exhibit significant Ca^{2+}/CaM-independent activity compared with the wild-type enzyme. Further truncation from residue 448 to 441 generated detectable but weak Ca^{2+}/CaM-independent activity, which is ~20–25% of fully constitutively active mutant (1–434). Interestingly, truncation at Ile^{441} resulted in complete constitutive activity of CaM-KK similar to that of 1–434 mutant. This result suggests that Ile^{441} is essential but residue 435–440 may not be absolutely critical for CaM-KK autoinhibition. Our previous result demonstrating a 10–20% constitutive activity of CaM-KK after triple Asp substitution between residues 435–440 was likely because of the indirect effect of the mutation on the function of residue 441. It is noteworthy that Ile^{441} is conserved in β-isoform (8, 9) and *C. elegans* CaM-KK (10, 11) (Fig. 1*A*).

To confirm the involvement of Ile^{441} in the regulatory mechanism of CaM-KK, we mutated Ile^{441} by Ala, Arg, or Asp in the

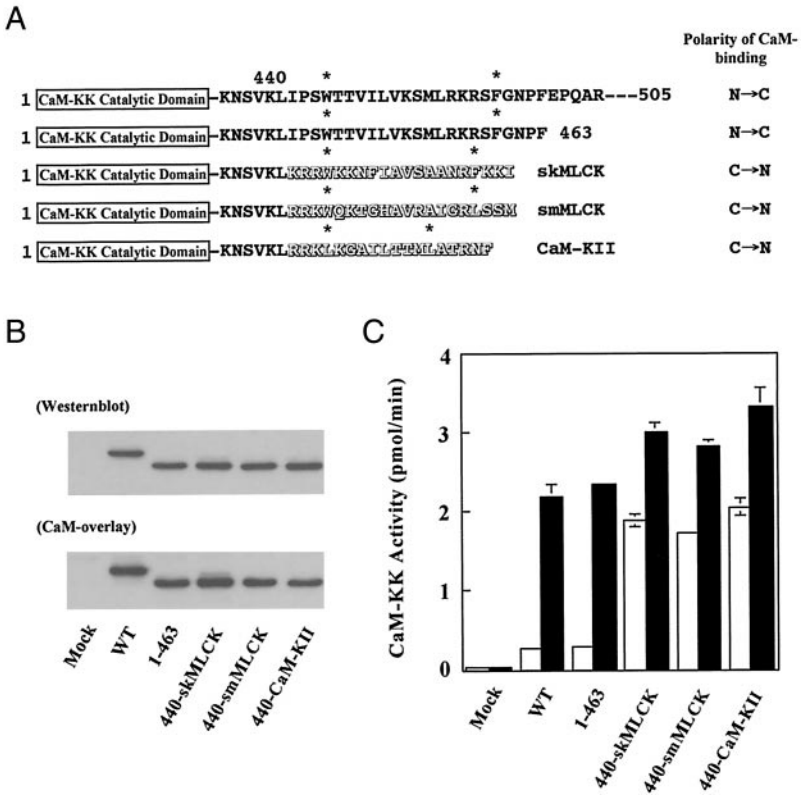

FIG. 4. **Characterization of CaM-KK chimera mutants.** *A*, schematic representation of wild-type, 1–463 mutant, and the chimera mutant of αCaM-KK containing the CaM-binding region of rabbit skeletal muscle MLCK (residues 577–596, 440-skMLCK) (39), chicken smooth muscle MLCK (residues 797–816, 440-smMLCK), (38) or rat αCaM kinase II (residues 296–313, 440-CaM-KII) (40, 41). Chimera CaM-KKs were constructed and expressed in COS-7 cells followed by partial purification as described under "Experimental Procedures." The binding direction of CaM to the CaM-binding sequence of each chimera mutant is indicated in the *right lane* based on previous structural analysis using NMR and crystallography (22, 35–37). Hydrophobic anchoring residues to N- and C-terminal domains of CaM are indicated by *asterisks*. *B*, partially purified CaM-KK chimera mutants (~2 μg of protein) including wild-type, 1–463 mutant, and Mock were analyzed by either Western blotting (*upper panel*) as shown in Fig. 2*B* or the CaM overlay method (*lower panel*). *C*, partially purified CaM-KK chimera mutants (~0.2 μg of protein) including wild-type, 1–463 mutant, and Mock were incubated with GST-CaM-KI-(1–293, K49E, 0.4 mg/ml) in the either absence (*open bar*) or presence (*closed bar*) of Ca^{2+}/CaM for 5 min at 30 °C as described under "Experimental Procedures." The experiment was performed in triplicate for each point, and the results are presented as the mean and S.E. of three experiments.

wild-type CaM-KK. Each mutant exhibited 40–60% of Ca^{2+}/CaM-independent activity and was further activated by Ca^{2+}/CaM (Fig. 3*B*). A similar result was obtained when these mutations were introduced into the CaM-KK 1–463 mutant (data not shown). The mutation of Ile^{441} by Asp significantly reduced the binding ability of CaM compared with either wild type or other Ile^{441} mutants (Fig. 3*A*, *lower panel*). This result is in accordance with the previous observation that the mutation of Val^{438}-Lys^{439}-Leu^{440} by triple Asp reduced the binding ability of Ca^{2+}-CaM complex (21). This is probably because of an intermolecular electrostatic repulsion, because there are many acidic residues in the region of CaM, which binds to the N terminus of the CaM-KK peptide (residues 438–463) (22). These results demonstrated that the CaM binding and autoinhibitory regions in CaM-KK are overlapped at Ile^{441}.

Extensive mutagenesis studies indicated that most of the elements required for CaM binding and autoinhibition of MLCK and CaM-KII were located within the combined segment but clearly separated because mutation of the CaM recognition region has little effect on autoinhibition of the kinases (28–33). Moreover, the CaM recognition sequence among MLCKs and CaM-KII can be interchangeable (28, 34). This may be guaranteed by the fact that MLCKs and CaM-KII contain basic amino acid clusters located at N-terminal of the first hydrophobic anchoring residue resulting in the same direction of CaM-binding (35–37). In contrast, the three-dimensional structure of the Ca^{2+}-CaM complex with the CaM-KK peptide (residues 438–463) has shown that the orientation of CaM binding to the peptide was opposite of that observed for MLCKs and CaM-KII. This difference can be due to the presence of a basic amino acid cluster (Lys^{451}, Arg^{455}-Lys^{456}-Arg^{457}) in the C-terminal region, which interacts with the negatively charged C-terminal channel outlet of CaM (22).

To determine whether the proper orientation of CaM binding to CaM-KK was required for the regulation of the enzyme, we constructed chimera CaM-KK mutants that contained CaM-binding segment of either chicken smMLCK (residues 797–816) (38), rabbit skMLCK (residues 577–596) (39), or rat αCaM-KII (residues 296–313) (40, 41) based on the position of an N-terminal anchoring residue to CaM (Trp^{444} in CaM-KK, Trp^{800} in smMLCK, Trp^{580} in skMLCK, and Leu^{299} in αCaM-KII) (Fig. 4*A*). The clusters of basic amino acids, which are critical for the orientation of the binding of CaM to the peptide, are located at the N terminus of the CaM-binding sequence of each CaM kinase. Therefore, we fused each CaM-binding segment with the catalytic portion of CaM-KK at Leu^{440}. These chimera enzymes were expressed in COS-7 cells and partially purified to analyze their enzymatic activities and Ca^{2+}/CaM binding by CaM overlay method (Fig. 4*B*). All of the chimeric enzymes retained the ability to bind Ca^{2+}/CaM as expected (Fig. 4*B*, *lower panel*). Unlike the wild-type and 1–463 mutant of CaM-KK, which are Ca^{2+}/CaM-dependent enzymes, all of the chimeric mutants expressed ~60% of constitutive activity (Fig. 4*C*). It is not surprising because Ile^{441}, which is critical for autoinhibition described above, was replaced by a basic residue (Arg or Lys) in all of the chimera mutants. In the presence of Ca^{2+}/CaM, all chimeric enzymes slightly enhanced the CaM-KK activity suggesting that the CaM-binding region of each CaM kinase slightly blocks the catalytic activity. This is consistent with that Ile^{441} mutants of CaM-KK, which exhibited 40–60% constitutive activities and were further activated by the addition of Ca^{2+}/CaM (Fig. 3*B*).

Finally we inserted Ile^{441} into the boundary between the catalytic domain and CaM-binding region of each chimeric CaM-KK to examine Ca^{2+}/CaM dependence of the mutants (Fig. 5*A*). As shown in Fig. 5*B*, all of the chimera CaM-KKs

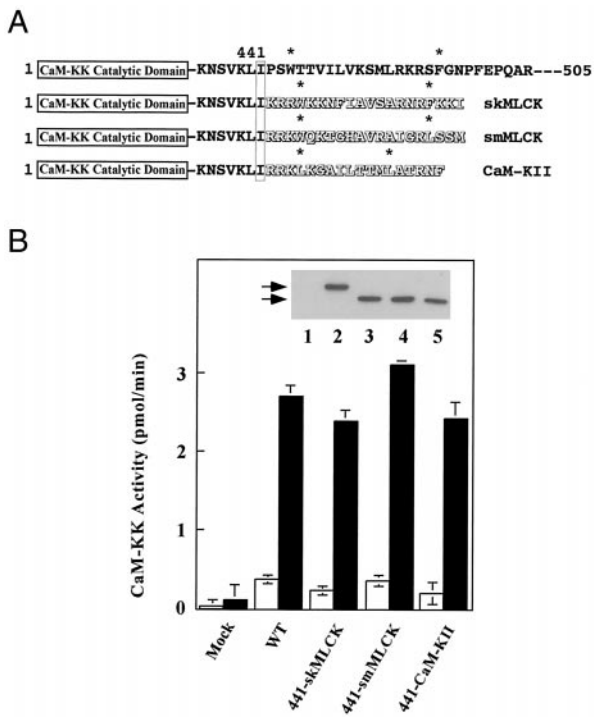

FIG. 5. **Effect of Ile^{441} insertion on the activities of CaM-KK chimera mutants.** *A*, schematic representation of wild-type and chimera mutant of αCaM-KK contained CaM-binding region of skeletal muscle MLCK (441-skMLCK), smooth muscle MLCK (441-smMLCK), or αCaM kinase II (441-CaM-KII) in which Ile^{441} is inserted. Chimera CaM-KKs containing Ile^{441} were constructed and expressed in COS-7 cells followed by partial purification as described under "Experimental Procedures." Ile^{441} in each chimera mutant is indicated by a *box*. Hydrophobic anchoring residues to N- and C-terminal domains of CaM are indicated by *asterisks*. *B,* partially purified CaM-KK chimera mutants (~0.4 μg of protein) including wild-type and Mock were incubated with GST-CaM-KI-(1–293, K49E, 0.4 mg/ml) in the either the absence (*open bar*) or presence (*closed bar*) of Ca^{2+}/CaM for 5 min at 30 °C as described under "Experimental Procedures." The experiment was performed in triplicate for each point, and the results are presented as the mean and S.E. of three experiments. *Inset*, partially purified CaM-KK chimera mutants (~3 μg pf protein) including wild-type and Mock were analyzed by Western blotting (indicated by *arrows*) as shown in Fig. 2*B*. *Lane 1,* mock; *lane 2,* wild-type CaM-KK; *lane 3,* 441-skMLCK; *lane 4,* 441-smMLCK; *lane 5,* 441-CaM-KII.

containing Ile^{441} are virtually inactive in the absence of Ca^{2+}/CaM and are activated by Ca^{2+}/CaM to the same extent to the wild-type CaM-KK. These results demonstrate that the orientation of Ca^{2+}/CaM binding to the CaM-binding region is not critical for the relief of CaM-KK autoinhibition.

Conclusion—The present mutagenesis identified Ile^{441} as a critical residue for autoinhibition of αCaM-KK located at −3 position from Trp^{444}, which is an anchoring residue to the N-terminal hydrophobic pocket of CaM. Three-dimensional structural analysis of Ca^{2+}/CaM binding (35–37) and sequence alignment of MLCKs and CaM-KII show that the residues at the −1 to −3 position from the N-terminal anchoring residue to CaM (Fig. 4*A*) are basic in all of them. These residues are critical for the determination of the direction in which CaM is binding, as they interact with more negatively charged C-terminal domain than N-terminal domain of CaM. In the case of CaM-KK, substitution of Ile^{441} by either Ala, Arg, or Asp resulted in increased constitutive activity (Fig. 3*B*), suggesting that a residue with a lower hydrophobicity at −3 position from Trp^{444} caused suppression of the autoinhibitory function. Thus, a basic residue should not be located at a key position for the autoinhibition in CaM-KK. In agreement with this, the cluster of basic amino acid is located at the C-terminal portion in

CaM-binding segment of CaM-KK (Lys^{451}, Arg^{455}-Lys^{456}-Arg^{457}), resulting in an opposite direction of Ca^{2+}/CaM binding compared with other CaM kinases such as MLCKs and CaM-KII (22), although the direction of Ca^{2+}/CaM binding itself is not critical for relief of the autoinhibition. This is supported by the result that all of the chimeric CaM-KKs lacking Ile^{441} exhibited enhanced Ca^{2+}/CaM-independent activity (Fig. 4). Kinetic analysis of CaM-KK peptide inhibition raises the possibility that Ile^{441} interacts with the ATP-binding domain directly and/or distort the ATP-binding site allosterically by interacting with the catalytic domain of CaM-KK. This might be analogous to Phe^{307} in the CaM-binding domain of CaM-KI that interacts with Phe^{31} in its glycine-rich ATP loop resulted in obstructing the nucleotide binding pocket (42). We propose that the Ca^{2+}/CaM-binding segment of CaM-KK with an opposite orientation of CaM binding to other CaM kinases plays a key role for its autoinhibitory function through Ile^{441}. Ile^{441} is likely the residue released from the catalytic domain upon binding of Ca^{2+}/CaM to the regulatory region. Future studies including structural determination will be necessary to establish the molecular mechanism of interaction between Ile^{441} and the catalytic domain of CaM-KK and to determine how this residue contributes to the autoinhibitory function of CaM-KK.

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