Substrate Recognition by Ca2+/Calmodulin-dependent Protein Kinase Kinase

ROLE OF THE ARG-PRO-RICH INSERT DOMAIN*

(Received for publication, September 17, 1998, and in revised form, February 15, 1999)

Hiroshi Tokumitsu‡§, Naomi Takahashi‡, Koh Eto‡, Shigetoshi Yano¶, Thomas R. Soderling¶, and Masa-aki Muramatsu‡‡

From the ‡Helix Research Institute, Inc., 1532-3 Yana, Kisarazu-shi, Chiba 292-0812, Japan, the ¶Vollum Institute, Oregon Health Sciences University, Portland, Oregon 97201, and the ¶Department of Biological Cybergenetics, Medical Research Institute, Tokyo Medical Dental University, Bunkyo-ku, Tokyo 113-8510, Japan

Mammalian Ca2+/CaM-dependent protein kinase kinase (CaM-KKK) has been identified and cloned as an activator for two kinases, CaM kinase I (CaM-KI) and CaM kinase IV (CaM-KIV), and a recent report (Yano, S., Tokumitsu, H., and Soderling, T. R. (1998) Nature 396, 584–587) demonstrates that CaM-KKK can also activate and phosphorylate protein kinase B (PKB). In this study, we identify a CaM-KKK from Caenorhabditis elegans, and comparison of its sequence with the mammalian CaM-KKK α and β shows a unique Arg-Pro (RP)-rich insert in their catalytic domains relative to other protein kinases. Deletion of the RP-domain resulted in complete loss of CaM-KIV activation activity and physical interaction of CaM-KKK with glutathione S-transferase-CaM-KIV (T196A). However, CaM-KKK autophosphorylation and phosphorylation of a synthetic peptide substrate were normal in the RP-domain mutant. Site-directed mutagenesis of three conserved Arg in the RP-domain of CaM-KKK confirmed that these positive charges are important for CaM-KIV activation. The RP-domain deletion mutant also failed to fully activate and phosphorylate CaM-KI, but this mutant was indistinguishable from wild-type CaM-KKK for the phosphorylation and activation of PKB. These results indicate that the RP-domain in CaM-KKK is critical for recognition of downstream CaM-kinases but not for its catalytic activity (i.e. autophosphorylation) and PKB activation.

Ca2+/calmodulin-dependent protein kinases (CaM-Ks) constitute a diverse group of enzymes which are involved in many aspects of calcium signaling such as neurotransmitter release, excitation-contraction coupling in muscle, and gene expression (1–4). Recent studies have demonstrated that two CaM-kinases, CaM-KI and -IV, are activated through phosphorylation by an upstream CaM kinase kinase (CaM-KKK) (5–12), analogous to other kinase cascades such as PKA/phosphorylase kinase (13), MAP kinase (14), and AMP kinase (15). CaM-KK is a recently cloned protein kinase that phosphorylates and activates CaM-KI and CaM-KIV, constituting the CaM-K cascade (11, 39, 47). Like other CaM-Ks, CaM-KK is negatively regulated by an intrastressor mechanism through its autoinhibitory domain (residue 436–441) and activated by the Ca2+/CaM complex (16). Ca2+/CaM binding to both CaM-KK and its downstream target CaM-Ks are required to activate the CaM kinase cascade (6, 7, 12). This CaM-K cascade has been functionally demonstrated for CaM-KIV activation in response to Ca2+ mobilization using transfected COS-7 cells (12), Jurkat cells (17), and cultured hippocampal neurons (18) and for CaM-KI activation in PC-12 cells upon membrane depolarization (19). The CaM-KK/CaM-KIV cascade can stimulate gene transcription through phosphorylation of Ser133 in cAMP response element-binding protein, and this may play an important physiological role in learning and memory (11, 18, 20–23). It has also been demonstrated that the MAP kinase pathways, especially JNK and p38, may be indirectly activated by the CaM-KK/CaM-KIV cascade (24). Recently, evidence has been provided that CaM-KKK may mediate the anti-apoptotic effect of modest elevations of Ca2+ through phosphorylation and activation of protein kinase B (PKB) (25). This result also indicates that multiple protein kinases might be phosphorylated and activated by CaM-KKK, resulting in regulation of a wide variety of functions.

The activation sites in CaM-KI (Thr117) (7), CaM-KIV (Thr196) (6, 12), and PKB (Thr208) (25) which are phosphorylated by CaM-KKK are located in their “activation loops” analogous to Thr197 in PKA (26) and Thr160 in cdk2 (27). For most protein kinases the substrate recognition determinants are located just NH2- and COOH-terminal of the phosphorylated Ser/Thr. For example, many substrates of PKA have basic residues at position 2 and 3 NH2-terminal of the phosphorylated Ser, and these basic residues are recognized by Glu127 and Glu170 in PKA (33–37). However, there is little sequence similarities in the NH2-terminal sequences of the activation sites of CaM-KI, CaM-KIV, or PKB. There is considerable COOH-terminal sequence identity, but these sequences are also conserved in many other kinases that have activation loops but are not phosphorylated by CaM-KKK. This suggests that CaM-KKK has specific mechanisms to recognize its target kinases other than the primary sequence surrounding the phosphorylated Thr. Although CaM-KKK can phosphorylate a synthetic peptide corresponding to the sequence in the CaM-KIV activation loop (KKKEQVLMKQ196VGGTY), very high concentrations of the peptide are required (28). In this report, we explored the mechanisms of selective substrate recognition of CaM-KKK by identifying a unique kinase insert domain which is involved in its interaction and activation of CaM-KI and IV but not PKB.
Experimental Procedures

Materials—CaM-KK cDNA (GenBank accession number L24810) was from a rat brain cDNA library (11). Recombinant CaM-KIV was expressed in SF9 cells and purified as described previously (21). Recombinant CaM-KKs were expressed in Escherichia coli BL-21 (DE3) pLysS (R) with pET16b vector and purified by CaM-Sepharose as described previously (12). CaM-KI was purified from bovine brain (29). CaM-KIV peptide (KKERHHQVMLGCTCTGPGY) (28) was synthesized by the Bio-Synthesis, Inc. Caenorhabditis elegans CaM-KK cDNA was cloned by RT-PCR by using a sense oligonucleotide (5'-TATTGGATTGGTACAATCCGGTTCCGAGGCT-3'), and an antisense oligonucleotide (5'-AGACTGTATTTACCTGGAATGTTGACCGCAA-CGGCTTGGCA-3') based on the sequence of C. elegans cosmid (Cos5H1.1) and reverse transcribed DNA from mRNA of N2 stage C. elegans as a template, which was kindly provided from Dr. Shouhei Mitani (Tokyo Woman's Medical College). A 1.1-kilobase pair of PCR fragment was digested with XhoI and SpeI and then inserted into pME18s vector. Nucleotide sequence of cloned C. elegans CaM-KK cDNA (GenBank accession number AB16838) was confirmed and the deduced amino acid sequence (357 amino acids) was completely matched with predicted coding sequence using the program Genefinder (Promega Co.) or QUANT-ESSENTIAL™ (QUANTUM Biotechnologies Institute, Inc.) or CaM-KK cDNA containing plasmid DNAs and 60 ng of pME18s plasmid DNA (DNAX Research Laboratories) was used as a template for Site-Directed Mutagenesis System (Promega Co.) or QUANT-ESSENTIAL™ (QUANTUM Biotechnologies Inc.) and mutagenic oligonucleotides as follows; P327E, 5'-CCTCCTGGAAGAAAGGAGTCAATGGAAGTGGC-3', P327V, 5'-CCTCCTGGAAGAAAGGAGTCAATGGAAGTGGC-3', P327A, 5'-CCTCCTGGAAGAAAGGAGTCAATGGAAGTGGC-3', S279E, 5'-AGGGACATCAAACGCCAGAAATCTGCCTCCTGGG-3', S279D, 5'-AGGGACATCAAACGCCAGAAATCTGCCTCCTGGG-3', S279G, 5'-AGGGACATCAAACGCCAGAAATCTGCCTCCTGGG-3', 278A, 5'-AGGGACATCAAACGCCAGAAATCTGCCTCCTGGG-3'. Nucleotide sequences of each mutant were confirmed by automated sequencing using an Applied Biosystems 377 DNA sequencer.

Transient Expression of CaM-KK Mutants—COS-7 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Cells were subcultured in 10-cm dishes 12 h before transfection. The cells were transfected by using LipofectAMINE Reagent (Life Technologies, Inc.) in 6 ml of medium. After 32-48 h incubation, the cells 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/ml leupeptin, 10 mg/ml pepstatin A, 1 ml/liter trypsin inhibitor) at 4°C. After centrifugation at 15,000 × g for 15 min, the supernatant was used for CaM-KIV activation assay and quantitated by Western blotting.

In Vitro Assay of CaM Kinase Activation by CaM-KK—Either COS-7 cell extract (0.6 μg) transfected with CaM-KK or E. coli expressed CaM-KK was incubated with either recombinant CaM-KIV (3.4 μM) or GST-CaM-KI (1 μM) at 50°C in 50 mM HEPES (pH 7.5), 10 mM MgAc2, 1 mM DTT, 400 μM ATP, and 2 mM CaCl2, 8 μM CaM. The reaction was terminated at the indicated time points by a 20-fold dilution at 4°C with 50 mM HEPES (pH 7.5), 2 mM mg/ml bovine serum albumin, 10% ethylene glycol, and 2 mM EDTA. CaM-KIV (34 nM) activity was measured at 30°C for 5 min in a 25-μl assay containing 50 mM HEPES (pH 7.5), 10 mM MgAc2, 1 mM DTT, 400 μM [γ-32P]ATP (1000–2000 cpm/pmol), 40 μM syntide-2, and 2 mM EGTA (Ca2+/CaM-independent activity). CaM-KI activity (1 μg/ml) was measured at 30°C for 5 min in a 25-μl assay containing 50 mM HEPES (pH 7.5), 10 mM MgAc2, 1 mM DTT, 400 μM [γ-32P]ATP (1000–2000 cpm/pmol), 40 μM syntide-2, and 2 mM CaCl2, 8 μM CaM. The reaction was initiated by the addition of CaM-KIV or CaM-KI and terminated by spotting aliquots (15 μl) onto phosphocellulose paper (Whatman P-81) followed by washing in 75 mM phosphoric acid (32). CaM-KK activity is expressed in terms of its ability to increase either Ca2+/CaM-independent activity of CaM-KIV or Ca2+/CaM dependent activity of CaM-KI under the defined assay conditions. The data of CaM-KIV activation are expressed as a percentage of the value of incubation of recombinant CaM-KIV with wild-type CaM-KK for 5 min which was calculated to be approximately 0.5 μmol/min/mg. The data of CaM-KI activation are expressed as a percentage of the value of incubation of recombinant CaM-KI with wild-type CaM-KK for 5 min which was calculated to be approximately 4.0 μmol/min/mg.

PKB Activation by CaM-KK—GST-rat PKB (α isoform) was transiently expressed in COS-7 cells with pME18s vector as described above and purified on glutathione-Sepharose as described previously (25). Purified GST-PKB (0.1 μg) was incubated for the indicated times with 50 mM HEPES (pH 7.5), 10 mM MgAc2, 1 mM DTT, 1 mM CaCl2, 3 μM CaM, 0.2 mg/ml histone H2B, 400 μM [γ-32P]ATP (1000 cpm/pmol), and approximately 100 ng of wild-type, mutant CaM-KK, or buffer. After terminating the reaction by addition of SDS-PAGE sample buffer, 32P incorporation into GST-PKB and histone H2B was analyzed by SDS-15% PAGE followed by autoradiography and then quantitated by densitometric scanning. The data of GST-PKB activity are expressed as fold of control value of 32P incorporation into histone H2B by recombinant GST-PKB with wild-type CaM-KK at 0 min.

CaM-KIV Peptide Phosphorylation Assay—CaM-KIV peptide phosphorylation activity of CaM-KIV was measured at 30°C for 10 min in a 25-μl assay containing 50 mM HEPES (pH 7.5), 10 mM MgAc2, 1 mM DTT, 400 μM [γ-32P]ATP (1000–2000 cpm/pmol), 400 μM CaM-KIV peptide and either one of 1 mM EGTA or 1 mM CaCl2, 4 μM CaM using 5 μl of partially purified CaM-KKs.

Autophosphorylation of CaM-KK—Autophosphorylation reaction was essentially the same as CaM-KIV peptide phosphorylation assay described above except for 100 μM [γ-32P]ATP (1000–2000 cpm/pmol) and 5 μg of CaM-KK peptide substrate. After terminating the reaction by addition of 5 μl of SDS-PAGE sample buffer, samples were loaded onto SDS-10% PAGE and then subjected to autoradiography.

Binding of CaM-KK with GST-CaM-KIV (T196A)—After 25 μl of glutathione-Sepharose (50 μl of 50% slurry) was loaded with either buffer or 0.5 μg of GST-CaM-KIV (T196A) which was purified by CaM-Sepharose, the resin was washed three times with 1 ml of phosphate-buffered saline and then washed three times with 1 ml of 50 mM HEPES (pH 7.5), 2 mM CaCl2, and 10 mM MgAc2. Equal amounts (approximately 200 ng) of either wild-type or mutant CaM-KK were applied to the resin in a solution (100 μl) containing 50 mM HEPES (pH 7.5), 2 mM CaCl2, 10 mM MgAc2, 5 μg CaM, and 1 mM ATP and then incubated for 1 h at room temperature. These resins were washed three times with 500 μl of 50 mM HEPES (pH 7.5), 2 mM CaCl2, 10 mM MgAc2, 1 μM CaM at 4°C and then GST-CaM-KIV was eluted with 80 μl of 50 mM Tris-HCl (pH 8.0) and 10 mM glutathione. Equal volumes of samples were subjected to SDS-7.5% PAGE followed by Western blotting by using both anti-CaM-KIV antibody or anti-CaM-KK antibody.

Others—Western blotting was carried out using antiserum (1/1000 dilution) against a phosphorylated synthetic substrate motif (residues 132–146 of CaM-KII), anti-CaM-KIV antibody (Transduction Laboratories), or anti-CaM-KK antibody (Santa Cruz Biotechnology, Transduction Laboratories), and the biotinylated-CaM overlay was done as described previously (10). Detection was performed by using chemiluminescence reagent (NEN Life Science Products Inc.). Protein concentration was estimated by Coomassie dye binding (Bio-Rad) using bovine serum albumin as a standard.

Substrate Recognition of CaM Kinase Kinase

Site-Directed Mutagenesis System (Promega Co.) using bovine serum albumin as a standard.

Western Blotting was carried out using antiserum (1/1000 dilution) against a phosphorylated synthetic substrate motif (residues 132–146 of CaM-KII), anti-CaM-KIV antibody (Transduction Laboratories), or anti-CaM-KK antibody (Santa Cruz Biotechnology, Transduction Laboratories), and the biotinylated-CaM overlay was done as described previously (10). Detection was performed by using chemiluminescence reagent (NEN Life Science Products Inc.). Protein concentration was estimated by Coomassie dye binding (Bio-Rad) using bovine serum albumin as a standard.
RESULTS

Identification and Characterization of *C. elegans* CaM-KK—A protein kinase gene was recently identified in the *C. elegans* genome data base that is highly homologous to rat CaM-KK (30, 47). To determine whether this clone encodes CaM-KK, cDNA of 1074 bp was cloned by RT-PCR from *C. elegans* mRNA (Fig. 1A). In order to ensure that the methionine at position 1 is the point of translation initiation, we also cloned the 5'-untranslated region by RT-PCR using two nucleotide sequences, 200 bp upstream in *C. elegans* genome sequence and 353 bp downstream from the first Met (ATG), as PCR primers (Fig. 1A, underlined). The nucleotide sequence of the PCR product (553 bp) includes 200 bp of the 5'-untranslated region containing three in-frame stop codons and the remaining 353-bp region is completely matched with its open reading frame from the first ATG. Since the antisense primer is located in the third exon, this PCR product is derived from *C. elegans* mRNA and the Met at position 1 is likely the translation initiation. The full-length clone encoded a protein of 357 amino acids with a calculated molecular mass of 40,701 (Fig. 1A). This protein is likely the *C. elegans* homologue of CaM-KK as it contains the unique RP-rich insert (residues 60-81) in the catalytic domain highly homologous to similar inserts in the α (11) and β (39, 47) isoforms of mammalian CaM-KK. The *C. elegans* clone was transiently expressed in COS-7 cells, and a CaM overlay in the presence of Ca$^{2+}$/CaM (Fig. 1B, inset panel) identified a 45-kDa protein which is smaller than the mammalian α CaM-KK. COS-7 cell extracts from mock transfected or transfected with plasmids expressing rat CaM-KK (●) or *C. elegans* CaM-KK (□). After terminating the reaction, Ca$^{2+}$/CaM independent activity of CaM-KIV was measured and is expressed as a percentage of the 10-min value for α CaM-KK. Each extract (18 μg) was subjected to SDS-10% PAGE, transferred onto nitrocellulose membrane (Hybond-C, Amersham), and analyzed by CaM overlay (panel B, inset). C, requirement of Ca$^{2+}$/CaM for *C. elegans* CaM-KK activity. Either wild-type (left panel) or constitutively active mutant (316FN-DD, right panel) of mouse CaM-KIV was activated with extracts from COS-7 cells either mock-transfected (●) or transfected with a plasmid expressing *C. elegans* CaM-KK (□) for 15 min as described in panel B in the absence (●) or presence (□) of 2 mM CaCl$_2$, 10 μM CaM. After terminating the reaction, Ca$^{2+}$/CaM independent activity of each CaM-KIV was measured and is expressed as a percentages of the value in the presence of *C. elegans* CaM-KK and Ca$^{2+}$/CaM. Results represent mean and S.E. of three experiments.

---

**FIG. 1.** Cloning and characterization of *C. elegans* CaM-KK. A, nucleotide and deduced amino acid sequence of *C. elegans* CaM-KK. *C. elegans* CaM-KK cDNA was cloned by RT-PCR using reverse transcribed DNA from mRNA of N2 stage *C. elegans* as a template (see “Experimental Procedures”). Nucleotide sequence of the 5'-untranslated region of the cDNA cloned by RT-PCR is combined. Underlines indicate the sequence of PCR primers for amplification of the 5'-untranslated region. In-frame stop codons are indicated with an asterisk. CaM-binding site is shown by a box (16). B, activation of CaM-KIV by *C. elegans* CaM-KK. Recombinant mouse CaM-KIV (3.4 μM) was incubated at 30 °C for the indicated times in the presence of Ca$^{2+}$/CaM (see “Experimental Procedures”) with extracts (0.6 μg) from COS-7 cells either mock-transfected (●) or transfected with plasmids expressing rat αCaM-KK (○) or *C. elegans* CaM-KK (□). After terminating the reaction, Ca$^{2+}$/CaM independent activity of CaM-KIV was measured and is expressed as a percentage of the 10-min value for α CaM-KK. Each extract (18 μg) was subjected to SDS-10% PAGE, transferred onto nitrocellulose membrane (Hybond-C, Amersham), and analyzed by CaM overlay (panel B, inset).
with vectors encoding mammalian αCaM-KK or C. elegans protein were tested for their abilities to activate recombinant mammalian CaM-KIV. The αCaM-KK protein gave activation of CaM-KIV which was about 2–3-fold less efficient than the mammalian CaM-KK in the initial rate of activation (Fig. 1B).

Next we tested Ca^2+/CaM dependence of the C. elegans enzyme by using both wild-type CaM-KIV (Fig. 1C, left panel) and constitutively active mutant of CaM-KIV (S199A, Fig. 1C, right panel) (10) which has shown to be activated by the constitutively active form of rat αCaM-KK (1–434) in a Ca^2+/CaM-independent manner (16). As shown in Fig. 1C, both wild-type and mutant CaM-KIVs are activated by the C. elegans enzyme in a complete Ca^2+/CaM-dependent manner suggesting that C. elegans enzyme requires Ca^2+/CaM for its activity as well as the mammalian CaM-KK (11). Furthermore, the COOH-terminal end of the C. elegans clone (residues 331–356, Fig. 1A) is 54% identical to the CaM-binding domain of αCaM-KK (16). We have also detected Ca^2+-dependent CaM binding of the syn-
thetic peptide corresponding to residues 331–356 in the \textit{C. elegans} clone (data not shown) similar to that of a CaM-binding peptide of aCaM-KK (16). These biochemical criteria confirm that the protein is the \textit{C. elegans} homologue of CaM-KK.

Acidic Residues Are Not Essential for CaM-KK Substrate Recognition—The mechanism of substrate recognition by CaM-KK has not been studied. To determine critical residues or domains for substrate recognition in CaM-KK, we first aligned and compared the amino acid sequences of the catalytic domains of mammalian \( \alpha \) and \( \beta \) CaM-KK and \textit{C. elegans} CaM-KK with mammalian CaM-KII and cAMP-dependent protein kinase (PKA), the best protein kinase for enzyme-substrate recognition mechanisms (Fig. 2A). According to the studies of: 1) the crystal structure of PKA with PKI (33, 34); 2) charged to alanine mutagenesis of the yeast homologue of PKA (35); and 3) labeling with a water-soluble carbodiimide (36), acidic residues Glu127 and Glu170 interact with the Arg in the P-2 and P-3 positions of PKA substrates. It has been shown that mutation of these Glu residues in PKA affect its \( K_m \) with the peptide substrate Kemptide (35, 37). Residues equivalent to Glu\(^{127} \) and Glu\(^{170} \) are conserved in Arg/Lys-directed CaM kinases (myosin light chain kinase, CaM-KI, -II, -IV, and phosphorylase kinase). Residues Pro\(^{237} \) and Ser\(^{279} \) in rat CaM-KK are analogous to Glu\(^{127} \) and Glu\(^{170} \) in PKA (Fig. 2A). Therefore, we initially mutated Pro\(^{237} \) to Glu, Val, or Ala and Ser\(^{279} \) to Glu, Asp, or Ala to test whether these residues are involved in CaM-KK activity. Mutant rat constructs, including wild-type \( \alpha \) CaM-KK, were transfected into COS-7 cells, and the expression level of CaM-KK in the cells were quantitated by immuno blotting (Fig. 2, panel B and C). All of the Pro\(^{237} \) and Ser\(^{279} \) mutants were expressed to an extent similar to wild-type enzyme. The same amount of CaM-KK mutants as wild-type enzyme were used for CaM-KIV activating assays in the presence of Ca\(^{2+}/\text{CaM} \). The time course of CaM-KIV activation by all the Pro\(^{237} \) and Ser\(^{279} \) CaM-KK mutants were similar to wild-type CaM-KK (\( t_{1/2} = 1 \text{ min} \)) (Fig. 2, B and C). Our results...
The RP-domain Is Essential for CaM-KIV Activation by CaM-KK—The function of the unique RP-domain insert in all cloned CaM-KKs (Fig. 2A) is completely unknown. To analyze its function, the RP-domain (residues 167–189) was deleted from αCaM-KK, and this mutant was expressed in COS-7 cells (Fig. 3A). As expected, deletion of the RP-domain from CaM-KK gave a slightly faster migrating species than wild-type enzyme (68 kDa) on SDS-PAGE. Surprisingly, the RP-deletion mutant was completely unable to activate CaM-KIV (Fig. 3A). The critical role of the RP-domain was further analyzed by site-directed mutation of Arg172 and Arg177, which are conserved among all three CaM-KKs. Mutation of both Arg172 and Arg177 to Glu resulted in significant reduction of CaM-KK activity. Ala mutation was less defective (t1/2 = 4 min) compared with either RP-domain deletion or Glu mutants (Fig. 3A). Single Glu mutation of either Arg172 and Arg177 revealed that both residues are required for the maximum activity of CaM-KK (Fig. 3B). Although mutation of Arg172 has a more significantly affect on the catalysis than that of Arg177, both residues seem to synergistically contribute to CaM-KK activity. Another Arg residue at 173 is also conserved in α and β isoforms of CaM-KK, but replaced by Gln66 in C.elegans enzyme (Fig. 2A). Since C. elegans CaM-KK is a 2–3-fold less efficient activator than rat CaM-KK toward mammalian CaM-KIV (Fig. 1B), we tested the possibility that Arg173 contributes to the catalytic efficiency (Fig. 3C). Glu mutation on Arg173 gave a significant reduction of the initial rate of CaM-KIV activation. However, when we mutated Gln66 in C. elegans CaM-KK to Arg, we could not detect a significant increase in CaM-KK activity (data not shown). This is consistent with the mutation of Arg173 in rat CaM-KK to a neutral amino acid residue, such as Ala, which caused little effect on CaM-KK activity.

Next we tested whether the RP-deletion mutant could physically interact with CaM-KIV using GST-CaM-KIV (T196A). The rational for using the T196A mutant, which cannot be activated by CaM-KK, is that it may capture the intermediate complex form between CaM-KK and CaM-KIV. GST-CaM-KIV (T196A) loaded glutathione-Sepharose was mixed with either the wild-type or RP-deletion mutant of CaM-KK, and a pull-down experiment was performed in essentially the same condition as an activation reaction with low ionic strength. As shown in Fig. 3D, association of wild-type CaM-KK with GST-CaM-KIV (T196A) was readily detected which was approximately 10% of applied CaM-KK, and the interaction occurred in a Ca2+/CaM-dependent manner (data not shown). Deletion of the RP-domain resulted in a loss of interaction to CaM-KIV compared with the wild-type CaM-KK.

RP-domain Mutants of CaM-KK Are Catalytically Active—To test whether these RP-domain mutants are catalytically defective, we measured their abilities to autophosphorylate. As shown in Fig. 4A, all of the RP-domain mutants showed Ca2+/calmodulin-dependent autophosphorylation similar to wild-type kinase although the R172A, R177A mutant gave a slightly weaker activity as compared with others. As a second test of catalysis, we determined their abilities to phosphorylate the CaM-KIV activation domain sequence (KKKEHQVLMKT-196′GCTGPGY) (28) which contains the phosphorylation-activation Thr196 in CaM-KIV. Again, all of the mutants including the RP-domain deletion could phosphorylate CaM-KIV peptide in a Ca2+/CaM-dependent manner (Fig. 4B). In these experiments, we used recombinant mutant enzymes expressed in E. coli and partially purified by CaM-Sepharose instead of using transfected COS-7 extract. CaM-KIV activating and phosphorylating activity of both wild-type and RP-domain deletion mutants of E. coli expressed CaM-KK was shown to be essentially the same as COS-7 cell expressed enzyme (see Fig. 5B). We also checked CaM-KIV activating activity of other E. coli-expressed CaM-KK mutants with the same conditions as shown in Fig. 3A with triplicate experiments and obtained essentially the same results as that obtained with COS-7 cell-expressed enzymes (data not shown). These results indicate that deletion and mutations on the RP-domain do not affect catalytic activity with regard to autophosphorylation and peptide phosphorylation. Also, the regulatory mechanism of CaM-KK, such as autoinhibition and CaM binding, remained intact with these mutations and deletions.

Requirements of the RP-domain for Activation of CaM-Kases but Not for PKB—Although the RP-domain mutants can catalyze Ca2+/calmodulin-dependent autophosphorylation and phosphorylation of a synthetic peptide, we wanted to test other physiological substrates. Two such substrates are CaM-KI and PKB. CaM-KI is phosphorylated on Thr177 resulting in its activation (7). As shown in Fig. 5A, recombinant GST-CaM-KI

![Image](330x332 to 532x729)

**Fig. 4.** Catalytic activity of CaM-KK RP-domain mutants. A, autophosphorylation of αCaM-KK. Equal amounts of E. coli-expressed wild-type or the indicated rat CaM-KK mutants were subjected to autophosphorylation conditions in the presence or absence of Ca2+/CaM (see "Experimental Procedures") and subjected to SDS-10% PAGE and autoradiography. B, E. coli-expressed rat αCaM-KK was partially purified by CaM-Sepharose and assayed (50 ng each) for phosphorylation of CaM-KIV peptide (400 µM) in the absence (open bar) or presence (solid bar) of Ca2+/CaM for 10 min (see "Experimental Procedures"). Results represent mean and S.E. of three experiments.
was activated and phosphorylated by wild-type CaM-KK as described previously (11). However, activation and phosphorylation of GST-CaM-KI by the RP-deletion mutant of CaM-KK was strongly reduced compared with wild-type (Fig. 5A) in a manner similar to CaM-KIV (Fig. 5B). PKB can be activated through phosphorylation of its activation loop Thr308 by PDK1 (40) and CaM-KK (25). In contrast to CaM-KI and CaM-KIV, PKB was activated and phosphorylated by the RP-deletion mutant of CaM-KK in a manner comparable to that obtained with wild-type CaM-KK (Fig. 5C) while the rate of activation of PKB by CaM-KI was lower than that of CaM-KI or CaM-KIV which is consistent with previous observations (25). This indicates that the RP-domain deletion mutant is catalytically active and recognizes PKB as its substrate.

**DISCUSSION**

In this study we demonstrated that the RP-domain of CaM-KK, which is conserved in all three known CaM-KKs, is required for selective substrate recognition. Thus, deletion of the RP-domain obviated CaM-KK activity toward its two classical substrates CaM-KI and CaM-KIV. Mutational analysis indicated that the three highly conserved Arg in rat CaM-KK, Arg172, Arg173 and Arg177 are essential in this substrate recognition. In contrast, the RP-deletion mutants showed normal autophosphorylation, phosphorylation of a synthetic substrate containing the activation site of CaM-KIV, and, more importantly, in phosphorylation and activation of PKB. Since these reactions were Ca2+/CaM-dependent, these results indicate that the RP-domain is involved in recognition of CaM-KI and CaM-KIV as substrates. This conclusion is substantiated by the fact that the GST fusion of CaM-KIV could pull down wild-type CaM-KK whereas the RP-domain deletion mutant was not able to interact with the GST fusion of CaM-KIV. The fact that RP-domain mutants could phosphorylate CaM-KIV peptide but not CaM-KIV itself is perhaps consistent with the report (28) that the CaM-KIV peptide (Km = 263 μM) is a very poor substrate for CaM-KK as compared with CaM-KIV (Km = 0.71 μM). Therefore, the recognition determinants of CaM-KK toward this peptide substrate are likely to be quite different than for the full-length CaM-KIV. The RP-domain of CaM-KK is localized between subdomains II and III in the catalytic domain, placing it between α-helices B and C in PKA. It is conceivable that this domain may recruit and stabilize the downstream CaM-KI and -IV to maintain proper orientation toward the catalytic cleft, resulting in high affinity and specific interaction between CaM-KK and these substrates. Interestingly, we could not detect significant interaction between CaM-KIV and a synthetic peptide corresponding to the RP-domain by using a direct binding assay or an inhibition assay for CaM-KIV activation, suggesting that domain addition to the RD-domain might be required for the interaction (data not shown).
CaM-KK has a Pro237 and Ser279 in positions analogous to positions in the activation loops of CaM-KI, -IV, and PKB, and however, there is no obvious consensus basic residues at those positions. Mutations in the activation loop of MAP kinases has little effect on phosphorylation and activation of CaM-KI and -IV. Based on a limited number of mutants, we cannot rule out the possibility that other residues or regions are involved in substrate recognition by CaM-KK. In this regard, our results are consistent with a study showing that mutations in the activation loop of MAP kinases has little effect on their recognition as substrates for their upstream activating MAP kinase kinases.

REFERENCES

1. Nairn, A. C., Hemmings, H. C., and Greengard, P. (1985) Annu. Rev. Biochem. 54, 931–970
2. Edelman, A. M., Blumenthal, D. K., and Krebs, E. G. (1987) Annu. Rev. Biochem. 56, 567–613
3. Braun, A. P., and Schulman, H. (1995) Annu. Rev. Physiol. 57, 417–445
4. Soderling, T. R. (1996) Biochim. Biophys. Acta 1297, 131–138
5. Lee, J. C., and Edelman, A. M. (1994) J. Biol. Chem. 269, 2158–2164
6. Selbert, M. A., Anderson, K. A., Huang, Q.-H., Goldstein, E. G., Means, A. R., and Edelman, A. M. (1995) J. Biol. Chem. 270, 17616–17621
7. Haribabu, B., Hook, S. S., Selbert, M. A., Goldstein, E. G., Tomhave, E. D., Edelman, A. M., Synderman, R., and Means, A. R. (1995) EMBO J. 14, 3679–3686
8. Okuno, S., and Fujisawa, H. (1993) J. Biochem. (Tokyo) 114, 167–170
9. Okuno, S., Kitani, T., and Fujisawa, H. (1994) J. Biochem. (Tokyo) 116, 923–930
10. Tokumitsu, H., Brickley, D. A., Glod, J., Hidaka, H., Sikela, J., and Soderling, T. R. (1994) J. Biol. Chem. 269, 28640–28647
11. Tokumitsu, H., Ensen, H., and Soderling, T. R. (1995) J. Biol. Chem. 270, 19320–19324
12. Tokumitsu, H., and Soderling, T. R. (1996) J. Biol. Chem. 271, 5617–5622
13. Walsh, D. A., Perkins, J. P., Brustrom, C. O., Ho, E. S., and Krebs, E. G. (1971) J. Biol. Chem. 246, 1968–1976
14. Nakielny, S., Cohen, P., Wu, J., and Sturgill, T. (1992) EMBO J. 11, 2123–2129
15. Weekes, J., Hawley, S. A., Corton, J., Shugar, D., and Hardie, D. G. (1984) Eur. J. Biochem. 139, 731–739
16. Tokumitsu, H., Wayman, G. A., Muramatsu, M., and Soderling, T. R. (1997) Biochemistry 36, 12823–12827
17. Park, I.-K., and Soderling, T. R. (1995) J. Biol. Chem. 270, 30464–30469
18. Bito, H., Deisserrth, K., and Tsien, R. W. (1996) Cell 87, 1203–1214
19. Alleta, J. M., Selbert, M. A., Nairn, A. C., and Edelman, A. M. (1996) J. Biol. Chem. 271, 29030–29034
20. Sun, P., Ensen, H., Myung, P. S., and Maurer, R. A. (1994) Genes Dev. 8, 2527–2539
21. Enslen, H., Sun, P., Brickley, D., Soderling, S. H., Klimo, E., and Soderling, T. R. (1994) J. Biol. Chem. 269, 15520–15527
22. Matthews, R. P., Guthrie, C. R., Waiers, L. M., Zhou, X., Means, A. R., and McKnight, G. S. (1984) Mol. Cell. Biol. 14, 6107–6116
23. Enslen, H., Tokumitsu, H., and Soderling, T. R. (1995) Biochem. Biophys. Res. Commun. 207, 1038–1043
24. Enslen, H., Tokumitsu, H., Stork, P. J. S., Davis, R. J., and Soderling, T. R. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 10803–10808
25. Tany, S., Tokumitsu, H., and Soderling, T. R. (1996) Nature 396, 584–587
26. Shoji, S., Titani, K., Demaillie, J. G., and Fischer, E. H. (1979) J. Biol. Chem. 254, 6211–6214
27. Desai, D., Gu, Y., and Morgan, D. O. (1992) Mol. Cell. Biol. 3, 571–582
28. Okuno, S., Kitani, T., and Fujisawa, H. (1997) J. Biochem. (Tokyo) 122, 337–343
29. Gopalakrishna, R., and Anderson, W. B. (1982) Biochem. Biophys. Res. Comm. 104, 380–386
30. Edelman, A. M., Mitchelli, K. I., Selbert, M. A., Anderson, K. A., Hook, S. S., Stapleton, D., Goldstein, E. G., Means, A. R., and Kemp, B. E. (1996) J. Biol. Chem. 271, 10806–10816
31. Cho, F. S., Phillips, K. S., Bogucki, B., and Weaver, T. E. (1994) Biochem. Biophys. Acta 1224, 156–160
32. Rozowski, R. (1985) Methods Enzymol. 99, 3–6
33. Knighton, D. R., Zheng, L., Ten Eyk, L. F., Ashford, V. A., Xiong, X. H., Taylor, S. S., and Sowadski, J. M. (1991) Science 253, 407–414
34. Knighton, D. R., Zheng, L., Ten Eyk, L. F., Xiong, X. H., Taylor, S. S., and Sowadski, J. M. (1991) Science 253, 414–420
35. Gibbs, C. S., and Zoller, M. J. (1991) J. Biol. Chem. 266, 8923–8931
36. Buechler, J. A., and Taylor, S. S. (1990) Biochemistry 29, 1957–1943
37. Takada, T., Murata, A., Ariy, K., and Muramatsu, M. (1995) Biochem. Biophys. Acta 1175, 333–342
38. Deleted in proof
39. Kitani, O., Okuno, S., and Fujisawa, H. (1997) J. Biochem. (Tokyo) 122, 243–250
40. Alessi, D. R., James, S. R., Downes, C. P., Hensley, S., Daffne, B. S., and Cohn, P. (1997) Curr. Biol. 7, 261–269
41. Puglisi, G., DeMagistris, P., Finocchiaro, G., Ognibene, S., Mignardi, G., and Macchiarini, G. (1999) J. Biol. Chem. 274, 16846–16853
42. Pullen, S. A., and Schulman, H. (1995) Science 270, 707–710
43. Lin, C. R., Kapfhol, M. S., Duriegan, K., Tomiato, K., Ruso, A. F., Hanson, H., Schulman, H., and Rosenfeld, M. G. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 5962–5966
44. Uhler, M. D., Carmichael, D. F., Lee, D. C., Chiriva, J. C., Krebs, E. G., and McKnight, G. S. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 1300–1304
45. Hanks, S. K., Quinn, A. M., and Hunter, T. (1988) Science 241, 42–52
46. Robinson, M. J., Cheng, M., Khokhlatcheva, A., Ebert, D., Ahn, N., Guan, K. L., Stein, B., Goldsmith, E., and Cobb, M. H. (1996) J. Biol. Chem. 271, 29754–29769
47. Anderson, K. A., Means, R. L., Huang, Q.-H., Kemp, B. E., Goldstein, E. G., Selbert, M. A., Edelman, A. M., Fremeau, R. T., and Means, A. B. (1998) J. Biol. Chem. 273, 31880–31889

---

S. Yano and T. R. Soderling, unpublished observation.