Ca^{2+}/Calmodulin-dependent Protein Kinase Cascade in Caenorhabditis elegans

IMPLICATION IN TRANSCRIPTIONAL ACTIVATION*

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Koh Eto‡§, Naomi Takahashi‡, Yoshishige Kimura‡, Yasuhiko Masuho‡, Ken-ichi Arai‡, Masa-aki Muramatsu‡, and Hiroshi Tokumitsu‡

From the ‡Helix Research Institute, Inc., 1532-3 Yana, Kisarazu-shi, Chiba 292-0812, the §Department of Molecular and Developmental Biology, Institute of Medical Science, University of Tokyo Shirokanedai, Minato-ku, Tokyo 108-0071, and the ¶Department of Biological Cybergenetics, Medical Research Institute, Tokyo Medical Dental University, Bunkyo-ku, Tokyo 113-8510, Japan

We have recently demonstrated that Caenorhabditis elegans Ca^{2+}/calmodulin-dependent protein kinase (CeCaM-KK) can activate mammalian CaM-kinase IV in vitro (Tokumitsu, H., Takahashi, N., Eto, K., Yano, S., Soderling, T.R., and Muramatsu, M. (1999) J. Biol. Chem. 274, 15803–15810). In the present study, we have identified and cloned a target CaM-kinase for CaM-KK in C. elegans, CeCaM-kinase I (CeCaM-KI), which has approximately 60% identity to mammalian CaM-KI. CeCaM-KI has 348 amino acid residues with an apparent molecular mass of 40 kDa, which is activated by CeCaM-KK through phosphorylation of Thr^{179} in a Ca^{2+}/CaM-dependent manner, resulting in a 30-fold decrease in the K_m of CeCaM-KI for its peptide substrate. Unlike mammalian CaM-KI, CeCaM-KI is mainly localized in the nucleus of transfected cells because the N-terminus six residues (PLFKKR) contain a functional nuclear localization signal. We have also demonstrated that CeCaM-KK and CeCaM-KI reconstituted a signaling pathway that mediates Ca^{2+}-dependent phosphorylation of cAMP response element-binding protein (CREB) and CRE-dependent transcriptional activation in transfected cells, consistent with nuclear localization of CeCaM-KI. These results suggest that the CeCaM-KK/CeCaM-KI cascade is conserved in C. elegans and is functionally operated both in vitro and in intact cells, and it may be involved in Ca^{2+}-dependent nuclear events such as transcriptional activation through phosphorylation of CREB.

Ca^{2+}/calmodulin-dependent protein kinases (CaM-K)^1 are implicated in the regulation of a wide variety of biological events mediated by intracellular Ca^{2+}, such as muscle contraction, neurotransmitter release, and gene expression (1–4). Recent studies have indicated that so-called multifunctional CaM-kinases, including CaM-KI, -II, and -IV, are regulated by phosphorylation either by itself or by upstream protein kinase (4, 5). In contrast to CaM-KI, which converts the Ca^{2+}/CaM-independent kinase by autophosphorylation, both CaM-KI and CaM-KIV are activated by CaM-KK through phosphorylation of a Thr residue (Thr^{177} in CaM-KI and Thr^{196} in CaM-KIV) in their “activation loops,” resulting in an increase in their catalytic efficiency (5–10).

The α isoform of CaM-KK was originally purified and cloned from rat brain as a regulatory protein kinase for CaM-KIV and was later demonstrated to be an activator for CaM-KI (6–8, 11). It has been shown that CaM-KK is regulated by an intracellular mechanism through its autoinhibitory domain (residue 436–441) and activated by binding of the Ca^{2+}/CaM-complex to the overlapping CaM-binding region (residue 438–463 in the α isoform) also common to other CaM-kinases (12–14) and conserved in the recently cloned β isoform (15, 16). Therefore, a dual action of Ca^{2+}/CaM binding to both CaM-KK and its downstream target CaM-Ks is required to activate the CaM-kinase cascade (10, 12). Recently, we have identified an Arg/Pro-rich insert region (the RP domain) in the catalytic domain of CaM-KK, which is involved in the recognition of target CaM-kinases (17). The RP domain is also conserved in the β isoform and in the Caenorhabditis elegans homologue. The CaM-kinase cascade has been functionally demonstrated in various mammalian cells such as transfected COS-7 cells (12), Jurkat cells (18), PC-12 cells (19), and cultured hippocampal neurons (20), which are strictly regulated by intracellular Ca^{2+}. One of the targets for the CaM-KK/CaM-KIV cascade has been demonstrated to be CREB, which plays a role in long term memories that depend on altered gene expression. Extensive studies have demonstrated that the CaM-KK/CaM-KIV cascade is involved in Ca^{2+}-dependent regulation of transcriptional activation through phosphorylation of CREB at Ser^{133} (20–24), which is consistent with nuclear localization of CaM-KIV (25). On the other hand, CaM-KI, another target for CaM-KK, is predominantly a cytoplasmatic enzyme (36), and the physiological function(s) of the CaM-KK/CaM-KI cascade is not well known. A recent study has shown that CaM-KK may mediate the anti-apoptotic effect of modest elevations of Ca^{2+} through phosphorylation and activation of protein kinase B (26). This result also indicates that multiple protein kinases might be phosphorylated and activated by CaM-KK, resulting in the regulation of a wide variety of functions. Most of the studies of the CaM-kinase cascade have been
**Fig. 1. Cloning and expression of C. elegans CaM-kinase I.** A, amino acid sequence comparison of C. elegans CaM-KI with mammalian CaM-KIs. CaM-KI cDNA (GenBank™ accession no. AB021864) was obtained from C. elegans N2 strain embryonic stage cDNA library in ZAP vector as described under “Experimental Procedures.” The putative initiation methionine is encoded by ATG at nucleotide 57. The termination codon TGA is denoted at nucleotide 1101. The deduced amino acid sequence of CeCaM-KI was aligned with those of rat (29, 30) and human CaM-KI (10). The respective amino acid numbers are shown at both sides. The positions where at least two of the three sequences are identical are indicated by the lighter shaded boxes. The catalytic domain is indicated by a solid line box. The ATP- and NH₂-terminal deletion mutant cDNA sequences. The phosphorylation site Thr⁵⁷⁹ in CeCaM-KI for activation by CaM-KK, equivalent to Thr⁶⁷⁷ in rat and human CaM-KI is indicated by an asterisk. A potential NLS of CeCaM-KI is overlaid with dashes. The regulatory region containing CaM-binding and autoinhibitory domains is indicated by black boxes (31). B, expression of recombinant CeCaM-KI and CeCaM-KK. Mock, CeCaM-KI, or CeCaM-KK cDNA in pME18s vector was transfected into COS-7 cells. Each cell extract (approx. 20 μg) was subjected to SDS-10% PAGE followed by CaM overlay as described under “Experimental Procedures.” C, expression of endogenous CeCaM-KI. CaM-binding proteins were enriched from the extract of C. elegans (mixed stage) by CeM- Sepharose column chromatography as described under “Experimental Procedures” and then subjected to SDS-10% PAGE followed by Western blotting using anti-CeCaM-KI antiserum (1/1000 dilution, right lane). The extract of COS-7 cells expressing wild type CeCaM-KI (as shown in B) was also analyzed in the left lane.

done in mammalian systems. The C. elegans homologue of CaM-KK (CeCaM-KK) was initially identified in the GenBank data base (27) and proven to activate mammalian CaM-KIV (CaM-KK) was initially identified in the GenBank data base (27) and proven to activate mammalian CaM-KIV (CaM-KK) was initially identified in the GenBank data base (27) and proven to activate mammalian CaM-KIV (CaM-KK) was initially identified in the GenBank data base (27) and proven to activate mammalian CaM-KIV (CaM-KK) was initially identified in the GenBank data base (27) and proven to activate mammalian CaM-KIV (CaM-KK) was initially identified in the GenBank data base (27) and proven to activate mammalian CaM-KIV (CaM-KK) was initially identified in the GenBank data base (27) and proven to activate mammalian CaM-KIV (CaM-KK) was initially identified in the GenBank data base (27) and proven to activate mammalian CaM-KIV (CaM-KK) was initially identified in the GenBank data base (27) and proven to activate mammalian CaM-KIV (CaM-KK) was initially identified in the GenBank data base (27) and proven to activate mammalian CaM-KIV (CaM-KK) was initially identified in the GenBank data base (27) and proven to activate mammalian CaM-KIV (CaM-KK) was initially identified in the GenBank data base (27) and proven to activate mammalian CaM-KIV (CaM-KK) was initially identified in the GenBank data base (27) and proven to activate mammalian CaM-KIV (CaM-KK) was initially identified in the GenBank data base (27) and proven to activate mammalian CaM-KIV (CaM-KK) was initially identified in the GenBank data base (27) and proven to activate mammalian CaM-KIV (CaM-KK) was initially identified in the GenBank data base (27) and proven to activate mammalian CaM-KIV (CaM-KK) was initially identified in the GenBank data base (27) and proven to activate mammalian CaM-KIV (CaM-KK) was initially identified in the GenBank data base (27) and proven to activate mammalian CaM-KIV (CaM-KK) was initially identified in the GenBank data base (27) and proven to activate mammalian CaM-KIV (CaM-KK) was initially identified in the GenBank data base (27) and proven to activate mammalian CaM-KIV (CaM-KK) was initially identified in the GenBank data base (27) and proven to activate mammalian CaM-KIV (CaM-KK) was initially identified in the GenBank data base (27) and proven to activate mammalian CaM-KIV (CaM-KK) was initially identified in the GenBank data base (27) and proven to activate mammalian CaM-KIV (CaM-KK) was initially identified in the GenBank data base (27) and proven to activate mammalian CaM-KIV (CaM-KK) was initially identified in the GenBank data base (27) and proven to activate mammalian CaM-KIV (CaM-KK) was initially identified in the GenBank data base (27) and proven to activate mammalian CaM-KIV (CaM-KK) was initially identified in the GenBank data base (27) and proven to activate mammalian CaM-KIV (CaM-KK) was initially identified in the GenBank data base (27) and proven to activate mammalian CaM-KIV (CaM-KK) was initially identified in the GenBank data base (27) and proven to activate mammalian CaM-KIV (CaM-KK) was initially identified in the GenBank data base (27) and proven to activate mammalian CaM-KIV (CaM-KK) was initially identified in the GenBank data base (27) and proven to activate mammalian CaM-KIV (CaM-KK) was initially identified in the GenBank data base (27) and proven to activate mammalian CaM-KIV (CaM-KK) was initially identified in the GenBank data base (27) and proven to activate mammalian CaM-KIV (CaM-KK) was initially identified in the GenBank data base (27) and proven to activate mammalian CaM-KIV (CaM-KK) was initially identified in the GenBank data base (27) and proven to activate mammalian CaM-KIV (CaM-KK) was initially identified in the GenBank data base (27) and proven to activate mammalian CaM-KIV (CaM-KK) was initially identified in the GenBank data base (27) and proven to activate mammalian CaM-KIV (CaM-KK) was initially identified in the GenBank data base (27) and proven to activate mammalian CaM-KIV (CaM-KK) was initially identified in the GenBank data base (27) and proven to activate mammalian CaM-KIV (CaM-KK) was initially identified in the GenBan...
from pT7Blue (Novagen) as a SacI-SalI fragment into pEGFP-C2.

**Mutagenesis—Site-directed mutagenesis of the CaM-KI cDNA in pBluescript SK(−) was performed using the GeneEditor* in vitro site-directed mutagenesis system (Promega) followed by subcloning into the expression plasmids described above. For construction of the point substitutions, mutagenic oligonucleotides are described as follows: T179A, 5′-GTAATGCCGTCACGCTTGTTG-3′; T179D, 5′-CAGG-GTAATTGCCGTCACGCTTGTTGAAACCCGGG-3′; K25A, 5′-CAGATGCT ATGACGATCCGATCCATTGTACAGACAAAAGCC-3′. The COOH-terminal truncated mutant of CaM-KI cDNA at position 295 was constructed by PCR using a sense oligonucleotide, 5′-CCGGAATCCTCCTTTTTTATGATC-3′, and an antisense oligonucleotide, 5′-CGCTGACACTCTACGGCCGACGATTTTCG-3′, and then subcloned into pGEX-4T-1. The nucleotide sequence of each mutant CaM-KI cDNA was confirmed.

**Cell Culture and Transient Transfection—**COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum at 37 °C in an atmosphere of 5% CO2. For transient transfection, cells grown in 10-cm-diameter dishes were transfected with 5–10 μg of plasmid DNA using 60 μg of LipofectAMINE (Life Technologies, Inc.) according to the manufacturer’s protocol. The cells were cultured in serum-free medium (Opti-MEM, Life Technologies, Inc.) for 5 h after transfection, followed by culture in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum for the indicated times. The supernatants were recovered for GFP fluorescence using a confocal microscope (Carl Zeiss) or lysed for purification of the recombinant CaM-KKs, CaM overlay assay, Western blotting, and CRE reporter gene assay.

**Expression and Purification of Reconstituent CaM-KI and CaM-KK—**E. coli (JM109) carrying the expression plasmid (pGEX-CaM-KI) was precultured in LB broth containing 100 μg/ml ampicillin at 37 °C overnight. An overnight culture of E. coli (1 ml) was added into 100 ml of LB broth containing 100 μg/ml ampicillin, the culture was continued to A600 of 0.8, and then 0.4 mM isopropyl-1-thio-β-D-galactopyranoside was added. After 4 h of culture, the E. coli was harvested by centrifugation. All of the purification steps described below were carried out at 4 °C. The bacterial pellet was resuspended in 10 ml of phosphate-buffered saline containing 0.5 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM DTT, 0.2 mM PMSF, 10 μg/liter leupeptin, 10 μg/liter pepstatin A, 10 μg/liter trypsin inhibitor, 50 mM sodium fluoride, 0.1 mM Na3VO4, and 1 M NaCl. After the column was washed with 10 ml of buffer A containing 0.5 mM CaCl2 and 1 M NaCl, the samples were subjected to SDS-10% PAGE followed by Western blotting for phosphorylated CREB, using anti-phospho-CREB antibody (Biolab), or phosphorylated CREB, using anti-CREB antibody (New England Biolab), or phosphorylated CREB, using anti-creph-phospho-CREB antibody.

**Activation and Phosphorylation of CaM-KI by CaM-KK—**GST-CaM-KI (0.5 μg) was incubated with recombinant CaM-KK (9 ng) expressed in COS-7 cell at 30 °C for 10 min in 10 μl of 50 mM HEPES (pH 7.5), 10 mM Mg(Ac)2, 1 mM DTT, and 400 μM ATP containing either 2 mM CaCl2, 8 μM CaM or 2 mM EGTA. The reaction was terminated by a 20-fold dilution at 0 °C with 50 mM HEPES (pH 7.5), 10 mM Mg(Ac)2, 1 mM DTT, and 400 μM γ-[32P]ATP (1000–2000 cpm/pmol). After a 10-min incubation at 30 °C, the reaction was terminated by adding 5 μl of SDS-PAGE sample buffer. Then the samples were subjected to SDS-15% PAGE followed by autoradiography.

**Enrichment of CaM-binding Proteins in C. elegans—**C. elegans (mixed stage) was harvested from four plates (6-cm-diameter dish) and extracted in 3 ml of lysis buffer (50 mM NaCl, 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 1% Nonidet P-40, 10% glycerol, 0.2 mM Tris, 10 μg/liter leupeptin, 10 μg/liter pepstatin A, 10 μg/liter trypsin inhibitor) containing 0.2 mM PMSF and lysed by sonication. After centrifugation at 15,000 × g for 15 min, the supernatant was loaded onto a 1-ml bed volume of glutathione-Sepharose (Amerham Pharmacia Biotech) affinity column. After washing the column with 20 ml of phosphate-buffered saline containing 0.2 mM PMSF, recombinant CaM-KI was eluted with 10 mM glutathione in 50 mM Tris-HCl (pH 8.0) and 0.2 mM PMSF and then dialyzed against 100 mM HEPES (pH 7.5), 1 mM EDTA, 1 mM EGTA, 2 mM DTT, and 0.2 mM PMSF. The recombinant protein was mixed with equal an volume of CaM-KK by lysis at 4 °C by adding 1 ml/10-cm-diam-
specific for CREB phosphorylated at Ser133 (New England Biolab). Detection of the immunoreactive band was carried out using an enhanced chemiluminescence reagent (Amersham Pharmaclia Biotech).

**Luciferase Assay**—COS-7 cells were transfected with 4 μg of a plasmid pFR-4xCRE-luciferase (Stratagene) and a combination of expression plasmids (pME18s) carrying either CeCaM-KI wild type or mutants (4 μg) and/or CeCaM-KK (2 μg). A cDNA (2 μg) of the catalytic subunit of PKA was also used as a positive control. After incubation for 40 h, the cells were deprived of serum for 6 h and then stimulated with 1 mM calcium ionophore and 10 mM CaCl₂ for 6 h. Then the cells were lysed with 1 ml of lysis buffer (25 mM glycyl glycine (pH 7.8), 8 mM MgSO₄, 1 mM EDTA, 1% Triton X-100, 5% glycerol, and 1 mM DTT), and the luciferase activity of each cell extract (10 μl) was measured by the luciferase assay kit (PicaGene, Toyo Ink).

**Anti-CeCaM-KI Antiserum**—After the recombinant GST-CeCaM-KI (wild type) was subjected to SDS-PAGE, electroeluted GST-CeCaM-KI from the excised gels was used to immunize a Japanese White rabbit (approximately 500 μg/injection). The rabbit received booster injections at 14-day intervals. The presence of anti-CeCaM-KI antibody was assayed by Western blotting using the extract of mock- and CeCaM-KI-transfected COS-7 cells. The antiserum was applied to a GST-bound glutathione-Sepharose column twice to remove anti-GST antibodies, and the flow-through fraction was collected and used for Western blotting and immunoprecipitation.

**Other Methods**—General techniques for the culture and handling of worms have been described (43). The *C. elegans* Bristol (N2) stock was used as the wild type strain. CaM overlay was performed as described previously (8). Anti-GFP antibody (CLONTECH) was used for detection of the GFP-fusion protein expressing in COS-7 cells. Protein concentration was estimated by Coomassie dye binding (Bio-Rad) using bovine serum albumin as a standard.

**RESULTS AND DISCUSSION**

**Cloning of CaM-KI Homologue from C. elegans**—Recent studies identified *C. elegans* CaM-KK (CeCaM-KK) in the data base (GenBank™ accession no. U11029) (27) and demonstrated that recombinant CeCaM-KK (GenBank™ accession no. AB016838) was able to activate mammalian CaM-KIV in a Ca²⁺-dependent manner (17). To identify the target(s) for CeCaM-KK, we searched the *C. elegans* genome data base and found a *C. elegans* cosmid (K07A9) containing a protein kinase catalytic domain that is highly homologous to mammalian CaM-KI. Because the cosmid does not contain a full-length sequence, we used a combination of reverse transcriptase-PCR, to amplify the portion of the protein kinase cDNA (residues 129–308), and screening of *C. elegans* λZAP phage cDNA library, using the reverse transcriptase-PCR product as a probe.

A cDNA of 1546 base pairs encoding 348 amino acid residues in the open reading frame was isolated, which was approximately 60% identical with rat (28, 30) and human CaM-KI (10) (Fig. 1A). We transfected the cDNA into COS-7 cells and detected an approximately 40-kDa CaM-binding protein on SDS-PAGE by the CaM overlay method; this is consistent with the calculated Mr value of 39,066 (Fig. 1B). Therefore, we have termed this gene product *C. elegans* CaM-KI (CeCaM-KI). Anti-CeCaM-KI antiserum recognized the endogenous *C. elegans* CeM-KII in the partially purified fraction from *C. elegans* extract by CaM-Sepharose column chromatography (Fig. 1C, right lane), which had the same mobility on SDS-PAGE as the overexpressed enzyme in COS-7 cells (Fig. 1C, left lane). We also detected 40-kDa CaM-binding protein by the CaM overlay method in the immunoprecipitated fraction from *C. elegans* extract with the anti-CeCaM-KI antiserum (data not shown). These results suggest that the isolated cDNA encodes full-length CeCaM-KI and the methionine at position 1 in CeCaM-KI is likely the translation initiation. CeCaM-KI contains a Thr residue (Thr179) in the catalytic domain equivalent to the phosphorylation-activation Thr177 in mammalian CaM-KI. It is noteworthy that a basic cluster (KRR) of a potential NLS is inserted at the NH₂-terminal region in CeCaM-KI but not in the mammalian CaM-KI, as described in detail below.

**Activation Mechanism of CeCaM-KI by CeCaM-KK**—We expressed and purified CeCaM-KI as a GST-fusion protein in *E. coli* to test its activity and activation by recombinant CeCaM-KK. As shown in Fig. 2, recombinant wild type CeCaM-KI has a Ca²⁺/CaM-dependent protein kinase activity toward the peptide substrate (syntide-2), and the activity is enhanced approximately 10-fold by CeCaM-KK treatment in a Ca²⁺/CaM-dependent manner. Furthermore, phosphorylation of CeCaM-KI by CeCaM-KK was strongly induced only in the presence of Ca²⁺/CaM, whereas basal CeCaM-KI underwent weak auto phosphorylation in a Ca²⁺/CaM-dependent manner, which did not induce the activity. We also observed that both the α and β isoforms of rat CaM-KK were able to activate CeCaM-KI in a manner similar to CeCaM-KK (data not shown). When we used the T179A mutant of CeCaM-KI, it was no longer activated and phosphorylated by CeCaM-KK, indicating that Thr179 is a primary phosphorylation-activation site for CeCaM-KK. This finding is also consistent with the observation that the mutation of
Thr\textsuperscript{179} to Asp resulted in an approximately 5-fold increase in the basal Ca\textsuperscript{2+}/CaM-dependent activity without activation. These results clearly demonstrated the activation of CeCaM-KI by CeCaM-KK through Ca\textsuperscript{2+}/CaM-dependent phosphorylation of Thr\textsuperscript{179} in vitro. Truncation at residue position 295 generated a constitutively active form of CeCaM-KI, which was incapable of binding Ca\textsuperscript{2+}/CaM (data not shown), suggesting the existence of an autoinhibitory domain and CaM-binding region in the COOH-terminal from position 295. The regulatory region of CeCaM-KI has 50% identity with that of mammalian CaM-KI (31). Based on the amino acid sequence comparison, Trp\textsuperscript{305} in CeCaM-KI is conserved in many CaM-binding proteins including mammalian CaM-KI (Fig. 1A) as one of the anchoring residues to the COOH-terminal hydrophobic pocket of CaM (32). According to NMR and x-ray structure determination of the CaM-MLCK peptide (M13) complex (33, 34) and the CaM-CaM-KI peptide complex (35), both skeletal and smooth muscle MLCK peptides have 14 residues between two key hydrophobic residues, and the CaM-KII peptide has 10 residues between them. Therefore Leu\textsuperscript{318} can be predicated as another anchoring residue in CeCaM-KI to the NH\textsubscript{2}-terminal hydrophobic pocket of CaM, which appears to be of the MLCK type. The truncation mutant was still activated and phosphorylated by CeCaM-KK in a complete Ca\textsuperscript{2+}/CaM-dependent manner, indicating that CeCaM-KK also requires Ca\textsuperscript{2+}/CaM for phosphorylation and activation of CeCaM-KI, consistent with previous observation by using a constitutively active mutant of mouse CaM-KIV (17).

**Effect of Activation on Kinetic Parameters of CeCaM-KI**—CeCaM-KI was incubated with activation reaction including Ca\textsuperscript{2+}/CaM, Mg-ATP, and either recombinant CeCaM-KK or buffer for 60 min. EDTA/EGTA-containing buffer was added to stop activation, and CeCaM-KK, Mg-ATP, and excess CaM were removed by glutathione-Sepharose column chromatography. Both basal and activated CeCaM-KIs were eluted by the addition of 10 mM glutathione followed by kinetic constants determination of both enzymes for syntide-2 and ATP. From the results shown in Fig. 3, it is clear that the main effect of activation by CeCaM-KK was to lower the $K_m$ for syntide-2. Phosphorylation of Thr\textsuperscript{179} by CeCaM-KK decreased the $K_m$ of CeCaM-KI for syntide-2 from 657 to about 20 $\mu$m with little effect on either the $V_{\text{max}}$ or $K_m$ for ATP (Fig. 3, A and B), which is similar to the activation mechanism of mammalian CaM-KIV by CaM-KK (7). However, the $V_{\text{max}}$ of recombinant CeCaM-KI (approximately 0.1 $\mu$mol/min/mg) for syntide-2 obtained in the present study was about 1–5% that of recombinant mammalian CaM-KI (2–12 $\mu$mol/min/mg (10, 44)) but comparable with that of CaM-KIV (0.15–0.5 $\mu$mol/min/mg (7, 45, 46)). This may be due to the structural difference of catalytic domain between C. elegans and mammalian CaM-KI, because approximately 25% of the amino acid residues in the catalytic domain are not identical between both CaM-KIs (Fig. 1A).

**Nuclear Localization of CeCaM-KI**—It has already been reported that mammalian CaM-KI is localized mainly in the cytoplasm (36). To visualize the subcellular localization of CeCaM-KI, we transfected GFP-fusion constructs of CeCaM-KI into COS-7 cells. Expression of the GFP-fusion protein of each CaM-KI was confirmed by Western blotting using anti-GFP antibody (Fig. 4D) and the CaM overlay method (data not shown). In contrast to rat CaM-KI localized in cytoplasm (Fig. 4C) consistent with a previous report (36), CeCaM-KI (wild type, Pro\textsuperscript{Ψ}-Ala\textsuperscript{148}) is highly concentrated in the nucleus (Fig. 4A). When we used a mutant CeCaM-KI lacking 6 residues (Pro\textsuperscript{Ψ}-Arg\textsuperscript{7}) at the NH\textsubscript{2}-terminal region, it was no longer staying in the nucleus (Fig. 4B), suggesting that the NH\textsubscript{2}-terminal 6 residues contain a potential NLS. This region includes the basic cluster Lys\textsuperscript{2}-Arg\textsuperscript{2}-Arg\textsuperscript{7}, which is similar to the NLS (KKKR) in the delta B isoform of CaM-KII (37), but it is lacking in the mammalian CaM-KI (Fig. 1A). We have detected GFP-CeCaM-KK localized in both the cytoplasm and the nuclei of transfected cells (data not shown).

**Transcriptional Activation by C. elegans CaM-kinase Cascade**—Nuclear localization of CeCaM-KI gave us an idea that the CaM-KK/CaM-KI cascade in C. elegans might be involved in the regulation of transcriptional activation analogous to the CaM-KK/CaM-KIV/CREB pathway in mammalian cells. It has been shown that CREB appears to be a good substrate for mammalian CaM-KI in vitro (38) and that overexpressed mammalian CaM-KI can stimulate CREB-dependent transcriptional activation (39). However, mammalian CaM-KI has been shown to be localized in the cytoplasm in intact cells (36), and therefore the involvement of this kinase in the activation of CREB-dependent transcriptional activation is still controversial. To analyze the C. elegans CaM-kinase cascade, we used mammalian cells because there is little information available about CREB and CREB-dependent transcriptional activation in C. elegans, although there is one predicted CREB gene in C. elegans (42). First, we tried to detect the phosphorylation of endogenous CREB at Ser\textsuperscript{133} upon stimulation with 1 $\mu$mol calcium ionophore in COS-7 cells, which was transfected with various combinations of plasmids carrying CeCaM-KI and/or CeCaM-KK (Fig. 5A). Detection of CREB phosphorylation was carried out using anti-phospho-CREB antibody. A 10-min stimulation with calcium ionophore induced significant phosphorylation of CREB only in the cells transfected with both CeCaM-KI wild type and CeCaM-KK as well as in PKA-transfected cells (Fig. 5A). We detected an immunoreactive band migrating faster than the phosphorylated CREB, which was also induced by co-transfection of the components of the C. elegans CaM-kinase cascade upon stimulation with calcium ionophore as well as PKA transfection. Because the antibody used for detection of the phosphorylated form of CREB also detects the phosphorylated form of the CREB-related proteins, activating transcription factor-1 (ATF-1) and cAMP response element binding modulator (CREM), the lower band is possibly ATF-1. This is consistent with a previous report that ATF-1 can

![Fig. 4. Subcellular localization of CeCaM-KI. COS-7 cells were transfected with GFP-fusion constructs carrying CeCaM-KI wild type (residue 2–348, A), the NH\textsubscript{2}-terminal deletion mutant, which lacks residue 2–7 (B), or rat CaM-KI wild type (C). After 20 h post-transfection, the cells expressing each GFP-fusion protein were observed for GFP fluorescence by a fluorescence microscopy and then lysed to monitor the expression level of each GFP-CaM-KI by Western blotting using anti-GFP antibody (panel D, lane a, CeCaM-KI wild type; lane b, NH\textsubscript{2}-terminal deletion mutant of CeCaM-KI; lane c, rat CaM-KI wild type). Results are representative of experiments repeated at least four times.](http://www.jbc.org/content/early/2000/10/22/jbc.C100017200/Fig4.jpg)
and subsequently phosphorylates CREB at Ser133, resulting in the activation of CRE-dependent transcription.

Conclusion—The results presented in this paper demonstrate the existence of a CaM-kinase cascade (CaM-KK/CaM-KI) in C. elegans; this cascade operates fundamentally both in vitro and in intact cells, as do its mammalian counterparts. Ca²⁺-dependent transcriptional regulation through a CaM-kinase cascade seems to be conserved in C. elegans, which is consistent with nuclear localization of CeCaM-KI. Therefore, the CaM-KK/CaM-KIV pathway, which is thought to be involved in Ca²⁺-dependent transcriptional regulation in mammalian cells (20–24, 41), may be replaced by the CaM-KK/CaM-KI cascade in C. elegans. This reasoning is also consistent with the fact that the CaM-KIV homologue cannot be found in the C. elegans genome data base. Identification and characterization of the components in the CaM-kinase cascade in C. elegans described in this paper provide useful tools for evaluating the physiological significance of this protein kinase cascade. The question of the physiological function(s) mediated by the CaM-KK/CaM-KI cascade in C. elegans still remains unanswered and is now under investigation with genetic approaches.

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