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13. ABSTRACT <i>(Maximum 200 words)</i> The mammalian calmodulin-dependent protein kinase kinases (CaMKKs) have been shown to phosphorylate and activate the calmodulin-dependent protein kinases I and IV (CaMKI, CaMKIV), leading to transcription of a variety of genes including c-fos, c-jun and cyclin A. Using a <i>C. elegans</i> homologue (ceCaMKK), we are investigating the importance of this proposed cascade to cell cycle, cell fate and development in the context of a well defined multicellular organism. To examine the cell-specific pattern of gene expression, transgenic worms have been generated which express β -galactosidase or green fluorescent protein under the control of the ceCaMKK promoter. These reporter constructs reveal expression in the excretory cell, vulval muscle cells and several neurons of adult hermaphrodites, as well as hypodermal cells of L1/L2 larvae. To compare the <i>C. elegans</i> and mammalian enzymes biochemically, we have cloned and expressed the ceCaMKK protein. Using human CaMKI as a substrate, ceCaMKK is Ca ²⁺ / calmodulin dependent and phosphorylates CaMKI on threonine 177, the residue phosphorylated by mammalian CaMKKs. These results indicate a functional homology between the mammalian and <i>C. elegans</i> CaMKKs, but further work remains to reveal the biological functions of this family of proteins.			
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FOREWORD

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Elucidating the Role of CaMKK in Cell Cycle and Cell Fate Using a *C. elegans* Model

Annual Report for Grant DAMD17-97-1-7331

Ethan E. Corcoran

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Introduction

Calcium and calmodulin (CaM) are ubiquitous signaling molecules, responsible for a wide variety of cellular functions, but specific mechanisms have been difficult to define. Ca^{2+} /CaM signals are implicated in G_0 reentry, G_1/S , G_2/M , and metaphase transitions in a variety of organisms. In one of the best studied examples, activation of calmodulin dependent kinase II (CaMKII) by the Ca^{2+} /CaM signal was shown to be responsible for resumption of meiosis in *Xenopus* oocytes. Even in this case, downstream targets for the kinase remain uncertain (1). More recently, a novel kinase cascade has been proposed connecting the action of CaM dependent kinase kinases (CaMKK) to transcription of key factors in cell cycle control (2). Understanding this cascade, from protein interactions to biological consequences, may help unravel the essential functions of calcium signals in the cell cycle.

Even before CaMKK was discovered, activation of the CaM dependent kinases I and IV (CaMKI, CaMKIV) by Ca^{2+} /CaM had been implicated in transcriptional control and cell cycle regulation (reviewed in 3). Both CaMKI and IV phosphorylate the cAMP response element binding protein (CREB), leading to transcription of immediate early genes, specifically *c-fos* and *c-jun* (4,5,6). CREB is also involved in the stimulation of cyclin A transcription during the G_1/S transition (7). Recently, transgenic mice with a dominant negative form of CaMKIV have been produced. Thymocytes from these animals were fewer in number, were less viable in primary culture and had reduced IL-2 production in response to a CD3 challenge (8). The behavior of these cells in culture correlates closely with those from the dominant negative CREB transgenic mouse (9). This proliferative defect indicates a role for both CREB and CaMKIV in the cell cycle progression of activated thymocytes.

However, *in vitro* activity of recombinant CaMKIV was unexpectedly low, significantly lower than the activity of purified rat brain CaMKIV, suggesting a more complex regulatory mechanism than Ca^{2+} /CaM alone (10). Based on the observation that the recombinant protein's activity improved by incubation with crude rat brain extract (11), CaMK kinase A (CaMKKA) was isolated and cloned (2,12). Since then, a second novel CaMKK has been cloned by this lab, CaMKKB, from both rat and human (13). Either CaMKK, in the presence of Ca^{2+} /CaM, can activate either CaMKI or CaMKIV by phosphorylation of a threonine residue, Thr177 and Thr196 respectively (11,12). Up to a 25 fold increase in activity is observed after treatment by the CaMKK, but mutation of the critical Thr in either CaMKI or CaMKIV to Ala completely blocks activation (12,14). Clearly, the CaMKKs are important regulators of CaMK activity.

The biological significance of the CaMKKs is unclear, although evidence is mounting to support their role in a kinase cascade. Experiments with COS7 cells transfected with both the CaMKKA and CaMKIV have confirmed that together, CaMKKA and CaMKIV can activate a CRE reporter gene *in vivo*, but the biological implications of activation remain untested (2). No proteins upstream in the pathway have yet been identified, but the CaMKKs all possess consensus SH3 binding sites (PXXP) (15). These sites could be binding sites for GAPs or other proteins, linking the CaMKK to membrane proteins such as transmembrane receptors. Evidence for such upstream links has been observed *in vivo*; in order to reach full CaMKIV activation in Jurkat cells, CD3 activation is required as well as a calcium influx (16). Identification of upstream proteins will be necessary to understand this novel Ca^{2+} /CaM dependent signaling pathway.

This project was designed to develop a model in which to assess the biological relevance of these relationships and of the CaMKK itself. The available evidence suggests this protein is part of a signaling cascade that regulates transcription of key factors in cell cycle control.

Using a *Caenorhabditis elegans* model, these possibilities can be fully examined, since any perturbation of normal cell fates can be identified (17,18,19). A BLAST search for proteins homologous to CaMKKA found a predicted *C. elegans* gene, CELC05H8.1, derived from an open reading frame search of sequence from the *C. elegans* genome project. This gene, with an amino acid sequence 66% similar to both rat CaMKKA and CaMKKB, has now been cloned. Its expression pattern in adult worms has been examined, and its functional homology to mammalian CaMKK has now been demonstrated *in vitro* (20). This gene provides an opportunity to explore the function of the CaMKKs in a genetically tractable, well studied model system. Using the advantages of genetic and molecular techniques available for this system, a new signaling pathway will be defined, and the role of calcium in cell cycle more completely revealed.

Methods

Objective 1: Cloning ceCaMKK

Total RNA isolated from mixed worm cultures was used for reverse transcriptase PCR (rtPCR, Stratagene kit) with the primers 5'-AAACTTGCGTACAATGAGGAGGAC-3' and 5'-GCGATGTCCCATAGCTTTCACC-3' to obtain all but the 5'-most 106 bp of the ceCaMKK cDNA. The resulting fragment was assembled with the clone previously isolated from the Barstead *C. elegans* cDNA library using a unique ClaI restriction site by standard methods (21, 22). To complete the cDNA, rapid amplification of cDNA ends (5' RACE, Gibco/BRL kit) was attempted using a variety of primers with total or poly-A RNA, but no clones extending into the missing 106 bp were obtained. Instead, a 180-mer synthetic oligonucleotide corresponding to the 5' sequence predicted by the *C. elegans* genome project was combined with the assembled cDNA in a PCR reaction. The resulting product was subcloned into PCR-Script (Stratagene), and sequenced.

Objective 2: Biological role of ceCaMKK

To study the cell-specific developmental patterns of gene expression, transgenic worms have been generated. The promoter region of the genomic DNA, defined as 2.5 kb of genomic DNA upstream of the translational start site, has been subcloned into a modular lacZ fusion vector which encodes the β -galactosidase (β -gal) gene fused to a nuclear localization signal (provided by A. Fire, 23). This construct, together with a plasmid containing the rol-6 gene, was microinjected into the uterus of gravid hermaphrodites. Transgenic worms were then selected based on the rol-6 phenotype, which is easily observed in live worms under a dissecting microscope. The pattern of ceCaMKK gene transcription was determined from the level of β -gal expression in the F3 generation. Nuclear localized β -gal was visualized following treatment with 5-bromo-4-chloro-3-indoyl- β -D-galactoside (x-gal), that results in a blue precipitate concentrated in the nucleus of each expressing cell. A second construct, expressing a green fluorescent protein (GFP) without a nuclear localization signal rather than nuclear localized β -gal, has also been used to generate transgenic worms by the same method (provided by A. Fire). Expression in these worms was visualized by GFP fluorescence in live worms.

Objective 3: Identify components of ceCaMKK pathway

CaMKI and CaMKIV sequences have been submitted to the *C. elegans* genome center for BLAST searches to find *C. elegans* homologs (24). These searches are periodically repeated as new genomic data becomes available.

The complete ceCaMKK cDNA was subcloned into the pET-30a His-tagged expression vector (Novagen), and used to generate recombinant protein purification over Ni-resin (Qiagen). Protein concentrations were determined by Bradford assay using reagents from BioRad. The resulting protein was tested for kinase activity against recombinant glutathione-S-transferase (GST)-CaMKI fusion proteins (either wild type, 1-294 truncation, or T177A mutant) that have been used in previous studies (provided by S. Hook, 14). Assays were carried out in 50 mM TrisCl pH 7.8, 1 mM dithiothreitol, 0.1% Tween-20, 10 mM MgCl₂, 250 μ M ATP, and 1 to 2 μ Ci γ -³²P-ATP at 30 degrees C for 20 minutes using 200 ng of GST-CaMKI and 500 ng of His-ceCaMKK. Where indicated, 2 mM CaCl₂ and 1 μ M calmodulin (CaM), or 5

mM EGTA are also included in the reaction. CaM-overlay was performed using ^{125}I -CaM in the presence of Ca^{2+} , as previously described (25).

Results and Discussion

Objective 1: Cloning ceCaMKK

While we have been able to clone the majority of the gene from worm RNA or cDNA libraries, the remaining 106 bp had to be synthesized. This has allowed other work to proceed, based on the assumption that the sequence predicted by the genome project for the mRNA transcript is correct. However, efforts are continuing to clone the 5' end from RNA by varying conditions used for RACE procedure. It is important to note that the sequence we have cloned differs from the predicted transcript by the presence of an additional exon at the 3' end, but is otherwise as predicted. It is 66% similar, 48% identical to rat CaMKKB (Figure 1).

Objective 2: Biological role of CeCaMKK

Transgenic lines expressing β -gal or GFP under the control of the CeCaMKK promoter were generated at the expected frequency and developed normally. Three lines of the β -gal line and two lines of the GFP line were generated independently and displayed similar expression patterns. Staining of adult hermaphrodite worms revealed protein expression only in pharyngeal neurons, the excretory cell and several perivulvar cells (Figure 2). These pharyngeal neurons are located in or near the neural ring, effectively the central nervous system for the worm. The GFP line was generated to allow more precise identification of these neurons. From the GFP pattern, which illuminates entire cells including the axonal processes, we have identified these cells as sensory neurons (Figure 3). The cells near the vulva have been identified as vulval muscle cells. Finally, preliminary observations of developmental regulation of expression have shown that there is expression in hypodermal cells of L1 larvae. Examination of the expression pattern in embryos and male worms is also planned.

The meaning of this expression pattern is not yet clear. The pattern of CaMKK expression has not been determined in other organisms, but the expression pattern of its substrate, CaMKIV, has been studied in rodents and humans. CaMKIV is found in subpopulations of cells in the brain, thymus, gonads and skin (3,26). The presence of the ceCaMKK in neurons, vulval cells, and hypodermal cells corresponds well with this pattern. It is not clear whether the protein would be serving the same functions in each of these cells, but transient expression in the hypodermal cells does indicate some role in development.

No mutants in the ceCaMKK gene have yet been isolated. The generation of a chemically mutagenized worm library is being undertaken in collaboration with four other groups at Duke to conserve resources. Genomic DNA from this worm library will be screened by PCR to identify deletions within our gene (27). This method does not suffer from the problems that have been found with transposon libraries (the method originally included in this project proposal). Transposons have been found to insert preferentially in hot spots rather than randomly, and even when they have been found inserted in the gene, they have been difficult to excise; unless excised, transposon insertion frequently has no phenotype. A newer method for repressing protein expression in worms, called RNAi, will also be tried (28). This method is based on the observation that injecting a double stranded RNA corresponding to a cDNA of interest can effectively block expression of that gene in the worm it was injected as well as one or two generations of progeny. This method has been demonstrated using cDNAs

corresponding to known mutants, to confirm penetrance and specificity, and has been used for two genes of unknown function published so far (28, 29, 30). The greatest advantage of RNAi is the ability to isolate lethal and sterile mutants, which are systematically selected against in the creation of a chemically mutagenized library. Once obtained by one of these methods, characterizing worms lacking ceCaMKK will be critical in interpreting the expression pattern and in defining the biological role of this protein.

Objective 3: Identify components of ceCaMKK pathway

Using the mammalian CaMKI and CaMKIV sequences to search the *C. elegans* genome, a homologue to CaMKI, the predicted gene K07A9.2, has been identified (24). This predicted protein is 58% identical to CaMKI, 44% identical to CaMKIV, and contains the conserved activation loop threonine 177, which is phosphorylated by the CaMKK (Figure 4). This predicted protein is an excellent potential substrate for the ceCaMKK; its cloning by rtPCR and cDNA library screening has begun. However, the predicted gene lacks the 5' end, since there is a gap in the genomic sequence at that position. Thus, the 5' end of this gene must be determined empirically and will require 5' RACE since the sequence is unknown. Once the cDNA has been obtained, it will be used to express protein and tested both as a kinase, using peptides that are good substrates for mammalian CaMKI, and as a substrate for the ceCaMKK.

The cDNA for ceCaMKK has been used to generate recombinant protein in order to study its biochemical properties and to confirm a functional homology with other CaMKKs. Initial experiments have used a His-tagged protein that has been enriched but not purified to homogeneity (discussed below). This protein is able to bind CaM, by CaM overlay (data not shown), and phosphorylates CaMKI(1-294) in a Ca^{2+} /CaM dependent manner (Figure 5). The truncated protein CaMKI(1-294) is used for these experiments since it lacks the CaM binding domain (14). Differences in the presence and absence of CaM can therefore be interpreted as effects of CaM on the CaMKK. Significant Ca^{2+} -independent activity of the ceCaMKK was observed in these experiments, but this has also been noted with the mammalian CaMKKs (13). Using the T177A mutant of CaMKI, it is clear that ceCaMKK phosphorylates CaMKI on T177, the site phosphorylated by mammalian CaMKKs to activate CaMKI (14, Figure 6). Testing with mammalian CaMKIV is in progress. While these results are not quantitative, they clearly demonstrate that the ceCaMKK is a functional homologue of mammalian CaMKK.

Several methods are being explored to obtain a homogeneously purified single protein. The relatively poor separation achieved with the His-tagged protein is due to the unusually low affinity of this particular fusion protein to the Ni resin; it elutes from the resin under conditions normally used during stringent washes, so a complete wash of the affinity column is impossible. Moreover, because the protein degrades even at low temperatures in the presence of protease inhibitors, additional purification steps have led to decreased rather than increased specific activity. To avoid these problems, we have tried using GST and MBP fusions of the ceCaMKK, which are purified over glutathione-sepharose and amylose affinity resins respectively. While these constructs provide larger yields, and can be purified to one or two bands on a Coomassie stained gel, neither fusion protein is active in kinase assays. So, for these preliminary studies, the less pure His-tagged protein has been used. However, it is not reasonable to attempt to define the exact biochemical parameters such as $k(\text{cat})$ or $k(\text{CaM})$ until a purer protein preparation is available. Currently, we are cloning the ceCaMKK into a vector appropriate for baculovirus expression, and are working on cleaving the GST and MBP fusion proteins with the Factor Xa protease. One of these methods should yield a purified, active protein that can be used in more detailed biochemical experiments.

Recommendations for Statement of Work

Objective 1: Cloning of ceCaMKK

We are continuing to attempt to clone the 5' end from RNA, but, using a synthetic oligonucleotide, we have obtained a complete cDNA sequence which can be used for the completion of the other objectives. Although cloning the 5' end from RNA would provide empirical evidence to validate the predicted sequence, the predicted sequence contains no known splice sites, has an ATG start codon, and has significant peptide similarity with the known CaMKKs. Thus, we believe that using the predicted sequence for the 5' end is a safe assumption, and should not impede work on the other objectives.

Objective 2: Biological role of ceCaMKK

The determination of expression patterns by transgenic methods is well underway. Confirmation of this pattern by in-situ hybridization using labeled riboprobes or antibodies has not yet been done. One of these methods must be employed to verify the results of the transgenic experiments, and also to look at the patterns in embryos (23,31,32). To this end, antibodies will be generated to a ceCaMKK peptide, and affinity purified. Because of the time required to obtain and purify antibodies, this portion of the project may take another six months.

As stated previously, no ceCaMKK mutants have been obtained, but several methods for generating mutants are currently planned. Chemical mutagenesis and RNAi will be used in place of the transposon library that was originally proposed. Depending on the success of these methods, this objective could be complete between six months and one year.

Objective 3: Identify components of ceCaMKK pathway

The search for interacting proteins by two hybrid screening, as defined in the original proposal, has not begun. However, using our knowledge of the CaMKK pathway in other organisms, we have been able to identify one likely substrate through analysis of information provided by the genome project. This predicted protein, K07A9.2, will be cloned and tested as a substrate of the ceCaMKK. We will continue to use the genome project database for homology searches, but plan to begin searching for interacting proteins on schedule as discussed in the original proposal.

Recombinant expression and basic biochemical characterization of the ceCaMKK has also begun. Difficulties with obtaining purified protein will require using alternate methods, as already discussed, but should remain well ahead of schedule.

Conclusions

The results obtained at this point in the project are preliminary. We have defined the expression pattern in adult hermaphrodites and larvae, and are working on extending our knowledge to embryos and males. The pattern we have observed corresponds to the pattern we would expect based on the distribution of the mammalian CaMKIV, a substrate of CaMKKs. However, to make any conclusions about this protein's function in these cells, we will need to generate and study ceCaMKK mutant worms.

The biochemical properties of recombinant ceCaMKK confirm functional homology with CaMKKs. The ceCaMKK binds CaM, phosphorylates CaMKI on threonine 177, and is Ca²⁺/CaM dependent although exhibiting significant Ca²⁺-independent activity. These results will be followed with a detailed biochemical analysis of kinetic parameters once the recombinant protein has been adequately purified.

Finally, the existence of a CaMKI homologue, K07A9.2, is evidence that a CaM-dependant kinase cascade does exist in *C. elegans*. By cloning and studying K07A9.2, as well as pursuing other methods for identifying interacting proteins, we will be able to define the CaMKK pathway in this model organism. Using the advantages of genetic and molecular techniques available with *C. elegans*, we can use this pathway to reveal the role of Ca²⁺ signaling in development and cell cycle.

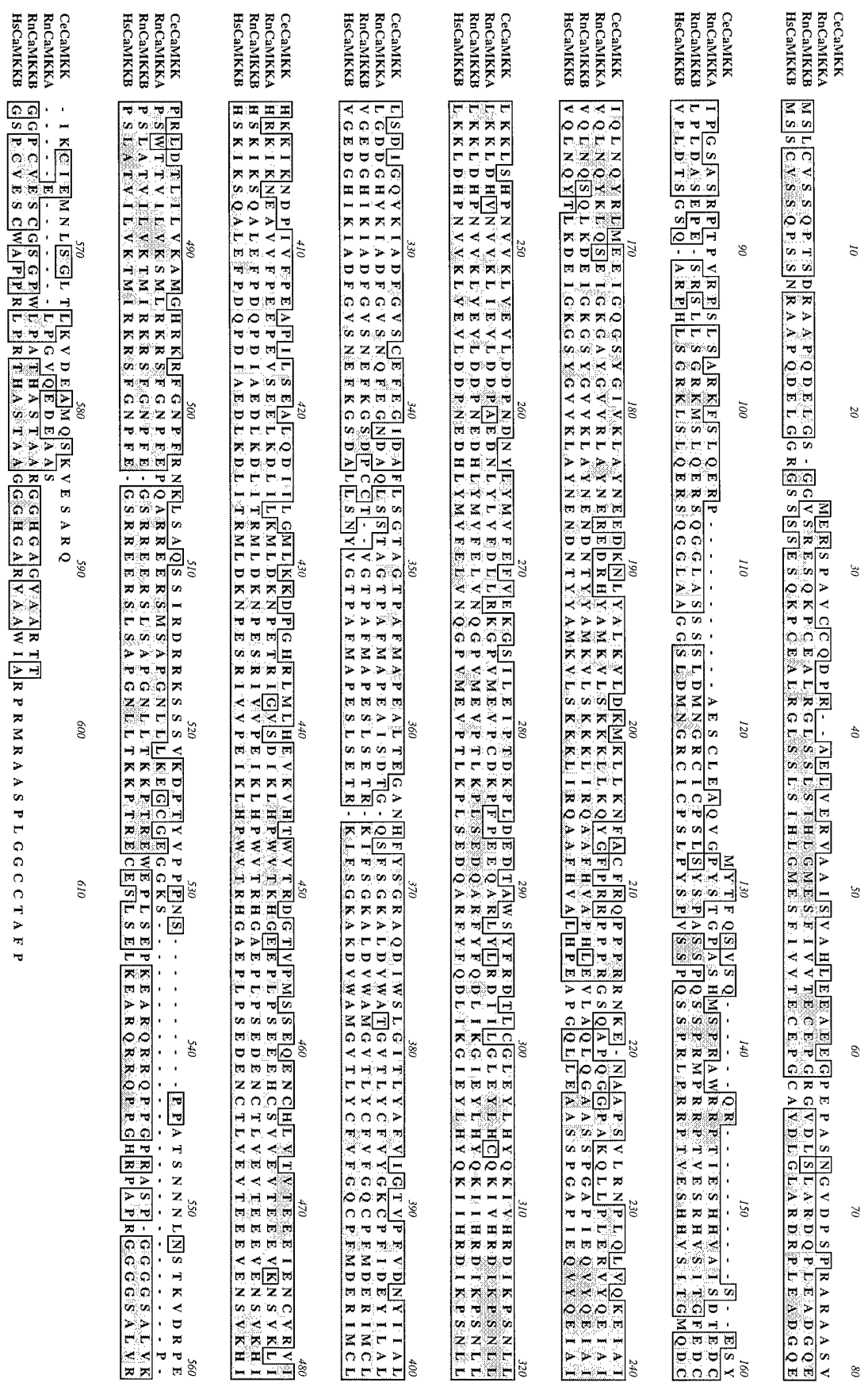


Figure 1: Alignment of ceCaMKK with *R. norvegicus* (rat) CaMKK A and B and *H. sapiens* (human) CaMKKB. ceCaMKK is 44% identical to human CaMKKB.

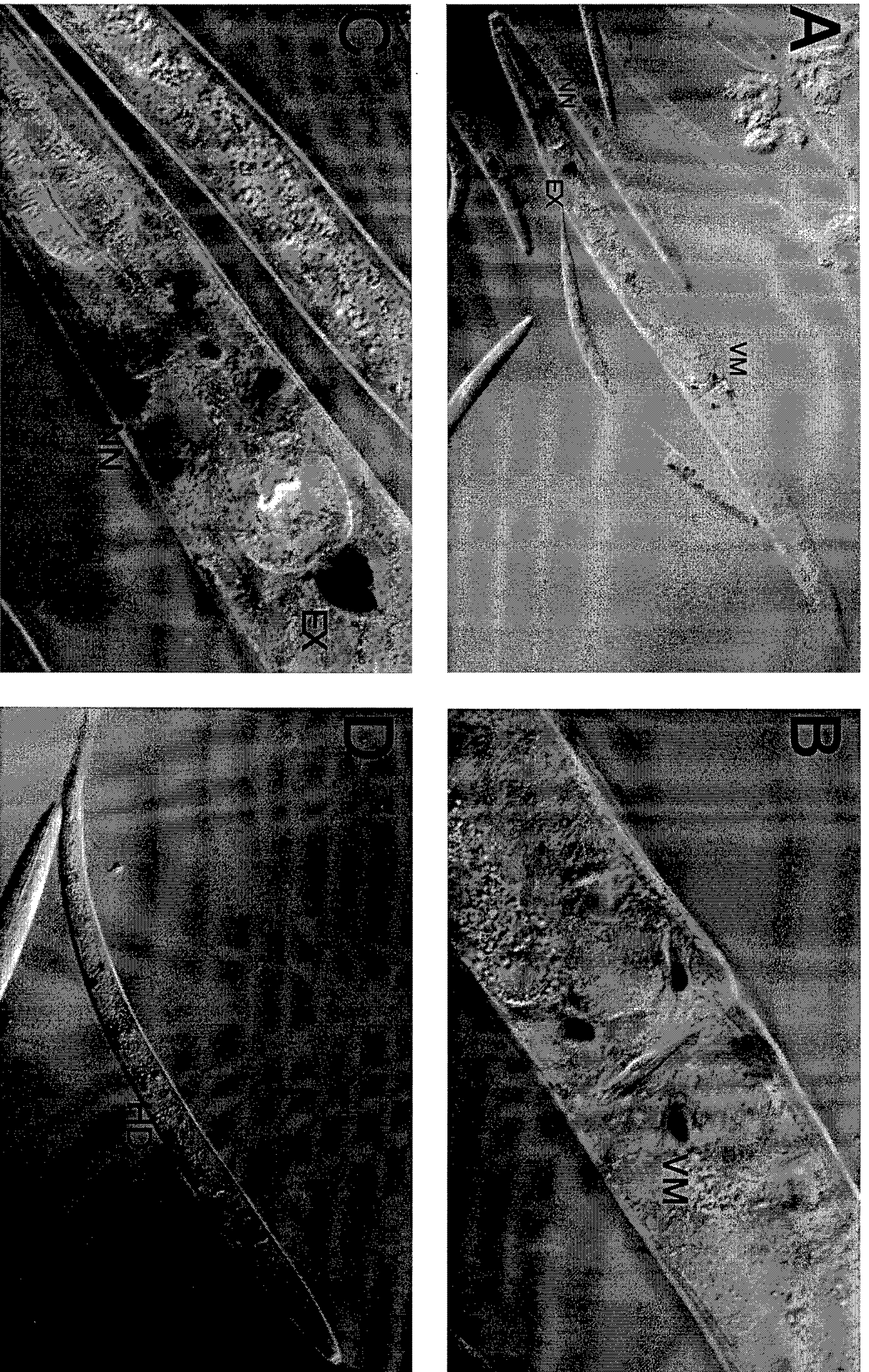


Figure 2: X-gal staining of worms expressing nuclear localized β -gal regulated by the ceCaMKK promoter. **A**, adult hermaphrodite (20X) demonstrating expression in neurons (NN), the excretory cell (EX), and vulval muscles (VM). **B**, higher magnification (100X) of vulval muscles. **C**, higher magnification (100X) of neurons. **D**, L1 larvae (40X) with expression in hypodermal cells (HD).

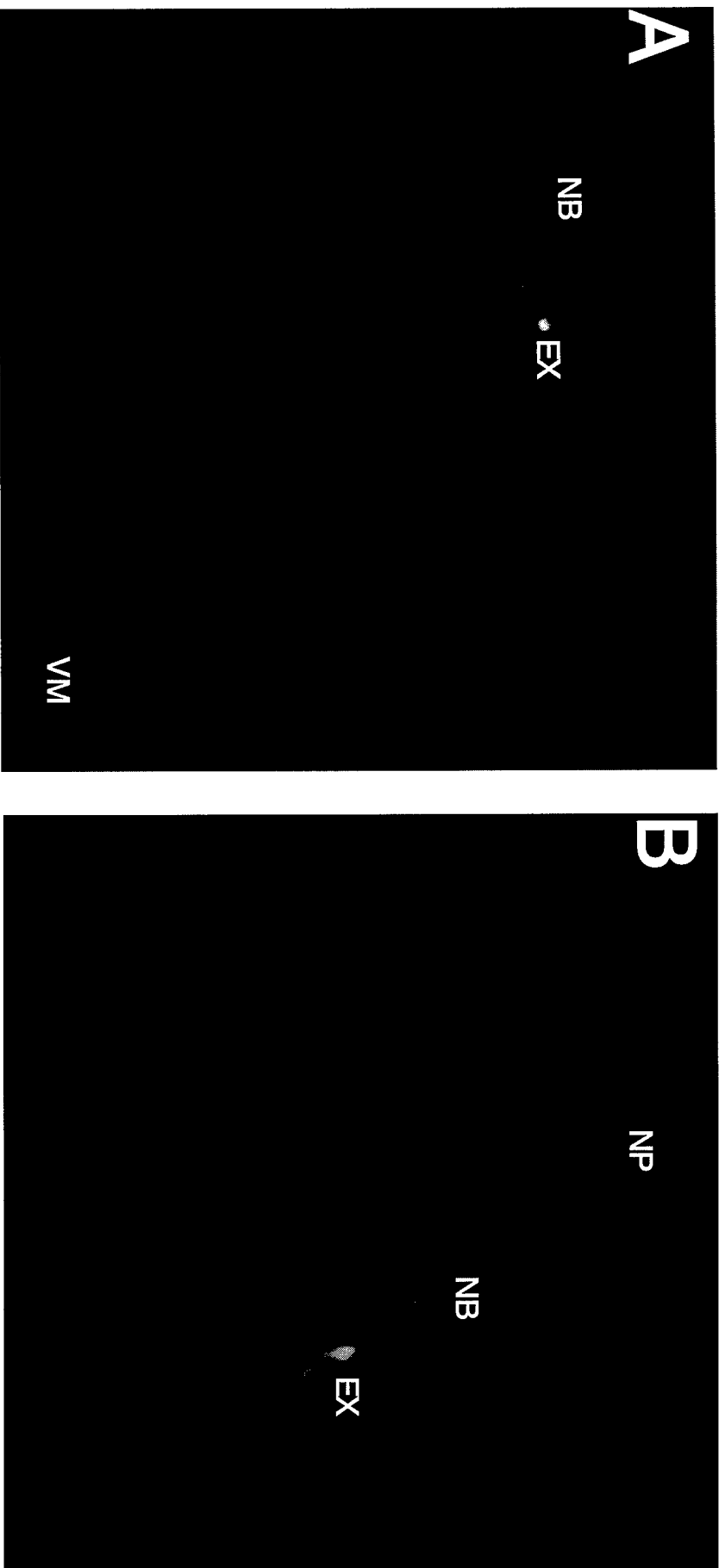


Figure 3: Fluorescence in transgenic worms expressing GFP regulated by the *ceCaMKK* promoter. A, adult hermaphrodite (20X) expressing GFP in the excretory cell (EX), vulval muscle cells (VM) and neurons (NN). B, higher magnification (100X) showing neuron cell bodies (NB) and nerve processes (NP) extending to the amphid.

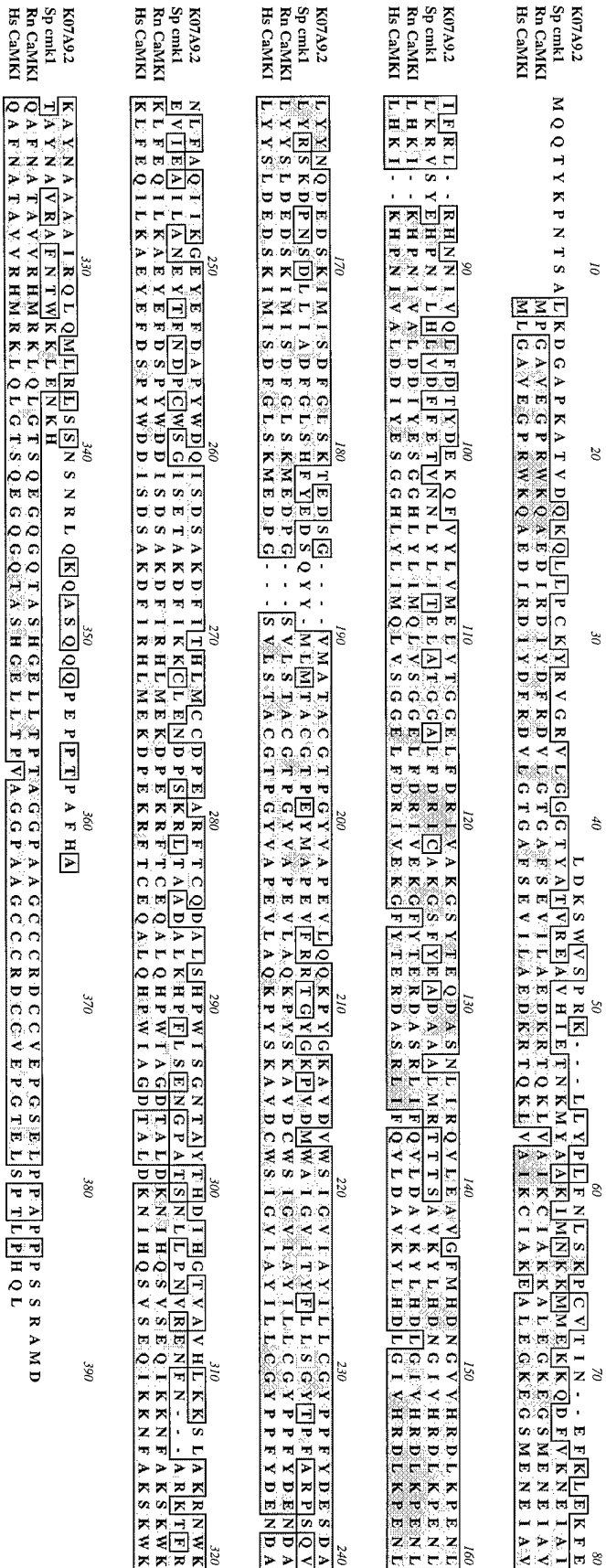


Figure 4: Alignment of K07A9.2 with *S. pombe* (fission yeast) cmk1 gene, *R. norvegicus* (rat) CaMKI and *H. sapiens* (human) CaMKI. The *C. elegans* predicted protein K07A9.2 is 58% identical to human CaMKI.

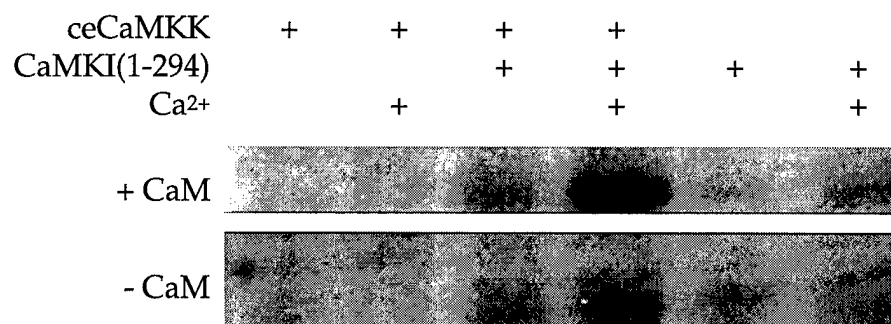


Figure 5: ceCaMKK phosphorylates CaMKI in a Ca²⁺/CaM- dependent manner. *In vitro* phosphorylation of GST-CaMKI(1-294) by ceCaMKK with ³²P- γ -ATP was carried out as described in Methods, with Ca²⁺ and/or CaM present where indicated. Maximum radioactive phosphate incorporation in CaMKI required both Ca²⁺ and CaM in the reaction.



Figure 6: ceCaMKK phosphorylates CaMKI on threonine 177. *In vitro* phosphorylation of GST-CaMKI (wt and T177A mutant) by ceCaMKK in the presence of Ca²⁺/CaM was carried out as described in Methods. Efficient incorporation of radioactive phosphate in CaMKI requires T177.

Acronyms/Abbreviations

ATP	adenosine triphosphate
β -gal	β -galactosidase
<i>C. elegans</i>	<i>Caenorhabditis elegans</i>
CaM	calmodulin
CaMKI	calmodulin dependent kinase I
CaMKII	calmodulin dependent kinase II
CaMKIV	calmodulin dependent kinase IV
CaMKKa	calmodulin dependent kinase kinase A
CaMKKb	calmodulin dependent kinase kinase B
cAMP	cyclic adenosine 3',5'-monophosphate
ceCaMKK	<i>C. elegans</i> calmodulin dependent kinase kinase
COS7	mouse fibroblast cell line
CRE	cAMP response element
CREB	cAMP response element binding protein
EDTA	(ethylenedinitrilo)tetraacetic acid
EGTA	ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid
FISH	fluorescent <i>in-situ</i> hybridization
GAP	GTPase activating protein
GFP	green fluorescent protein
GST	glutathione-S-transferase
IL-2	interluekin 2
MBP	maltose binding protein
PCR	polymerase chain reaction
PKA	protein kinase A; heart muscle cAMP dependent protein kinase
RACE	rapid amplification of cDNA ends
SH3	src-homology 3 domain
X-Gal	5-bromo-4-chloro-3-indoyl-b-D-galactoside

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