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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 important in cell cycle regulation. Using a *C. elegans* 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. By generating transgenic worms with reporter proteins controlled by the ceCaMKK promoter, gene expression has been demonstrated in the excretory cell, vulval muscle cells and several neurons of adult hermaphrodites, in the hypodermal cells of L1/L2 larvae, and in several male-specific tail cells. To examine this pathway biochemically, we have cloned the ceCaMKK and ceCaMKI cDNAs, and produced recombinant proteins by prokaryotic expression methods. Both mammalian and *C. elegans* CaMKKs can phosphorylate either species' CaMKI homologue specifically on the activation loop (T177 in human, T179 in *C. elegans*) *in vitro*. These results indicate a functional homology between the mammalian and *C. elegans* calmodulin dependent kinases, and demonstrate the existence of a calmodulin dependent kinase cascade in the worm. We have used these proteins to begin a screen for cascade targets, but further work remains to reveal the biological functions of this signaling pathway.

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## Introduction

Calcium and calmodulin (CaM) are ubiquitous signaling molecules, implicated a wide variety of cellular functions including cell cycle control (1). $Ca^{2+}$/CaM signals are critical in $G_0$ reentry, $G_1$/S, $G_2$/M, and metaphase transitions in a variety of organisms (2). 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, through the phosphorylation and activation of calmodulin dependent kinases I and IV (CaMKI and CaMKIV) (3-7). In addition, mammalian CaMKK has now been implicated in the regulation of protein kinase B and of the mitogen-activated protein kinase (MAPK) cascade, both of which have significant established roles in cell cycle control and apoptosis (8, 9). This project was designed to assess the biological relevance of these relationships and of the CaMKK itself. Using a *Caenorhabditis elegans* model, any perturbation of normal cell division or cell fates can be identified (10, 11). We have now cloned cDNAs for *C. elegans* isoforms of both CaMKK and CaMKI (ceCaMKK and ceCaMKI), examined their biochemical homology using recombinantly expressed proteins, and used transgenic techniques to determine the developmental expression pattern of ceCaMKK. We are currently pursuing the downstream effectors of this cascade through an *in vitro* screen for kinase substrates of either protein, and attempting to isolate null mutants for these genes. Both of these efforts will make use of the unique genetic and molecular biology tools available to the *C. elegans* model, but rely upon these initial characterizations and comparisons with the mammalian kinases in order to extrapolate from our results insights into the mammalian cascade. Understanding this signaling cascade, from protein interactions to biological consequences, may help unravel the essential functions of calcium signals in the cell cycle.

## Results

Objective 1: Cloning and Biochemical Characterization of ceCaMKK and ceCaMKI

The first step in establishing a CaMK cascade in *C. elegans* was cloning the CaMKK and CaMK proteins. The initial lead came from BLAST searches of the *C. elegans* genome, which identified C05H8.1 as a potential CaMKK homologue. A portion of this genomic region was amplified by PCR from cosmid C05H8, and used to probe approximately 100,000 plaques of the Barstead lambda cDNA library. Three positive clones were obtained which were identical except in the length of their extension to the 5' end. They included unexpected sequence at the 3' end, and a polyadenylation site, but lacked 500 bp of the 5' end of the predicted cDNA. This region was eventually confirmed by a combination of rtPCR and 5'-RACE. Overall, the competed cDNA sequence includes more than 500 bp of sequence 3' to the predicted stop site, as well as the entire predicted coding sequence. The new sequence can be aligned with genome data from the 3' cosmid, F54E7, indicating that two exons have been. As predicted, the deduced amino acid sequence has very high homology with the mammalian enzymes, demonstrating 65% similarity to rat CaMKKα, 46% to CaMKKβ, and 39% to AnCaMKK overall with greater conservation within the catalytic domain (Figure 1). The appended sequence includes the end of the CaM-binding domain, and improves homology up to that point, but also appends sequence diverging from the rest of the CaMKK family and without significant homology to any reported proteins.

The search for a CaMKI homologue to complete the cascade also began by BLAST searches. Although the predicted gene K07A9.2 lacked an ATP binding motif, and so would not have been expected to be a functional kinase, it was otherwise an excellent match for CaMKI. The remaining 5' exons were identified in the upstream genomic sequence (Y39C2) using tBLASTn to find potential alignments with the mammalian CaMKI ATP-binding motif. Identifying the missing exons in this way allowed the complete cDNA to be cloned by rtPCR. RACE confirmed the correct end of the gene, and provided the sequence of the 3' UTR. Overall, the translated sequence is more similar to human CaMKI (69% overall, 82% within the catalytic domain) than CaMKIV (43% overall, 67% catalytic domain) or the Aspergillus homologue, anCaMKB (48% overall)(Figure 2).

To functionally compare the *C. elegans* proteins with their mammalian homologues, recombinant proteins were made from the ceCaMKK and ceCaMKI cDNAs by bacterial expression of $His_6$- and GST- fusion proteins respectively. As expected from their sequence homology, ceCaMKI and ceCaMKK bind CaM in a calcium dependent manner by CaM-overlay (Figure 3A). Binding by ceCaMKI is comparable to human CaMKI. However, the recombinant ceCaMKK was poorly expressed, and only amenable to partial purification, so it is difficult to determine relative binding to CaM compared with recombinant rat MBP-CaMKKβ (rnCaMKKβ). Recombinant ceCaMKK requires $Ca^{2+}$ and CaM for maximal phosphorylation of recombinant, human GST-CaMKIα(1-294) (hsCaMKI(1-294)), but does have some $Ca^{2+}$/CaM-independent activity (Figure 3B). ceCaMKK phosphorylates both hsCaMKI and ceCaMKI, but not the activation loop mutants (Thr177Ala and Thr179Ala, respectively, Figure 4A). Recombinant ceCaMKI is also phosphorylated by rat CaMKKβ on its activation loop at Thr179 (Figure 4B). Addition of either ceCaMKK or rnCaMKKβ to either ceCaMKI or hsCaMKI, activates the CaMK activity toward site-1 peptide (Figure 4C), although the recombinant rnCaMKKβ is more efficient. Neither ceCaMKK or rat CaMKKβ show measurable activity toward site-1 peptide on their own.

Objective 2.1: Expression Pattern and Subcellular Localization of ceCaMKK

To compare with the localization of both ceCaMKI and mammalian CaMKKs, and to predict possible biological roles, we examined the expression patterns and subcellular localization of ceCaMKK. To obtain the cell-specific developmental expression patterns of the ceCaMKK gene, a variety of transgenic nematodes were generated. The promoter region of the genomic DNA, arbitrarily defined as 2.4 kb 5' of the predicted translational start site, was subcloned into a modular transgenic vector encoding either the β-galactosidase (β-gal) fused to a nuclear localization signal (NLS) or green fluorescent protein (GFP) gene (12). Each construct, together with a plasmid containing the *rol-6* gene, was microinjected into the uterus of gravid hermaphrodites, and transgenics were selected based on the *rol-6* phenotype. Transgenic were generated at the expected frequency and developed normally. Three independent lines for each construct displayed similar expression patterns.

Staining of transgenic adult hermaphrodites expressing β-gal revealed protein expression only in peripharyngeal cells, the excretory cell and vulval muscle cells (Figure 5). In addition, the promoter was active in hypodermal cells of L1/L2 larvae and in male tail cells (Figure 6). Using the GFP derivative of this transgenic construct, which lacks the nuclear localization sequence, we have confirmed that the peripharyngeal cells are neurons, most likely sensory, which extend processes to the amphid (data not shown).

To determine the subcellular localization of the ceCaMKK in living nematodes, a transgenic construct was created fusing the cDNA with GFP under control of the same 2.4 kb promoter used for the reporter constructs. These transgenic animals were produced from injections at unusually low frequency, and few were found to transmit the transgene to progeny. Many were sterile with morphological changes in the ovaries and spermatheca. As a result, rolling animals in the F1 generation, which were mosaic for the transgene, were examined. Fluorescence was observed in the vulval muscle cells and the amphid sensory neurons as with the other constructs, and was generally uniform throughout the cells but had significantly reduced fluorescence in the nucleus (Figure 7). Decreasing the concentration of injected DNA appeared to improve the survival of these transgenic animals, and has permitted the establishment of several lines.

Objective 2.2: Gene suppression and search for null mutants

The second focus of objective two, obtaining and characterizing null mutants of ceCaMKK, is still in progress and has been extended to include the ceCaMKI homologue in parallel. Two methods have been employed - PCR screening of mutagenized worm libraries and RNAi. PCR screening of mutant libraries requires the construction of a mutagenized deletion worm library, which can be generated using well-established techniques (13). Our lab participated in the construction of a library of chemically mutagenized worms in collaboration with four other labs at Duke, however, PCR screening of this library did not yield any relevant mutants. The same basic strategy has been adopted by the Sanger Center's *C. elegans* Gene Knockout Program, with the goal of eventually obtaining knockouts of every known worm gene (14). We have requested that they screen their libraries for deletions of both ceCaMKK and ceCaMKI genes. Recently, after the term of this grant, we have received a deletion mutant in the CeCaMKI gene, and are currently finishing the back-crossing with wild type worms before beginning to characterize the phenotype. The Sanger Center is working on methods to improve the library generation and screening procedures, and will continue to search for deletions in the CeCaMKK loci.

The alternate method for obtaining null mutants in a gene involves the poorly understood phenomenon of RNAi(15). Using Bluescript vector containing either complete cDNAs or 5' fragments of the cDNAs as the template, we have generated dsRNA corresponding to both ceCaMKK and ceCaMK cDNA's by *in vitro* transcription. The purified, annealed, dsRNAs were injected into early larvae and embryos in gravid adults, however no phenotype was observed. This could indicate that there is no developmental consequence of ceCaMKK or ceCaMKI gene suppression, that the consequence is not obvious without appropriate testing, or that the gene has not been successfully suppressed by the dsRNA. We have tried simultaneous injection of both ceCaMKK and ceCaMKI dsRNA, since if these genes are in the same pathway as we expect, a more severe phenotype might be expected with partial suppression of both compared with either alone. No phenotype was obvious following these injections. We have been able to use the unc-22 gene as a positive control to generate the expected twitching phenotype with reasonable penetrance, indicating effective use of the RNAi technique.

Objective 3: Targets of the CaMK Cascade

With the identification and expression of the ceCaMKI, the focus for screening for ceCaMKK pathway targets has turned to finding substrates of the kinase cascade. Following the methods successfully applied by Tony Hunter and colleagues in the screen for MAPK substrates (16), we have been developing an *in vitro* screen for protein substrates of this kinase cascade. We have a unidirectional cDNA library based on the lambda-ZAP-XR vector (Stratagene); this type of library has been used for antibody expression screens. The basic strategy involves inducing a growing phage cDNA library with IPTG impregnated nitrocellulose membranes. The filters bind proteins from the bacterial lysates made by the lytic phage infection, including the expressed cDNA specific to each phage. The filters are blocked, washed and incubated with ATP (to avoid isolating ATP-binding proteins), before they are exposed to activated kinase in the presence of $\gamma$-$^{32}$P-ATP. After further washes to remove unincorporated isotope, the filters are exposed to film, and positives appearing on both duplicate filters can be selected for further screening. The reaction conditions have been adapted to accommodate the calmodulin-dependent protein kinases by including calcium, calmodulin and increased ATP in the reaction buffer and by preincubating the kinase with kinase kinase at high ATP concentrations to activate the kinase. The ceCaMKK has been difficult to purify and is only produced in small quantities, so recombinant mammalian CaMKKB is used to activate ceCaMKI used for the screen. Samples of the final kinase buffer are very reactive against purified substrates.

We tested and optimized these methods using a lambda-ZAP (Stratagene) control phage containing a convenient protein substrate sequence as an insert (the 1-117 amino acid fragment of p300, referred to as $\lambda$117) (17). Bacteria infected with $\lambda$117 have been used in comparison with bacteria infected with wild type phage ($\lambda$WT) to test conditions for the screen. Although we had no trouble identifying $\lambda$117 and $\lambda$WT derived bacterial extracts in a crude kinase assay resolved by SDS-PAGE, with a very strong band corresponding to the 1-177 p300 fragment, the background on the filter lifts remained unexpectedly high. By changing detergents, shortening the kinase reaction time, and increasing the specific activity of our radioactive ATP mix, we were able to reduce background to the point that a full scale screen was attempted. From duplicate lifts of five plates, with approximately 5000 plaques each, we followed 20 putative positives. Secondary screens reduced the number to 8, which we then excised from the phage and expressed as bacterial fusions. None of the 8 were substrates for the cascade *in vitro*.

Following these disappointing results from plaque lifts, we decided to try another approach for identifying substrates. Based on our success at picking phosphorylated 1-117 fragment out of bacterial extract on a gel, we decided to develop a screen using in vitro phosphorylation of raw extracts. This approach became feasible through a collaboration with Tim Haystead, who has used two dimensional gel electrophoresis (2DE) and microsequencing techniques to identify signaling targets in whole cells (18). Using our experience from developing the first screen, we optimized kinase reaction conditions to favor the exogenous kinase cascade (ceCaMKI and KKB added to worm extract) over endogenous kinases. The reactions are run with $\gamma$-$^{32}$P-ATP and separated by 2DE, and transferred to a sequencing compatible membrane. Many proteins were seen on the autoradiogram that were phosphorylated in the presence of exogenous kinases but not in extract alone, however these targets were in too low abundance for microsequencing. We enriched the extract for these targets by fractionation with anion exchange chromatography before electrophoresis, and ran reactions with and without exogenous activated kinase. These reactions were run on standard SDS-PAGE gels, and again allowed identification of many differences in phosphorylation patterns (Figure 8). Five bands were sequenced, four were mixtures too complex for unique identification, but the last was

identified as vitillogenin-5. To improve separation, kinase reactions using each fraction were further resolved by 2DE. From these membranes, several more substrates have been identified: aldehyde reductase, paramyosin, PKC Substrate and Splicing factor-associated 32K chain (Figure 9). All of these substrates have potential CaMK sites in their predicted primary sequence, but none have yet been confirmed using recombinant proteins.

**Discussion**

Objective 1: Cloning and Biochemical Characterization of ceCaMKK and ceCaMKI

The first project objective of cloning ceCaMKK has been completed. In addition, during the course of the project, we also identified and cloned a homologue to the downstream kinase CaMKI. Although arguably part of Aim 3 in the original statement of work, it is essential to characterize both pathway members together, and compare them as a pathway with the mammalian homologues, so this result is probably more appropriately included in Aim 1. Both of these genes were independently cloned and subsequently published by a competing group (19, 20), although their seqeuence for the ceCaMKK is not complete due to an oversight in the rtPCR based cloning strategy (19). The cloning alone confirms conservation of CaMK cascade elements in nematodes, and they appear to be the only CaMKI and CaMKK homologues in the genome. The next best matches from BLAST searches of the wormpep database of known and predicted nematode proteins are unc-43, the CaMKII homologue for ceCaMKI (40% identity), and a predicted AMP-dependent protein kinase homologue, T01C8.1 for ceCaMKK (31% identity). Although the predicted exons were basically correct, both predicted genes were incomplete based on the final cloned cDNA, so as powerful as *in silico* cloning has become, it cannot replace empirical evidence. Interestingly, the missed exons in both cases were located in the YAC/cosmid adjacent to the cosmid containing the predicted gene. The exon identification problem may have stemmed from lack of continuity in the genomic sequence used for analysis. The omissions made by the GeneFinder program in these cases can be used to improve the next generation of genomic analysis software.

It is still worth considering, given the frequency of alternative splicing in *C. elegans*, that other gene products may exist. Multiple gene products have become a frequent finding for CaMKs in mammalian genomes; mammalian CaMKKβ (21), CaMKI (22, 23), CaMKII (24) and CaMKIV (25) genes all appear to produce multiple isoforms with differences in tissue distribution, and in some cases, with functional distinctions such as changes in subcellular localization. In the case of ceCaMKK, there was no evidence for a second transcript after multiple cDNA library screens, and no other cDNAs have been sequenced from that region of the genome. However, the first intron of the ceCaMKI gene is unusually large, and could easily harbor a promoter analogous to that of calspermin in the CaMKIV gene (25). This region remains an area of uncertainty in the genomic sequencing data, although it has been partially resolved by further 5' sequencing on cosmid K07A9, which now includes the entire ceCaMKI gene. It contains several large repeats and regions of low complexity sequence. The cDNA database contains multiple ESTs with sequence homology to this intron, but all have matches to other genomic sequences as well making them difficult to specifically attribute to this region. The complete ceCaMKI cDNA we have cloned has since been reproduced in the sequence tags of yk747c11, and the work of a second lab (20). In addition, western blots using polyclonal anti-ceCaMKI antibodies revealed only a single immunoreactive band in nematode extract enriched

for CaM-binding proteins by CaM-sepharose (20). Unfortunately, northern blotting, the most direct way to address the question of multiple transcripts, was not sufficiently sensitive to detect either message in total mRNA (data not shown). This is not unexpected given recent experiments using oligonucleotide arrays in which the relative expression levels of every gene or predicted gene in the *C. elegans* genome were analyzed (26). Both C05H8.1 (ceCaMKK) and K07A9.2 (ceCaMKI) were found at or below the minimal level of detection in all developmental stages. We have generated transgenic expression constructs that include various regions of the ceCaMKI promoter and first introns to ascertain if there is an internal promoter which might regulate a second gene product.

Sequence homology does not guarantee similarity in function. To confirm a functional homology, the full length ceCaMKK and ceCaMKI cDNAs have been used for recombinant expression to study the cascade biochemistry. ceCaMKK has been difficult to express by both bacterial and baculovirus methods, and cannot be purified to a single band by SDS-PAGE. In constrast, ceCaMKI is readily produced in bacteria as a GST fusion protein, and can be purified to near homogeneity. When used for kinase assays, these enzymes are remarkably similar to the mammalian forms. They bind CaM in overlay experiments and are $Ca^{2+}$/CaM dependent for kinase activity. In standard *in vitro* kinase assays, ceCaMKI is phosphorylated and activated by either recombinant mammalian CaMKKB or ceCaMKK, and similarily ceCaMKK is able to phosphorylate and activate mammalian CaMKI as well as ceCaMKI. Mutation of threonine 179 to alanine in the activation loop (analogous to T177A in the human form) abolishes phosphorylation by kinase kinases and prevents activation. One notable difference from recombinant hsCaMKI was autophosphorylation. The site(s) have not been determined, but an excellent candidate is Ser10, which is in a context similar to many known CaMK substrate sequences ($^{1}$MPLFKRRDG<u>S</u>GPAP$^{14}$). Since phosphorylation of N-terminal serines is required to relieve a unique second autoinhibitory mechanism during the activation of hsCaMKIV (27), this region deserves more exploration for possible regulatory functions. Interestingly, the same sequence of ceCaMKI has been identified as a putative nuclear localization signal, so its phosphorylation state could serve multiple regulatory functions.

Finally, interchangeability of ceCaMKK and ceCaMKI with the mammalian enzymes has been demonstrated in transfection experiments, using CREB activation as a reporter (20). Both CREB phosphorylation, determined by anti-phospho CREB antibodies, and CRE-reporter gene transcription were stimulated by ionomycin treatment following co-transfection with ceCaMKK and ceCaMKI. This confirms the ability of these proteins, at least in the context of transient overexpression, to reproduce functions of the mammalian cascade.

<u>Objective 2.1: Expression Patterns and Subcellular Localization</u>

Determining localization, particularly for proteins believed to act in concert, is essential for attributing biological roles. Transgenic expression techniques have been used because the low mRNA levels make *in situ* hybridization impractical (and unsuccessful in several attempts), and no antibody for the ceCaMKK is available. The reporter constructs make use of a presumed promoter region, and thus cannot be assumed to represent the actual protein distribution. However, in other studies of this type, the only frequent differences between the transgenic results and actual protein distribution involved expression in the embryonic stages where transgene reporters are generally not expressed. It is also possible, since the transgene is carried in an extra-chromosomal array, that the nematodes are mosaic. This possibility was reduced by

analyzing several lines of transgenics, and waiting until the F3 generation for analysis. Both nuclear localized and non-localized reporters were used to analyze the cellular expression pattern in order to facilitate identification of specific cells.

The expression of ceCaMKK is limited, but, like it's mammalian counterparts, it is found in subsets of neurons but not exclusively neuronal, is developmentally regulated, and overlaps with the expression of its putative target, ceCaMKI. In adult hermaphrodites, the transgene was only expressed in three cell types - the vuvlal muscles, the excretory cell, and amphid neurons. At first analysis, these cell types appear to have little in common, but in fact they all adapt to environmental conditions. Vulval muscles modify responses to acetycholine-induced egg laying according to serotonin and neuropeptide stimulation. These responses are essential in the switch from quiescent to active modes of egg laying, and require several $Ca^{2+}$ signaling proteins including an L-type voltage gated $Ca^{2+}$ channel and a PKC homologue (28). Similarly, the excretory cell is required for osmoregulation, and is thus involved in adaptive responses to the environment. Finally, the ciliated amphid sensory neurons use processes extending from cell bodies in the region of the nerve ring to the amphid to gain access to the external environment, allowing them to respond and adapt to chemosensory stimuli.

The expressing cells in the male tail are more difficult to specify. Based on their position near the preanal ganglion, they could also be sensory neurons (29), which would be functionally consistent with the amphid neuron expression. Young L1/L2 nematodes of both sexes express the transgene in hypodermal cells, which are still undergoing cell divisions and development. However, they are not precursors to any of the cells observed expressing the reporter in the adult.

The expression pattern of ceCaMKI has also been recently determined in hermaphrodites, both using transgenic methods and immunohistochemistry.[1] Both methods find expression in amphid sensory neurons, but the antibody also indicates vulval expression, and the transgenic GFP reporter vector expresses in several other neurons that have not yet been specifically identified. Thus, ceCaMKI expression overlaps with that of ceCaMKK at least in sensory neurons, but possibly also in the vulval muscles. Differences between the expression patterns could exist where these proteins serve other functions, or where the expression pattern is incompletely described due to the limitations of the methods used. This limited distribution of ceCaMKI is unlike the ubiquitous mammalian CaMKI, but similar to the limited, primarily neuronal and endocrine expression of CaMKIV. Nonetheless, overlapping expression of ceCaMKK and ceCaMKI in sensory neurons is of particular interest because most of the known elements in CaMK regulation of CRE-dependent expression have been reported in amphid sensory neurons, including *C. elegans* homologues of CREB (30) and CaMKII (31). Since the sensory neurons can be easily tested for function and adaptive changes, this presents an attractive experimental approach for dissecting the functional significance this pathway *in vivo*.

The subcellular localization of ceCaMKK was investigated by fusing the ceCaMKK cDNA to GFP in the transgenic vector, allowing observation of subcellular localization in live animals. The images are not confocal, so it is not absolutely clear whether the GFP is completely excluded from the nucleus, or is simply more concentrated in the cytoplasm. This construct has the potential for analysis of changes in subcellular localization in living nematodes in response to stimuli or drug treatment. Initially, unlike the other transgenic constructs, no nematodes passed on the transgene to the F2 generation. Further investigation found that most of the F1 progeny were sterile, and had morphologic changes in their gonads. The rest of the construct had been successfully used for other lines of transgenic animals, so these effects were

[1] H. Tokumitsu, unpublished observations

not likely to be a result of promoter interference, and were probably attributable to the overexpression of the ceCaMKK cDNA. Since the standard method of injection creates an extrachromosomal array containing hundreds of transgene copies, and alternative methods integrating low copy numbers have been able to establish useful transgenic lines by reducing the relative overexpression (32), it is not surprising that decreased concentrations of DNA allowed the establishment of transgenics capable of reproduction. The phenotypes observed with higher levels of overexpression may be significant indicators of potential ceCaMKK functions. In addition to localization experiments, these cDNA-GFP transgenic nematodes will be a valuable asset in determining the success of RNAi suppression of gene expression. dsRNA injections have not produced any apparent phenotypes, but there is currently no way to evaluate the degree of suppression before proceeding with more detailed phenotypic analysis. These transgenics will provide the necessary readout for gene suppression, since dsRNA injected should suppress both the endogenous gene and the cDNA/GFP fusion (15).

The discrepancy between CaMKK and CaMKIV subcellular localization has been a difficult problem to explain, since apparently cytoplasmic CaMKKs can increase nuclear CaMKIV-dependent transcription. The *C. elegans* proteins suffer from the same discontinuity, with ceCaMKI localized to the nucleus at least in cell culture experiments (20), and ceCaMKK is apparently concentrated in the cytoplasm of living nematodes, if not excluded from the nucleus. There have been reports that CaMKKs do reside in the nucleus, including a recent immunohistochemistry paper using electron microscopy that found CaMKKβ exclusively cytoplasmic in neurons of some areas of the rat brain, but in the nucleus of neurons in other areas (33). We currently have no mechanism to explain these differences, but the significance for modulating potential CaMK cascade effects is obvious.

Objective 2.2: Gene suppression and search for null mutants

The second focus of objective two, obtaining and characterizing null mutants of ceCaMKK, is still in progress and has been extended to include the ceCaMKI homologue in parallel. After many screening efforts both independently and through the Gene Knockout Consortium, we have recently obtained (after the term of this grant) a deletion mutant in the ceCaMKI gene. We look forward to characterizing the phenotype once the back-crossing has been completed, and hope to find a similar mutant in the ceCaMKK gene. However, since many librbaries have already been screened for that locus, we may also return to RNAi for ceCaMKK suppression. The effectiveness of the expression block varies with the gene, the dsRNA used, the cells involved and the stage of development. It is most effective during early development, but has been observed to cause adult phenotypes and sometimes persists into another generation (15). All of our attempts to apply these methods to repress ceCaMKI and ceCaMKK expression have failed. Being unable to confirm the presence or absence of either RNA message or protein in a worm makes it difficult to interpret attempts to use RNAi suppression of the gene, but we believe the cDNA-GFP fusion transgenics will provide a way around this problem. Using ionizing radiation to incorporate the transgenic extrachromosomal array, we will generate worms that stably express the GFP-cDNA fusion. When these worms are treated with RNAi, the GFP fluorescence should be blocked as well as the endogenous ceCaMKK expression, providing a visual confirmation that RNAi has been successful. Once we are certain that we have suppressed gene expression, we will challenge the worms with a variety of tests to try to determine the phenotype, including responses to pheromones that depolarize the amphid sensory neurons.

Interestingly, reports in the Worm Breeder Gazette of the worm CREB homologue, a likely target of this cascade, indicate that null mutants have a constitutive dauer phenotype when they are grown at 27°C, but appear unaffected at normal temperatures. We will likely need to use tests of this sort to reveal phenotypes after suppressing the CaMK cascade. The search for null mutants of ceCaMKK, and attempts to analyze the phenotypes will continue to be a major focus of our investigations.

Objective 3: Targets of the CaMK Cascade

The final objective of the project is to identify other components of the ceCaMKK pathway. We have tried several methods over the course of the project. Using molecular biological techniques to generate protein expression libraries is an attractive way to avoiding issues of relative abundance of proteins. These methods involve expressing substrates in a phage library, binding them to a membrane, and carrying out phosphorylation in solid phase to maintain separation (16). In our experience using CaMKI with this type of screen, it was difficult to obtain reliable signal over background even when using the 117(wt) recombinant protein expressed in a phage. In addition, this type of assay restricts the proteins that are tested to those which can be produced in bacteria, can be phosphorylated in solid phase, and do not require any post-translational modifications.

As an alternative, we developed a kinase assay using extracts of mixed stage worms as a substrate. By comparing phosphate incorporation in the presence and absence of exogenous, activated kinase, differences were readily identified that were likely due to direct phosphorylation by ceCaMK. Fractionating *C. elegans* extract before the kinase reaction, we were able to significantly increase sensitivity toward less abundant substrates. The methods employed are simply the application of well established activity-directed purification strategies to a general screen, and, although labor intensive, can overcome problems of abundance and are amenable to automation. Unlike traditional biochemical purification, we only required one fractionation step, along with two-dimensional resolution of proteins on a gel, to identify several possible substrates. These substrates need further study in order to assess their significance. Nonetheless, by combining traditional biochemistry with microsequencing, we can achieve sensitivity that rivals molecular biological techniques without any of the caveats those methods introduce. And, regardless of the physiologic relevance of each individual substrate, further biochemical characterization and identification of specific target sequences favored by CaMKI will provide a better idea of determinants required to be a target of the CaMK cascade. By comparing expression patterns reported in the *C. elegans* database with those of the CaMK cascade, we will be able to further limit the number of candidates for physiologic substrates, which will require more extensive investigation.

## Key Research Accomplishments

1. Cloning of the *C. elegans* homologue of the calmodulin dependent protein kinase kinase (ceCaMKK)
2. Prokaryotic expression of recombinant ceCaMKK
3. Confirmation of biochemical homology between mammalian CaMKK and ceCaMKK
4. Determination of the developmental and cell specific expression pattern of ceCaMKK in *C. elegans* using transgenic methods
5. Cloning of the *C. elegans* homologue of the calmodulin dependent protein kinase I, ceCaMKI
6. Development of transgenic vectors for visualizing the subcellular localization of CeCaMKK in intact worms.
7. Prokaryotic expression of recombinant ceCaMKI as a GST fusion protein
8. Confirmation of biochemical homology between mammalian CaMKI and ceCaMKI
9. Development of *in vitro* screening methods for identifying substrates of the CaM-dependent kinases
10. Identification of several potential substrates of ceCaMKI

## Reportable Outcomes

1. Corcoran, E.E., Freedman, J.F., Means, A.R. Elucidating the Role of CaMKK in Cell Cycle and Cell Fate Using a *C. elegans* Model. Abstract for 10th International Symposium of Calcium-Binding Proteins and Calcium Function in Health and Disease, June 17-21, 1997.
2. Corcoran, E.E., Freedman, J.F., Means, A.R. Elucidating the Role of CaMKK in Cell Cycle and Cell Fate Using a *C. elegans* Model. Abstract for Era of Hope, Department of Defense Breast Cancer Research Program Meeting, June 8-11, 2000.
3. Corcoran, E. E., Means, A. R. Defining Ca2+/calmodulin-dependent protein kinase cascades in transcriptional regulation. *J Biol Chem* 276, 2975-8. (2001).
4. Corcoran, E.E. Identification and Characterization of a Calcium/Calmodulin-Dependent Protein Kinase Cascade in the Nematode, *Caenorhabditis elegans*. Ph.D. Thesis, Department of Pharmacology and Cancer Biology. Durham, NC: Duke University, 2001:164.
5. Means, A.R., renewal for NIH grant GM33976-13

## Conclusions

In this project, we have identified and cloned a calmodulin dependent protein kinase cascade in *C. elegans*. Using recombinant expression, we have confirmed biochemical homology between the *C. elegans* and mammalian CaMKK and CaMKI, and are continuing to search for targets of the cascade by *in vitro* screening methods. To define biological functions of this cascade, we have investigated the expression patterns and gene regulation of these kinases, and applied the available reverse genetic techniques to try to obtain null mutants. These results form the necessary foundation for further exploration of this kinase cascade in the *C. elegans* model organism, by providing clues about where to look for mutant phenotypes and by validating comparisons between the *C. elegans* and mammalian pathways. We have continued

to work toward the project goals, as their completion will be a significant advance in our understanding of the biological roles of calcium, calmodulin and calmodulin dependent protein kinases.

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## Appendices

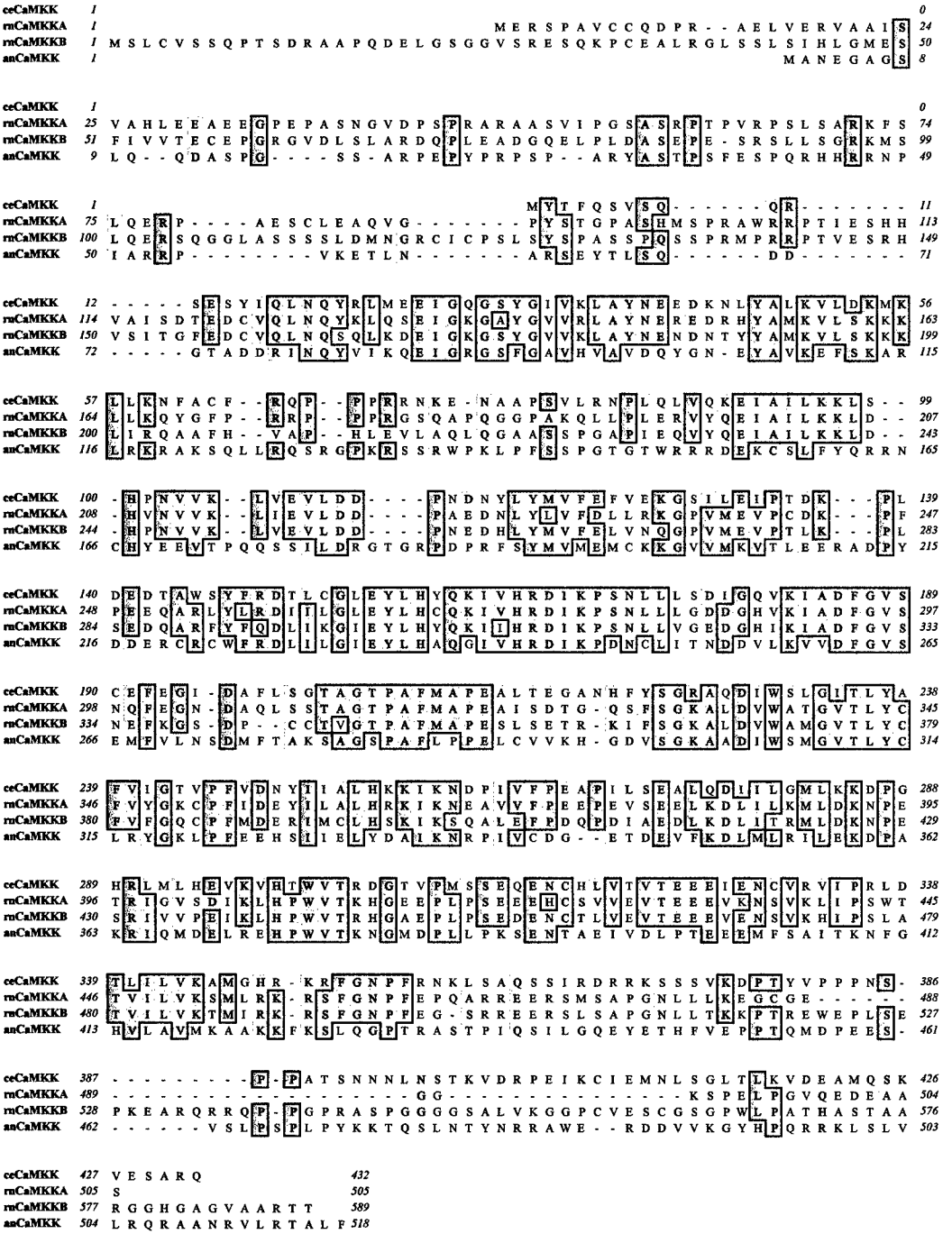



**Figure 1:** Alignment of CaMKK family members

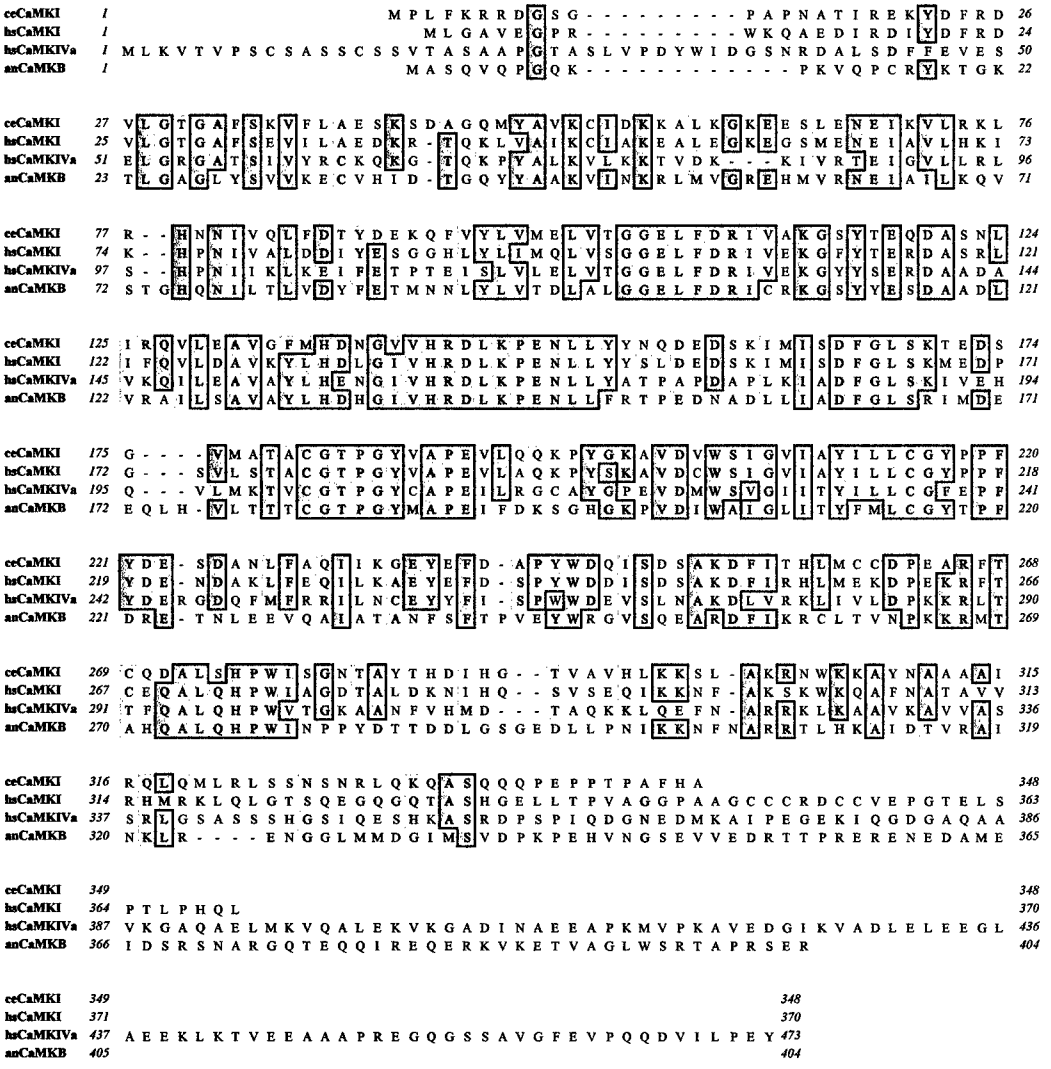



**Figure 2:** Alignment of CaMK family members.

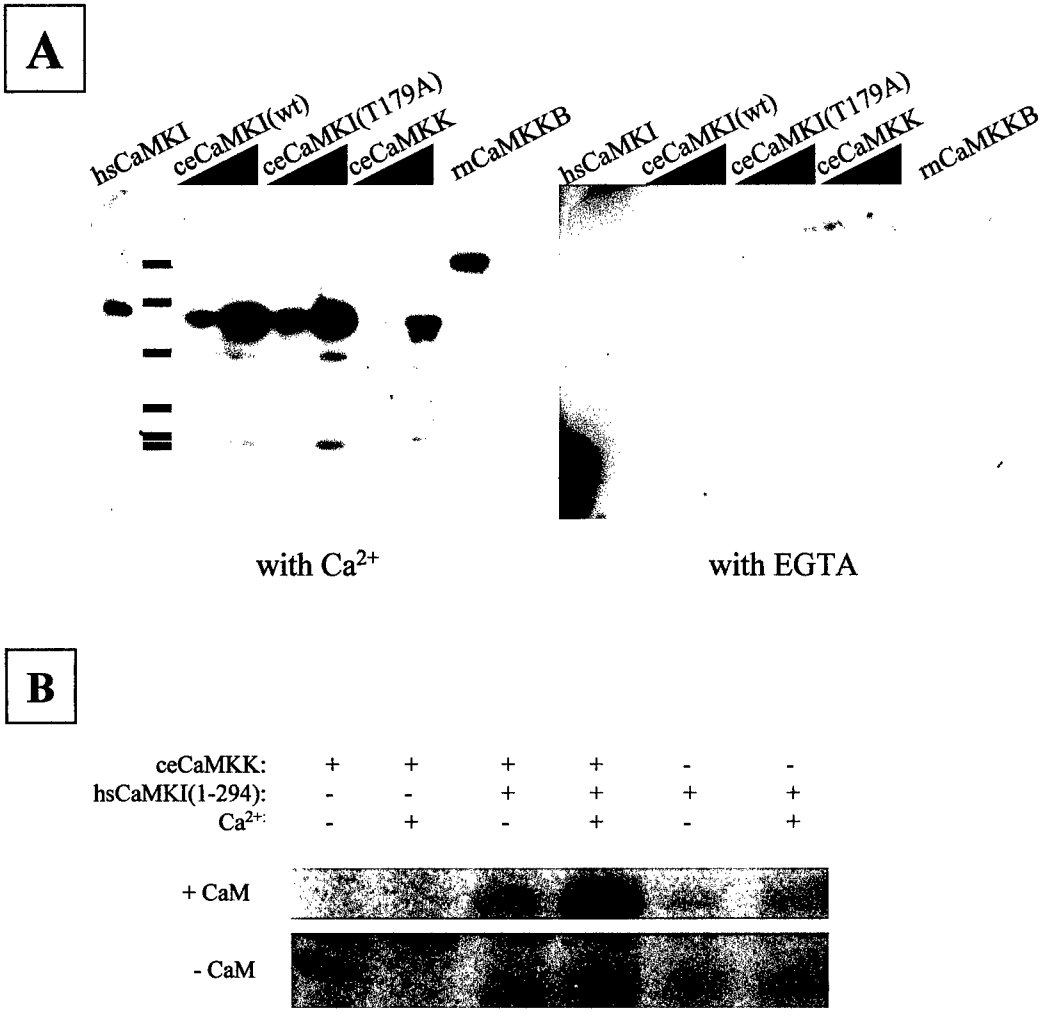



**Figure 3:** $Ca^{2+}$/CaM binding and regulation of ceCaMKK. **A,** $^{125}$I-CaM binds to ceCaMKK and ceCaMKI in a calcium-dependent manner. 500 ng of hsCaMKI and rnCaMKKß positive controls were loaded. Both 500 ng and 2.5 µg of ceCaMKI(wt), ceCaMKI(T179A) and ceCaMKK were loaded as indicated by the ramps. **B,** maximal phosphorylation of hsCaMKI(1-294) by ceCaMKK requires $Ca^{2+}$/CaM. Indicated reaction mixtures (500 ng ceCaMKK, 250 ng CaMKI(1-294)) were incubated with ?-$^{32}$P-ATP for 20 m, resolved by SDS-PAGE and exposed to film.

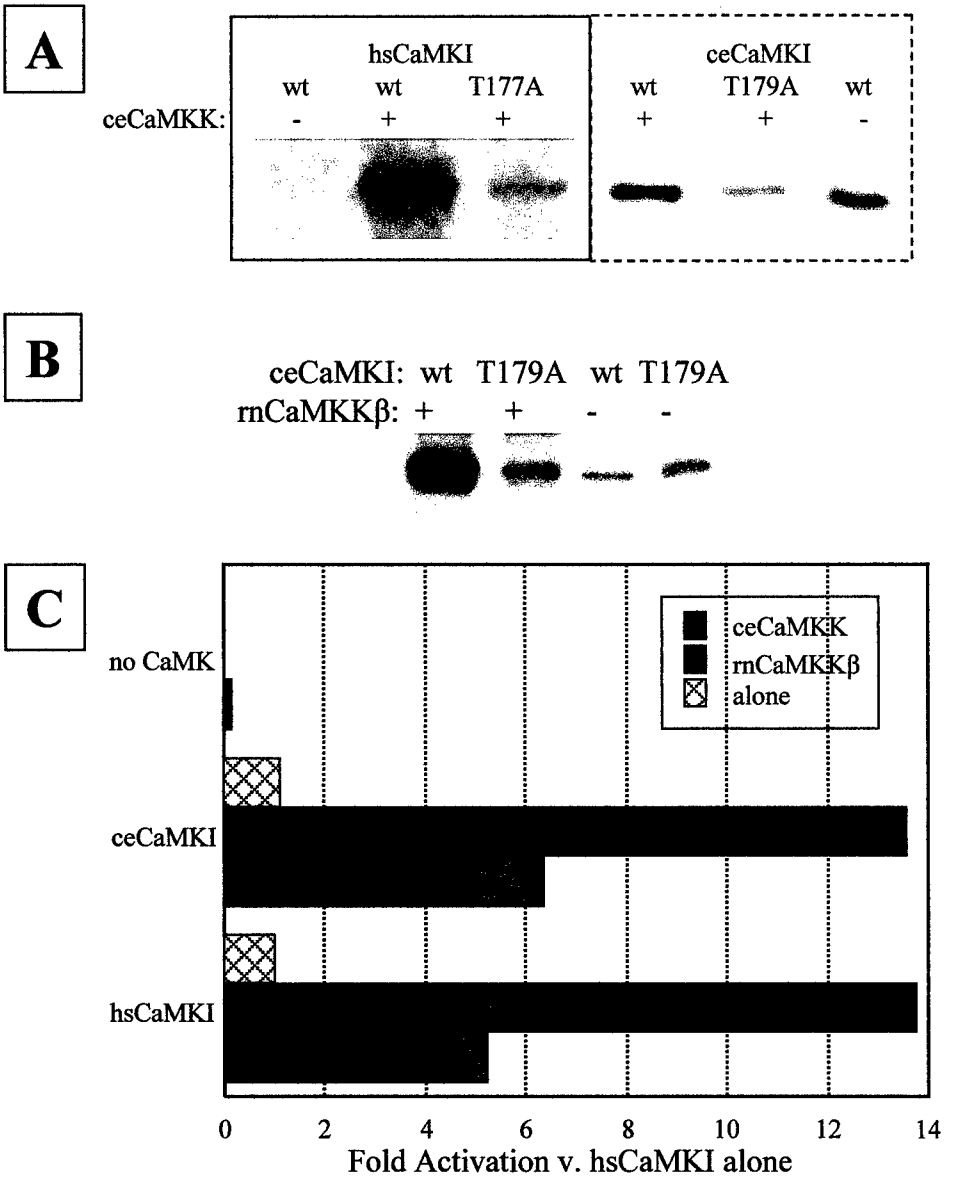



**Figure 4:** Activation of CaMKs by CaMKKs (all at 30°C). **A**, ceCaMKK (250 ng) phosphorylates either hsCaMKI or ceCaMKI (100 ng) on the activation loop (Thr177 and Thr179 respectively, 20 min rxn). **B**, ceCaMKI (100 ng) is phosphorylated by rnCaMKKβ (250 ng) on its activation loop Thr179 (20 min rxn). **C**, in a 2 min DE81 filter peptide assay with 200 μM site-1 (LRRRLSDANF) as substrate, preincubation with ceCaMKK (50 ng) enhances ceCaMKI and hsCaMKI (10 ng) activity but not as efficiently as rnCaMKKβ

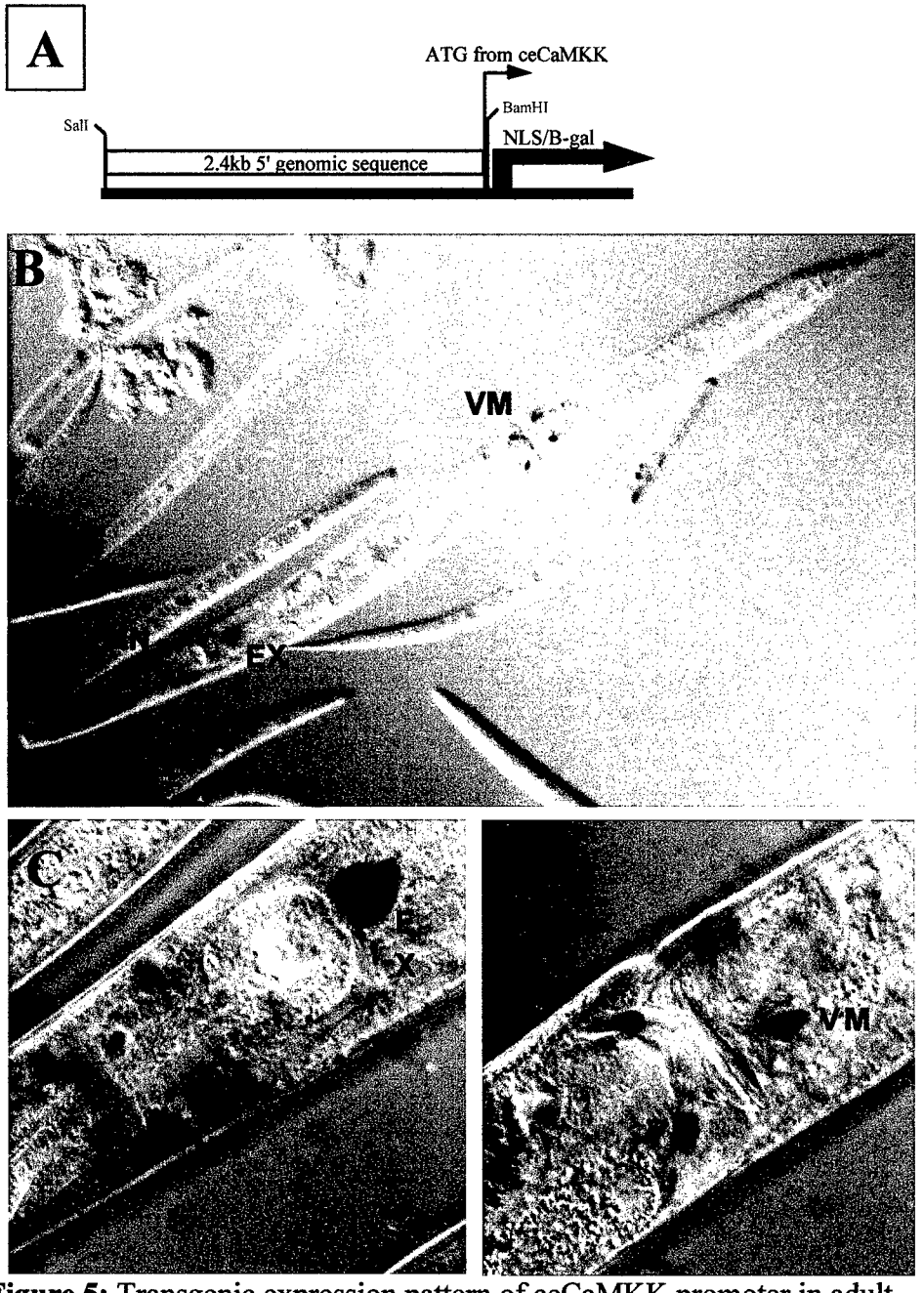



**Figure 5:** Transgenic expression pattern of ceCaMKK promoter in adult hermaphrodites. **A**, schematic representation of the vector used to generate these transgenic nematodes. A 2.4 kb piece of genomic DNA 5' of the ceCaMKK start codon was used to drive a β-gal reporter with an NLS. Restriction sites relevant to cloning are shown. **B**, 20X image of an adult hermaphorodite transgenic nematode stained for β-gal expression. Notice signal in the vulval muscles (VM), the excretory cell (EX) and neurons in the amphid (NN). **C**, 100X image of the neurons and excretory cell. **D**, 100X image of the vulval muscles.

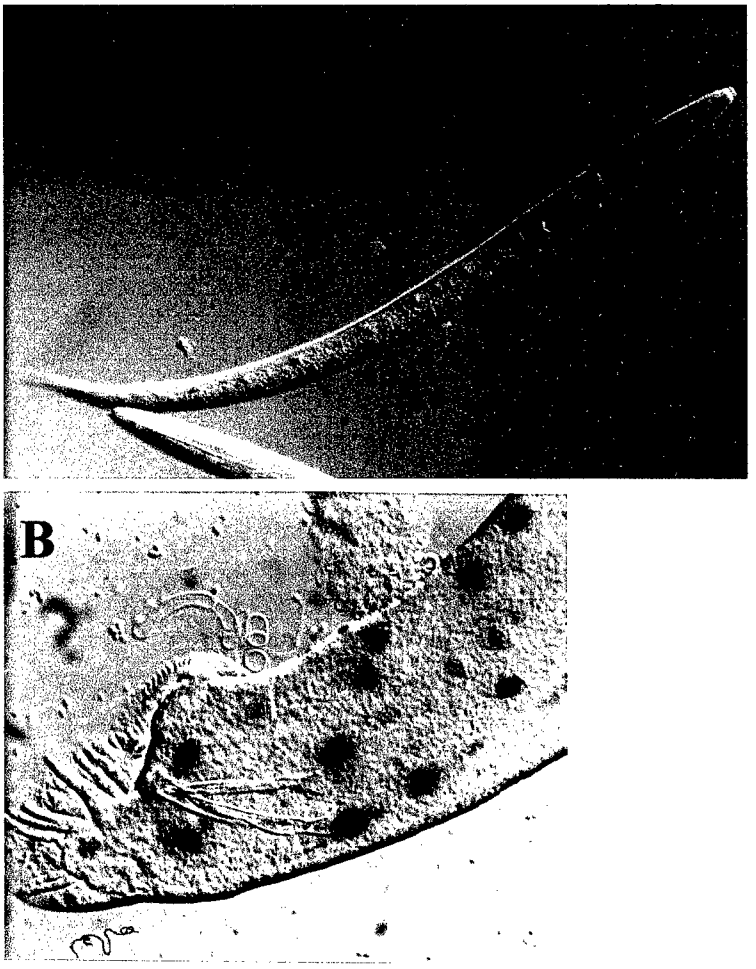



**Figure 6:** Transgenic ceCaMKK expression pattern in the male tail and L1/L2 larvae. Using the NLS-LacZ transgenic reporter (Figure 2A), expression of the ceCaMKK protmoter was examined in males and larvae. **A**, 40X image of expression in larval hypodermal cells. **B**, 100X image of stained male tail with unidentified cells expressing the reporter.

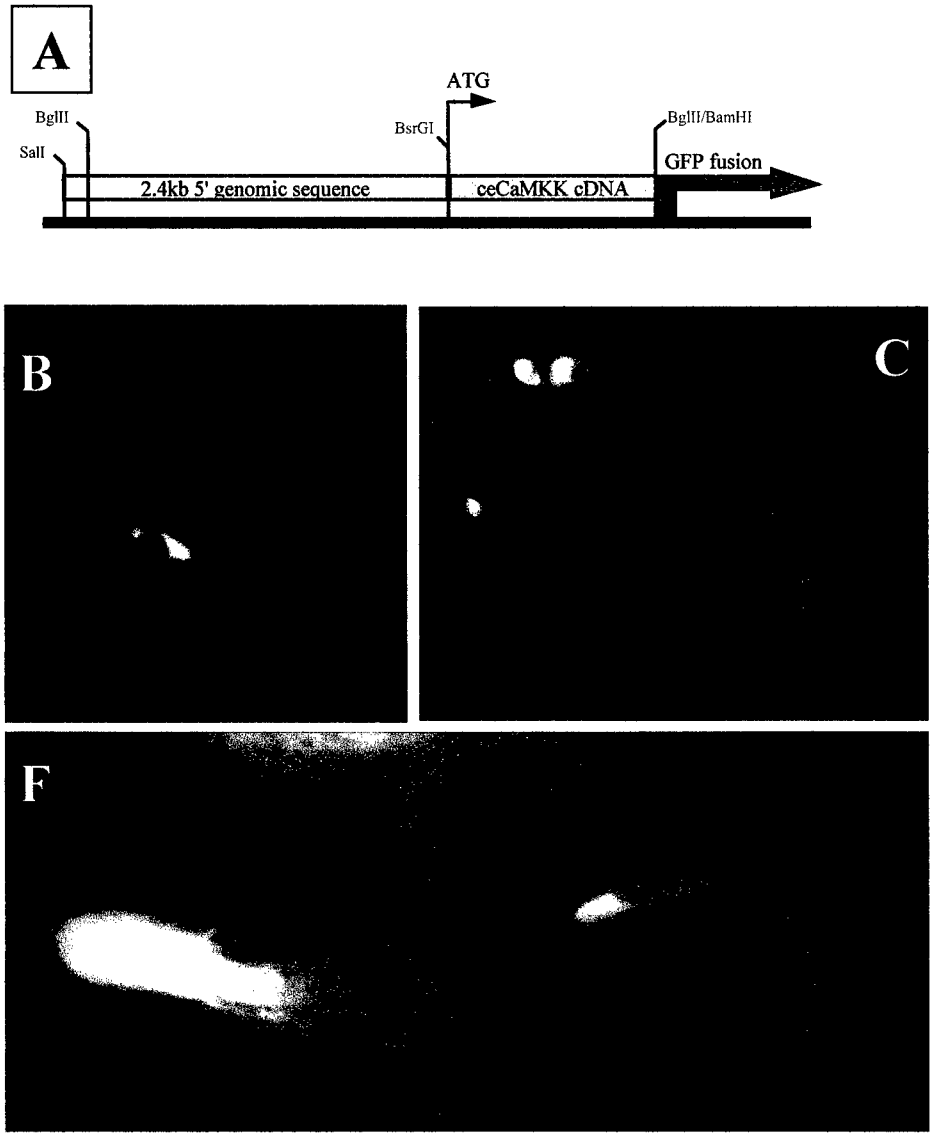



**Figure 7:** Subcellular localization of ceCaMKK-GFP in live nematodes. **A**, schematic representation of the vector used to generate these transgenic nematodes. The ceCaMKK has been inserted in the an unlocalized GFP vector so it will form a ceCaMKK-GFP fusion protein when expressed. **B**, 100X fluorescent image of an anterior neuron expressing GFP in the cytoplasm, but little if any in the nucleus. **C**, 40X fluorescent image with more anterior neurons. **E**, 100X fluorescent image of vulval muscles showing reduced signal in the nucleus.

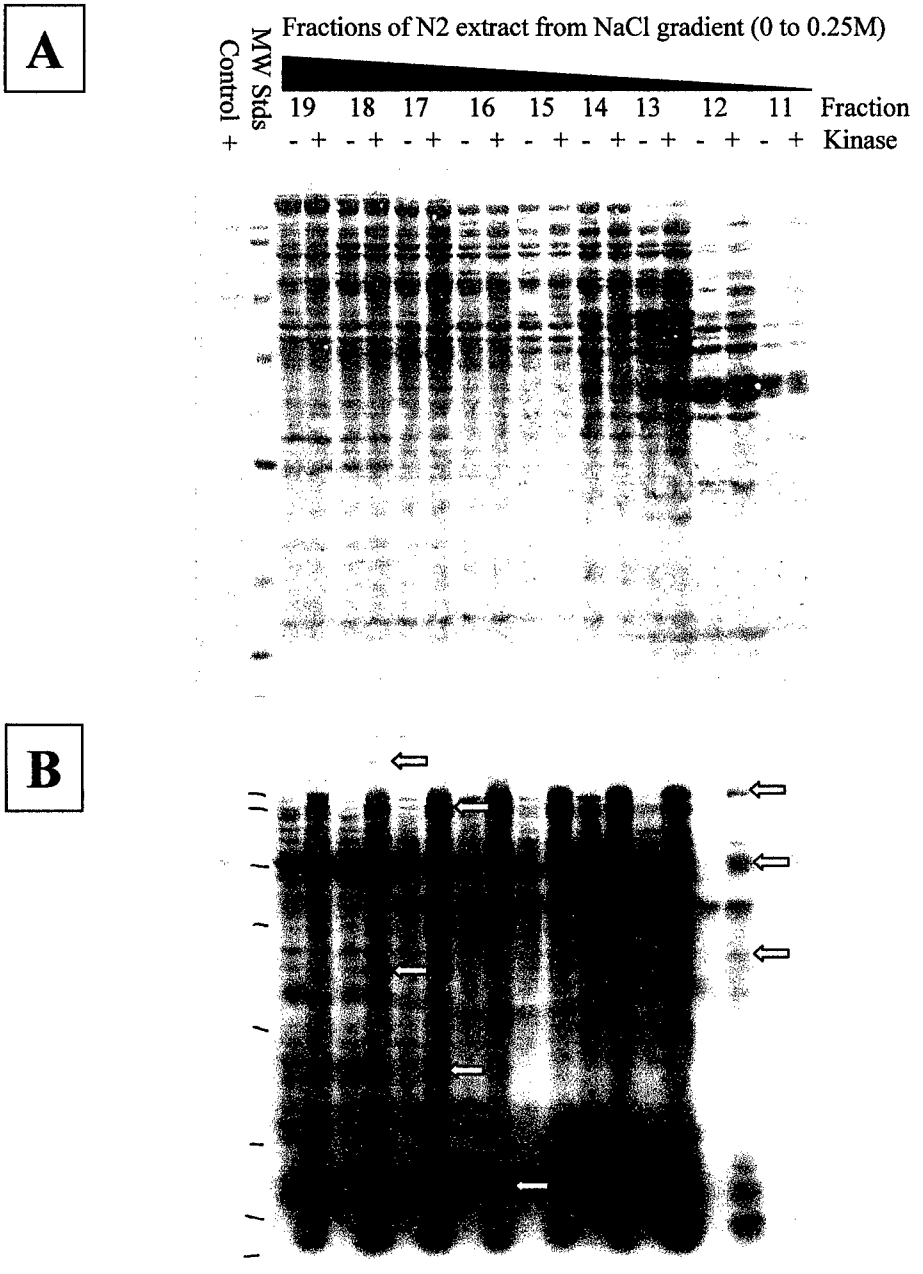
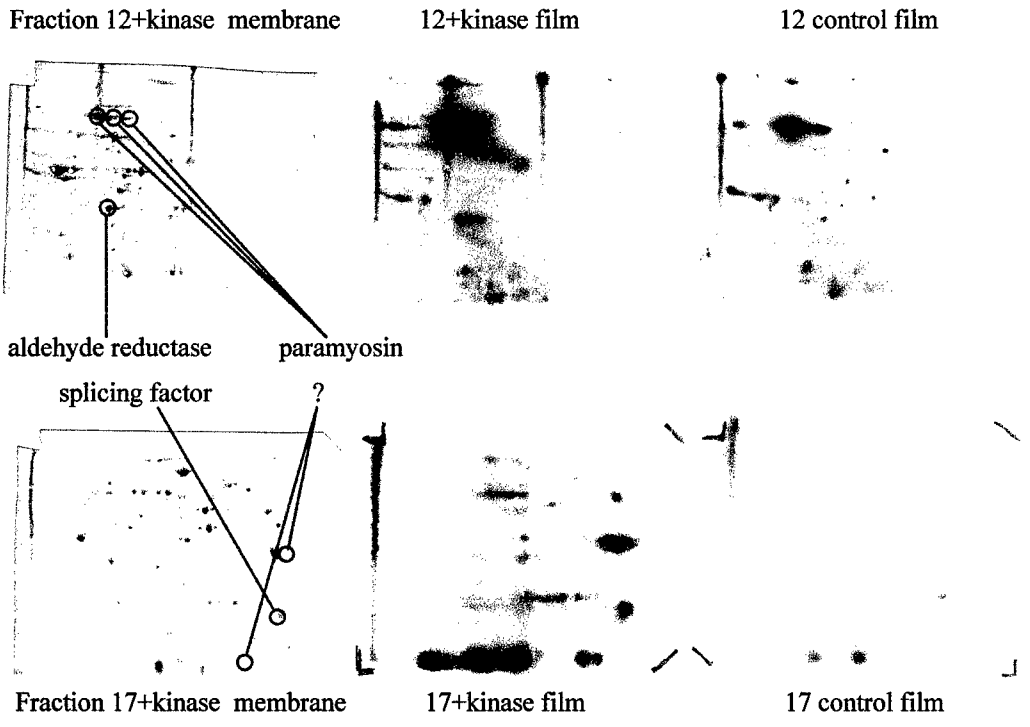



**Figure 8:** Fractionation and kinase assay of mixed stage *C. elegans* extract. Nematode extract was fractionated by MonoQ anion exchange chromatography over a NaCl gradient. Fractions were dialyzed into the same salt-free buffer, and used for kinase reactions with rnCaMKKβ activated ceCaMKI. **A**, reactions were resolved by 10% SDS-PAGE, transferred to PVM membrane and stained with amido black. Adjacent lanes represent fractions with and without exogenous kinase. **B**, autoradiogram of the above membrane demonstrating several differences specific to the addition of kinase (yellow arrows).

**A**




**B**

| *Best FASTF match* | *E Value* | *AceDb Locus* | *Pred MW* | *Pred pI* |
|---|---|---|---|---|
| aldehyde reductase | 2.70E-09 | C07D8.6 | 35191 | 5.42 |
| paramyosin | 2.80E-18 | F07A5.7 | 100634 | 5.35 |
| PKC Substrate | 1.20E-13 | ZK1307.8 | 58063 | 4.65 |
| Splicing factor-associated 32K chain | 1.20E-34 | F59A2.3 | 26411 | 4.94 |

**Figure 9:** 2D Gels of N2 fraction kinase assays and microsequencing results. **A**, fractions described in Figure 8 were further resolved on 2D gels (pH 3-10 1st dimension, 10% SDS-PAGE 2nd dimension) before transfer to membrane and staining. Samples excised for microsequencing are indicated, along with the best match identification where possible. **B**, matches obtained from microsequencing of specifically labeled proteins from the nematode extract are listed, each with match fit estimation, the locus in the *C. elegans* database, and the molecular weight and pI predicted from the primary sequence.

## Acronyms/Abbreviations

| | |
|---|---|
| 2DE | 2-dimensional gel electrophoresis |
| ATP | adenosine triphosphate |
| *C. elegans* | *Caenorhabditis elegans* |
| CaM | calmodulin |
| CaMKI | calmodulin dependent kinase I |
| CaMKIV | calmodulin dependent kinase IV |
| CaMKKB | calmodulin dependent kinase kinase B |
| ceCaMKK | *C. elegans* calmodulin dependent protein kinase kinase |
| ceCaMKI | *C. elegans* calmodulin dependent protein kinase I |
| CREB | cyclic adenosine 3',5'-monophosphate response element binding protein |
| GFP | green fluorescent protein |
| GST | glutathione-S-transferase |
| IEF | isoelectric focusing (1st dimension of 2DE) |
| IPTG | isopropyl β-D-thiogalactopyronoside |
| λ117 | lambda-ZAP phage expressing the 1-117 amino acid fragment of p300 |
| λWT | wild type lambda-ZAP phage |
| MAPK | mitogen-activated protein kinase |
| MBP | maltose binding protein |
| PCR | polymerase chain reaction |
| PKB | protein kinase B |
| RACE | rapid amplification of cDNA ends |
| RNAi | double stranded RNA mediated inhibition (see ref 21) |

## Abstracts

1. Corcoran, E.E., Freedman, J.F., Means, A.R. Elucidating the Role of CaMKK in Cell Cycle and Cell Fate Using a *C. elegans* Model. Abstract for 10th International Symposium of Calcium-Binding Proteins and Calclium Function in Health and Disease, June 17-21, 1997 attached, p. 14).
2. Corcoran, E.E., Freedman, J.F., Means, A.R. Elucidating the Role of CaMKK in Cell Cycle and Cell Fate Using a *C. elegans* Model. Abstract for Era of Hope, Department of Defense Breast Cancer Research Program Meeting, June 8-11, 2000 (attached, p. 15).

## Manuscripts

1. Corcoran, E.E., Means, A.R. (2001). "Defining Ca2+/calmodulin-dependent protein kinase cascades in transcriptional regulation. [review]" J Biol Chem 276(5): 2975-8. (attached)

**10th International Symposium of Calcium-Binding Proteins and Calclium Function in Health and Disease**
**June 17-21, 1997**

## Elucidating the role of the calmodulin-dependent protein kinase kinase in cell cycle and cell fate using *Caenorhabditis elegans*

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**Introduction:** The role of calcium in the cell cycle is not well understood. One potential pathway involves the calmodulin-dependent kinases I and IV (CaMKI and CaMKIV) and the recently discovered calmodulin-dependent protein kinase kinases (CaMKKs). CaMKIV has been shown to phosphorylate the cAMP-response element binding protein, stimulating transcription of a variety of genes including c-fos, c-jun and cyclin A. Moreover, CaMKK enhances transcription of CRE reporter genes when cotransfected with CaMKIV. These results support the existence of a kinase cascade, connecting calcium influx with transcriptional activation of genes important in cell cycle control, through the $Ca^{2+}$/CaM dependent activation of CaMKIV by CaMKK.

Using a *Caenorhabditis elegans* homologue of CaMKK, we are investigating the role of this pathway in cell cycle, cell fate and development. *C .elegans* is an ideal model to address these questions, since the fate and lineage of every cell from egg to adult worm has been mapped. The *C. elegans* homologue of CaMKK, 56% identical to rat CaMKKA, was identified from the *C. elegans* genome project. This system provides an opportunity to explore the function of CaMKK in the context of a well-defined multicellular organism.

**Materials and Methods:** The cosmid containing genomic DNA for the CaMKK gene was obtained from the *C. elegans* genome project. A probe generated from the cosmid by PCR was used to screen a lambda-ZAP cDNA library, yielding an 800 bp clone. Subsequent screening identified a second cDNA, 150 bp longer. In pursuit of the remaining 5' portion of the gene, total RNA isolated from a mixed age culture of worms is being used for PCR based strategies that take advantage of our knowledge of the predicted sequence.

To study the cell-specific developmental patterns of gene expression, transgenic worms are being generated. The promoter region of the genomic DNA, defined as 2.5 kb 5' 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. Worms will be transformed by microinjection of this construct. The pattern of CaMKK gene transcription will be determined from the level of β-gal expression. Nuclear localized β-gal will be visualized following treatment with 5-bromo-4-chloro-3-indoyl-b-D-galactoside (x-gal), that results in a blue precipitate concentrated in the nucleus of each expressing cell.

**Results and Discussion:** We have partially cloned the *C. elegans* homologue of CaMKK. The cDNA sequence so far obtained exactly matches the predicted gene, including three intron/exon junctions. Using the promoter region of the genomic DNA in a reporter construct, we are generating transgenic worms to determine the transcription pattern through all stages of development. Future experiments will use anti-sense RNA to generate mutant worms with knockout phenotypes. This information, together with the extensive knowledge of *C. elegans* biology, will help reveal the biological function of CaMKK *in vivo*.

**Era of Hope, Department of Defense Breast Cancer Research Program Meeting**
**June 8-11, 2000**

# Elucidating the Role of The CaMK Cascade Using a *C. elegans* Model

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In a variety of models, from *Xenopus* oocytes to *Aspergillus* to cell culture, proper progression of the cell cycle has been related to calcium and calmodulin ($Ca^{2+}$/CaM). However, because $Ca^{2+}$/CaM signaling is so ubiquitous, the mechanisms by which it modulates the cell cycle and cell fate have been difficult to dissect. One pathway involves the calmodulin dependent kinases (CaMKs), which can activate the cAMP response element binding protein (CREB) by phosphorylation and thus activate transcription of several factors important to the cell cycle, including c-fos, c-jun and cyclin A. The CaMK kinase (CaMKK) family has been found to stimulate CaMKI and IV, and thereby enhance transcription. These facts suggest the existence of a calcium dependent kinase cascade that links the $Ca^{2+}$/CaM signal with cell cycle and cell fate through the CaMKK.

Using *C. elegans* homologues of the CaMKK (ceCaMKK) and CaMK (ceCaMK), 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. By generating transgenic worms with reporter proteins controlled by the ceCaMKK promoter, gene expression has been demonstrated in the excretory cell, vulval muscle cells and several neurons of adult hermaphrodites, in the hypodermal cells of L1/L2 larvae, and in several male-specific tail cells. To examine this pathway biochemically, we have identified and cloned the ceCaMKK (48% amino acid similarity to human CaMKKB) and ceCaMKI (69% amino acid similarity to human CaMKI) cDNAs, and produced recombinant proteins by prokaryotic expression methods. Both mammalian and *C. elegans* CaMKKs can phosphorylate either species' CaMKI homologue specifically on the activation loop (T177 in human, T179 in *C. elegans*) *in vitro*. These results indicate a functional homology between the mammalian and *C. elegans* calmodulin dependent kinases, and demonstrate the existence of a calmodulin dependent kinase cascade in the worm. To further define biological functions of this cascade, we are investigating the expression patterns and gene regulation of these kinases and attempting to generate null mutants of these genes using the available reverse genetic techniques.

The U.S. Army Medical Research and Materiel Command under DAMD17-97-1-7331 supported this work.

*Minireview*

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# Defining $Ca^{2+}$/Calmodulin-dependent Protein Kinase Cascades in Transcriptional Regulation*

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Calcium is a well established regulator of transcription. Modulation of responses to this ubiquitous second messenger can occur by superposition of coincident $Ca^{2+}$-independent signals, but there is also growing evidence that the strength, frequency, source, and location of the $Ca^{2+}$ signal are determinants for specific transcriptional results. These complex variations must be translated into changes in protein function that preserve and process the information conveyed by the original signal. The $Ca^{2+}$ receptor calmodulin (CaM)[1] is involved in many of these changes through its effects on a variety of CaM-binding proteins (1). Among these, the multifunctional $Ca^{2+}$/calmodulin-dependent protein kinases (CaMKs) are notable for their effects on components of transcription complexes, directly connecting $Ca^{2+}$ with changes in gene expression. The highly homologous CaMKI and CaMKIV are distinct from the multimeric CaMKII, although all have broad and overlapping substrate preferences, because their activation is greatly enhanced following phosphorylation catalyzed by "upstream" kinases in a manner analogous to the mitogen-activated protein kinase cascade. Based on an evolving understanding of CaMKI/IV regulation and cloning of the CaMKI/IV kinases (CaMKKs), a $Ca^{2+}$/CaM-dependent protein kinase I/IV cascade (CaMK cascade) has been proposed (2, 3). This review will discuss the biochemical and physiologic basis for the existence of this cascade and its potential for mediating $Ca^{2+}$ regulation of transcription.

## *Identification and Biochemical Characterization of a CaMK Cascade*

CaMKI and CaMKIV are closely related protein kinases with many similarities in mode of activation and substrate preferences *in vitro* but with different tissue distributions. The kinases are regulated by $Ca^{2+}$/CaM binding, which relieves intramolecular steric inhibition of the active site by a C-terminal autoinhibitory domain (Fig. 1). A second autoinhibitory mechanism unique to CaMKIV is relaxed by the autophosphorylation of Ser-12 and Ser-13 (4). In addition to deinhibition, CaMKI and CaMIV are activated 10–50-fold by *trans* phosphorylation on a single Thr residue in the activation loop. Once activated, CaMKIV acquires $Ca^{2+}$/CaM independence, whereas CaMKI remains $Ca^{2+}$/CaM-dependent (5). Dephosphorylation and inactivation of activated CaMKI/IV can be catalyzed *in vitro* by PP1, PP2A, calcineurin (CaN), and the CaMK phosphatase, but the relevant phosphatase(s) *in vivo* is still unclear (6, 7). CaMKI is ubiquitously expressed, whereas CaMKIV has a more limited distribution, although both enzymes are strongly expressed in the brain.

Recognition of the ability of kinases in brain extract to phosphorylate and activate CaMKI/IV led to the cloning of two upstream kinases, CaMKK$\alpha$ and CaMKK$\beta$ (8, 9). In addition to the brain, where both CaMKKs are highly expressed, CaMKK$\alpha$ mRNA is found in thymus and spleen, whereas CaMKK$\beta$ is present at lower levels in all tissues that express CaMKIV. Although derived from distinct genes, rat CaMKKs are 80% similar, and either CaMKK can phosphorylate and activate CaMKI and CaMKIV *in vitro*. Both CaMKKs bind and are positively regulated by $Ca^{2+}$/CaM *in vitro*, and although their CaM binding site is different from the other CaMKs, the autoinhibitory mechanism functions in a similar manner to that of other $Ca^{2+}$/CaM-dependent kinases (10). Importantly, $Ca^{2+}$/CaM binding to CaMKI/IV is a prerequisite to phosphorylation by the CaMKKs (5). Thus, in theory $Ca^{2+}$/CaM could regulate CaMKI/IV activity on many levels.

The substrate preferences of CaMKI and CaMKIV are similar and intersect with CaMKII. *In vitro,* all three can phosphorylate synapsin I, cAMP response element-binding protein (CREB), and activating transcription factor 1 (11, 12). Their minimum consensus sequence Hyd-*X*-R-*X*-*X*-(S/T) (where Hyd is any hydrophobic amino acid), determined through peptide studies, provides only a rough template common to many protein kinases (11). Additional specificity is provided by residues adjacent to the phosphorylation site of the substrate, producing differences in substrate preference among these kinases. The differences can have important transcriptional implications; for example, although all three can phosphorylate CREB on the activating site Ser-133, only CaMKII phosphorylates an additional inhibitory site, Ser-142 (13).

Interestingly, the presence of two additional basic amino acids 6 and 7 residues N-terminal to the phosphorylation site in certain peptide substrates allows phosphorylation by CaMKI/IV equally well with or without activation by a CaMKK (14). This "activation independence" has not yet been demonstrated toward protein substrates. Nonetheless, because CaMKI/IV requires $Ca^{2+}$/CaM for deinhibition in addition to activation loop phosphorylation, substrates of this type would not be phosphorylated by CaMKI/IV until a $Ca^{2+}$ signal was initiated and so could represent $Ca^{2+}$-dependent but activation-independent signaling targets. Subsequent activation by a CaMKK would increase the number of available substrates by enhancing CaMKI/IV activity toward a second set of substrates (Fig. 1).

## *Does the Cascade Function in Cells?*

The first reconstruction of a CaMK cascade in cells used transient transfection experiments with CREB as the transcriptional target. CaMKI and CaMKIV phosphorylate CREB on its activating Ser-133 *in vitro* and stimulate Gal4-CREB-dependent transcription in response to a rise in intracellular $Ca^{2+}$ when cells are cotransfected with Gal4-CREB and a Gal4 reporter gene (15). Additional cotransfection with a CaMKK increases reporter activity more than 10-fold (8, 9). Mutation of the CaMKIV activation loop T to A abolishes CaMKK enhancement.

To be a signaling cascade, kinase activation loop phosphorylation must be dependent upon induction of CaMKK activity. Phosphate incorporation into endogenous CaMKIV in Jurkat cells is induced rapidly following T-cell receptor stimulation and is blocked by chelation of extracellular $Ca^{2+}$. This is accompanied by 8–14-fold increases in immunoprecipitated CaMKIV activity that is refractory to further activation by exogenous CaMKK and can be reversed by *in vitro* treatment with PP2A (16). Recombinant CaMKIV transfected into BJAB cells, which lack endogenous CaMKIV, demonstrates similar activation following anti-IgM stim-

* This minireview will be reprinted in the 2001 Minireview Compendium, which will be available in December, 2001. This is the second article of four in the "$Ca^{2+}$-dependent Cell Signaling through Calmodulin-activated Protein Phosphatase and Protein Kinases Minireview Series." Our research was supported by United States Army Grant DAMD17-97-1-7331(to E. E. C.) and National Institutes of Health Grants HD07503 and GM33976 (to A. R. M.).

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[1] The abbreviations used are: CaM, calmodulin; CaMK, $Ca^{2+}$/calmodulin-dependent protein kinase; CaMKK, CaMKI/IV kinase; CaN, calcineurin; CREB, cAMP response element-binding protein; CBP, CREB-binding protein; CRE, cAMP response element; dn, dominant negative; ROR, retinoic acid-related orphan receptor; LBD, ligand binding domain.

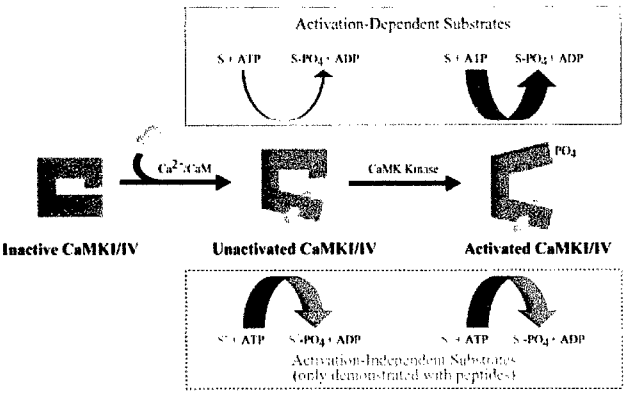


FIG. 1. **Stages of CaMKI/IV activation and effects on activation-dependent (*S*) and activation-independent (*S'*) substrates.** For CaMKIV, autophosphorylation is also required in the first step but is not depicted.

ulation that is abrogated by mutating the activation loop T to A (4). Likewise, CaMKI phosphorylation is induced in PC12 cells, coincident with increased CaMKI activity and reduced activation of CaMKI by exogenous CaMKK (17). Collectively, these experiments provide strong evidence for an inducible, $Ca^{2+}$-dependent activation of CaMKI and CaMKIV in intact cells via activation loop phosphorylation. However, the fact that activation loop phosphorylation requires $Ca^{2+}$ does not confirm that the physiologic activator is itself $Ca^{2+}$-dependent, because $Ca^{2+}$/CaM binding to CaMKI or CaMKIV is required before these enzymes can be phosphorylated by the known CaMKKs.

The issue of subcellular localization is a confounding one for any model of the CaMK cascade regulating transcription. There are several lines of evidence that CaMKK$\alpha$ and -$\beta$ are both cytoplasmic. In contrast to an early study using a polyclonal antibody, immunohistochemistry of rat brain slices using monoclonal antibodies able to distinguish between the $\alpha$ and $\beta$ isoforms found exclusively cytoplasmic immunoreactivity for both (18). Furthermore, green fluorescent protein-tagged CaMKK$\alpha$ and -$\beta$ are cytoplasmic in NG108 cells even after depolarizing stimulation (18). This holds true for overexpressed CaMKK$\beta$ in Jurkat and BJAB cells.[2] Similarly, CaMKI appears to be excluded from the nucleus in brain slices as well as in cells overexpressing the protein, yet CaMKI immunoreactivity has recently been observed to translocate to the nuclei of hippocampal neurons during long term potentiation (19, 20).[3] In contrast, CaMKIV is predominantly nuclear but can also be found in neuronal soma and dendritic processes, where it could interact with a cytoplasmic CaMKK (21).

The CaMK cascade then is well positioned to affect cytoplasmic events, but it is more difficult to explain its effects on transcription. There could be changes in subcellular localization, an unidentified nuclear CaMKIV activator, or cytosolic CaMK cascade targets that modulate nuclear events. Whether full-length CaMKI enters the nucleus or affects transcription through a cytoplasmic intermediate has not been well studied, but it can stimulate transcription of reporters in transient transfection assays (15). Consequently, although the biochemistry is unambiguous and transient transfections appear to reconstruct a cascade, the mechanism for transcriptional regulation by a CaMK cascade in cells is more complicated than expected.

Several other pathways may influence or be influenced by the CaMK cascade. CaMKI and protein kinase A can phosphorylate CaMKKs on multiple inhibitory sites *in vitro*, and forskolin stimulation reduces CaMKI/IV activation in several cell lines (22). Likewise, nuclear localization of CaMKII$\alpha_B$ and $\delta_B$ isoforms is inhibited by phosphorylation of their nuclear localization sequence by CaMKI or CaMKIV, presumably counteracting direct CaMKII effects on transcription (23). In addition, *in vitro* experiments indicate CaMKK can phosphorylate and activate both AMP kinase and protein kinase B, and the AMP kinase kinase can phosphorylate and activate CaMKI, although all of these effects are far less substantial than those of the accepted activators AMP kinase kinase, phosphoinositide-dependent kinase 1, and CaMKK, respectively (24–26). Finally, Ras-independent activation of the mitogen-activated protein kinases ERK2, p38, and JNK1 was observed in cells transfected with constitutively active CaMKIV and further enhanced by cotransfection with CaMKK$\alpha$ (27). The physiologic significance of interacting signal cascades is still unclear, but these examples do serve as a reminder of the intricacy of signal processing.

### *Is the Cascade Physiologic?*

The evolutionary conservation of CaMKI/IV and CaMKK suggests a fundamental biological role. From *Aspergillus nidulans* to *Caenorhabditis elegans* to mammals, the cascade members are highly conserved and biochemically interchangeable in gross assays of cascade function *in vitro* (28, 29). Potential homologues have also been identified in *Schizosaccharomyces pombe* (30, 31) and *Drosophila melanogaster* (32). Investigating the CaMK cascade in genetically tractable systems will help determine whether it is a physiologic pathway and what biological functions it might regulate.

However, to assess the biological significance of the cascade, cellular targets must be identified. CREB is a likely possibility, as it can be activated by the CaMK cascade in transfection experiments (Fig. 2), but many kinases can be induced to phosphorylate CREB (33, 34). It seems likely that the physiologic kinase(s) depends on the situation. For example, expression of catalytically inactive, dominant negative CaMKIV (dnCaMKIV) specifically in developing thymocytes blocks CREB phosphorylation on Ser-133, leading to effects including decreased interleukin-2 production that are reminiscent of thymocytes from mice expressing dnCREB S133A (35). Yet, in initial experiments with thymocytes from CaMKIV-null mice, Ser-133 phosphorylation is subtly decreased but not prevented, and total interleukin-2 production appears normal.[4] The most parsimonious explanation for these conflicting observations is that the dnCaMKIV prevented CREB phosphorylation not only by CaMKIV but also by other thymic CREB kinases. In contrast, CREB phosphorylation is severely reduced in both cerebellar extracts and stimulated hippocampal neurons of CaMKIV knockout mice but is unaffected in the testis (36–38). These results suggest that CaMKIV functions as a CREB kinase in some but not all tissues. Evaluation of CREB phosphorylation in the absence of CaMKKs awaits development of appropriate genetic models.

Situation-specific signal regulation could be accomplished, in part, through regulated association of CaMKIV with other cellular components. An avid association between CaMKIV and PP2A has been demonstrated by copurification and coprecipitation; immunoprecipitated CaMKIV is nearly stoichiometrically associated with PP2A, although the reverse is not true because of the large excess of PP2A (39). Inhibition of PP2A with adenovirus small t antigen or okadaic acid increases CaMKIV-dependent activation of Gal4-CREB transcription, which could result from blocking dephosphorylation of CaMKIV, CREB, and/or CBP. In contrast to the apparently constitutive association of CaMKIV with a potential deactivator, attempts to coprecipitate CaMKIV and CaMKK have been unsuccessful, suggesting that the preformed signaling complex may not include the known activators.[2] Although its significance is not understood, biochemical fractionation of testis extract indicates that a hyperphosphorylated form of CaMKIV is associated with the nuclear matrix (40). Targeting CaMKIV to appropriate loci in a preformed signaling complex could be an important factor in its effects on transcription. As the example of CREB phosphorylation in CaMKIV-null mice poignantly demonstrates, we cannot yet predict when a transcription factor that can be regulated by CaMKIV in transfected cells will be regulated by CaMKIV *in vivo* and how that specificity is determined.

### *Other Transcriptional Targets of CaMKIV*

CaMKIV has been linked with the regulation of many transcription factors other than CREB, including AP-1, serum response

[2] E. E. Corcoran and A. R. Means, unpublished data.
[3] S. S. Hook and A. R. Means, unpublished data.
[4] K. A. Anderson and A. R. Means, unpublished data.

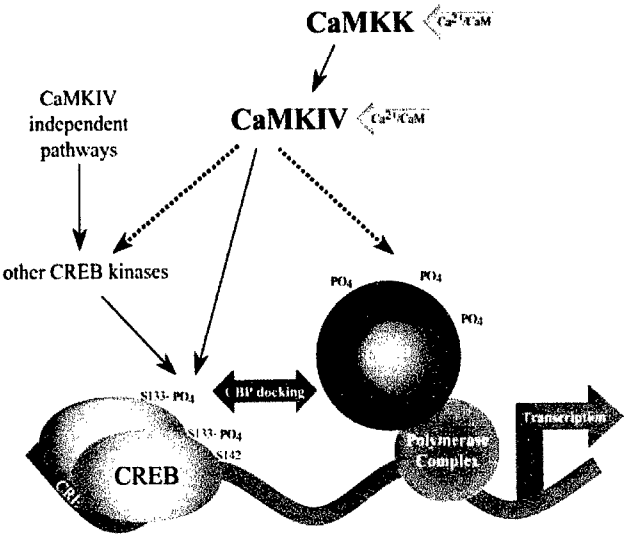


FIG. 2. **Modes of CREB regulation by CaMKIV.**

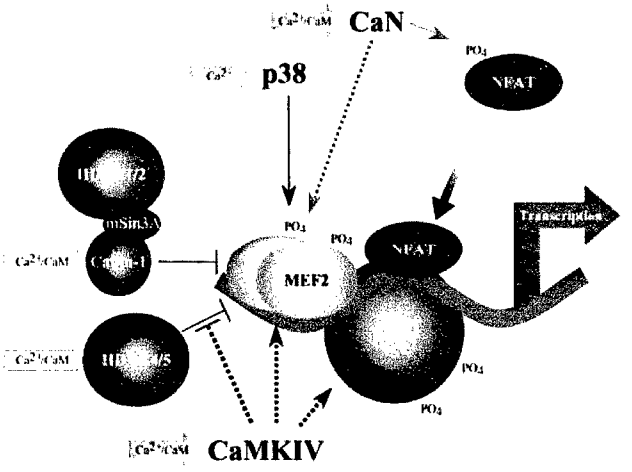


FIG. 3. **Regulation of MEF2 by $Ca^{2+}$ signaling and possible roles for CaMKIV.**

factor, and activating transcription factor 1, and may have multiple modes of regulating CREB-dependent transcription (Fig. 2) (12). CREB transcriptional activation occurs through its binding to the coactivator protein CBP/p300, which links many transcription factors to components of the general transcriptional machinery. Phosphorylation of CREB on Ser-133 increases its affinity for CBP but whether CREB Ser-133 phosphorylation is sufficient to induce CRE-dependent transcription without other regulatory signals to CBP itself is controversial. Microinjection of phosphoserine 133-CREB or transfection with the Y134F CREB mutant that is constitutively phosphorylated induces CRE-dependent reporter plasmid transcription without additional stimuli (41, 42). Seemingly contradictory experiments demonstrate that induction of CRE-mediated transcription by depolarization of AtT20 cells requires nuclear $Ca^{2+}$ but not increased CREB Ser-133 phosphorylation (43). Moreover, reporter gene transcription driven by a CREB-independent Gal4-CBP fusion is enhanced by constitutively active CaMKIV. Sites in CBP are inducibly phosphorylated, which has led to the hypothesis that CaMKIV regulates CBP by direct phosphorylation, but this remains to be demonstrated.

CaMKIV stimulation of transcription by the orphan receptor ROR$\alpha$ may also be related to effects on coactivators. A CaMKIV effect on ROR was investigated because of the similarities in phenotypes between CaMKIV and ROR$\alpha$ knockout mice. In transfection assays, CaMKIV induces a 20–30-fold increase in ROR$\alpha$-dependent transcription of a reporter plasmid (44). The ROR$\alpha$ ligand binding domain (LBD) is not a substrate for CaMKIV *in vitro*, but LBD binding peptides that disrupt LBD association with endogenous coactivators abrogate the CaMKIV effect. Although the mechanism remains to be elucidated, these results and the CaMKIV effects on CBP suggest a new role for CaMKIV in recruiting or stabilizing coactivator-containing transcriptional complexes.

CaMKIV has also been implicated in the $Ca^{2+}$-dependent regulation of MEF2 family transcription factors (Fig. 3). In cardiomyocytes and neurons, $Ca^{2+}$ influx leading to MEF2 activation correlates with activation of p38, which phosphorylates MEF2 on multiple sites *in vitro* including those in its activation domain (45, 46). MEF2 has also been reported to be a substrate for CaMKIV *in vitro* (47). Constitutive forms of CaMKI/IV and CaN independently activate MEF2 reporter genes but synergize when cotransfected. There is evidence for two complementary mechanisms for the effect of CaN: dephosphorylation of NFAT, promoting its nuclear translocation and allowing it to synergize with MEF2 to activate reporter genes; and dephosphorylation of MEF2, which enhances its DNA binding (48). The coactivator CBP/p300 can bind both NFAT and MEF2 and may be involved in stabilizing a NFAT-MEF2 complex on the promoter and/or may itself be a target of a $Ca^{2+}$-dependent stimulatory signal (49). Furthermore, Cabin-1, originally identified as a CaN inhibitor, also binds and suppresses MEF2 transcriptional activity in T-cells (50). Cabin-1 binding to MEF2 is competitive with p300 and is relieved by $Ca^{2+}$/CaM binding to Cabin-1 (50, 51). Because Cabin-1 also associates with mSin3a and histone deacetylases 1 and 2 (HDAC1/2), its dissociation from MEF2 may replace a repressing complex with an activating complex (51). In a like manner, MEF2 binds and is repressed by HDAC4 but is released by $Ca^{2+}$/CaM binding to HDAC4 (52). Association of MEF2 with HDAC4/5 can also be inhibited by cotransfection with a constitutively active fragment of CaMKI/IV, which may provide an indirect mechanism for CaMKI/IV enhancement of MEF2 transcription (53). Clearly, $Ca^{2+}$ regulation of MEF2 is multifaceted, involving both stimulatory phosphorylation/dephosphorylation and dissociation from transcriptional repressors.

Missing from many studies of CaMKIV and transcription is a characterization of CaMKK effects and differentiation between CaMKI and CaMKIV. Unfortunately, the kinase inhibitors currently available, KN62 and KN93, inhibit CaMKI, II, and IV similarly and so they cannot be used alone to define a role for a specific CaMK (54). Moreover, these drugs block voltage-dependent $K^+$ currents at concentrations comparable with those used for CaMK inhibition, so results from the use of these inhibitors should be interpreted cautiously (55). Truncations of the kinases to form constitutively active forms are also frequently used to study transcription, but because this removes domains of the kinase whose functions are unknown, these proteins could be inappropriately localized or regulated. For example, the CaMKII$\alpha$-(1–290) activating truncation also removes the association domain and thus both changes its activation biochemistry and permits inappropriate nuclear entry (2, 11). For CaMKI and CaMKIV, functions of the C-terminal region are not as well defined but may also affect subcellular distribution and substrate preference. One alternative approach that circumvents these pitfalls but has not yet been extensively employed is to use mutations in the autoinhibitory domains identified for CaMKII, CaMKIV, and CaMKKs that allow $Ca^{2+}$/CaM-independent activity without truncation (10, 56, 57). Finally, because understanding of the cascade is still so skeletal, it is not reasonable to assume that transcriptional effects attributed to CaMKIV are also regulated by the CaMKK without directly testing that hypothesis. These caveats should be kept in mind in interpreting the results of experiments designed to implicate one of the CaMKs in a CaMK cascade.

### *Evidence for a CaMK Cascade*

The evidence for a working CaMK cascade regulating $Ca^{2+}$-dependent transcription in cells is largely favorable but still circumstantial. CaMKI and CaMKIV are excellent substrates of the CaMKKs and are dramatically activated by activation loop phosphorylation that occurs following stimulation of intact cells. The tissue distributions of the CaMKKs appropriately overlap those of CaMKI and CaMKIV, but the question of subcellular localization

still needs to be resolved. Whether the CaMKKs so far identified are the only kinases capable of activating CaMKI and CaMKIV is unknown.

If this cascade is physiologic, does it function as a signal integrator or as an amplification circuit? Of course, inducible phosphorylation by CaMKKs provides a mechanism for amplification. However, like CaMKII, these kinases have elaborate activation mechanisms that rely on a $Ca^{2+}$ signal for multiple steps. For CaMKII, this complex activation biochemistry has been shown to differentiate stimulation frequencies (58), but no such evidence yet exists for the CaMK cascade. The suggestion from peptide experiments that CaMKI/IV might exhibit different substrate specificities before and after activation offers tantalizing possibilities for signal processing beyond amplification. Elements of the cascade also interact with a variety of other pathways, and the CaMKKs may have substrates other than CaMKI/IV. Therefore, both amplification and integration are possible roles for a CaMK cascade. The intricate regulation of these kinases provides many interesting possibilities for transmitting $Ca^{2+}$ signals.

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MCMR-RMI-S (70-1y) 21 Feb 03

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLLIS M. RINEHART
Deputy Chief of Staff for
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