Calcineurin, a Calcium/Calmodulin-dependent Protein Phosphatase, Is Involved in Movement, Fertility, Egg Laying, and Growth in *Caenorhabditis elegans*

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Calcineurin is a Ca^{2+} -calmodulin-dependent serine/threonine protein phosphatase that has been implicated in various signaling pathways. Here we report the identification and characterization of calcineurin genes in *Caenorhabditis elegans* (*cna-1* and *cnb-1*), which share high homology with *Drosophila* and mammalian calcineurin genes. *C. elegans* calcineurin binds calcium and functions as a heterodimeric protein phosphatase establishing its biochemical conservation in the nematode. Calcineurin is expressed in hypodermal seam cells, body-wall muscle, vulva muscle, neuronal cells, and in sperm and the spermatheca. *cnb-1* mutants showed pleiotropic defects including lethargic movement and delayed egg-laying. Interestingly, these characteristic defects resembled phenotypes observed in gain-of-function mutants of *unc-43*/ Ca^{2+} -calmodulin-dependent protein kinase II (CaMKII) and *goa-1*/ G_α -protein α -subunit. Double mutants of *cnb-1* and *unc-43(gf)* displayed an apparent synergistic severity of movement and egg-laying defects, suggesting that calcineurin may have an antagonistic role in CaMKII-regulated phosphorylation signaling pathways in *C. elegans*.

INTRODUCTION

Calcineurin (CaN), a protein phosphatase 2B (PP2B), is a serine/threonine phosphatase under the control of Ca^{2+} /calmodulin (Klee *et al.*, 1979; Stewart *et al.*, 1982; Klee *et al.*, 1998). Although CaN is a member of a family of protein phosphatases, it is structurally and functionally distinct from alkaline and acid phosphatases (Cohen, 1989; Guerini and Klee, 1991). Calcineurin is a heterodimer of an ~ 60 -kDa catalytic subunit, calcineurin A (CNA), and a 19-kDa regulatory subunit, calcineurin B (CNB; Klee *et al.*, 1988), and the two-subunit structure is well conserved from yeast to human. CaN is abundantly expressed in the brain and broadly distributed in nonneural tissues as well (Kincaid, 1993).

Among its several functions in controlling intracellular Ca^{2+} signaling, CaN participates in gene regulation and external signal-mediated biological responses in many organisms and in many cell types (Crabtree, 1999).

CaN functions have been extensively studied in the yeast, *Saccharomyces cerevisiae*. To investigate the biological role of CaN, immunosuppressant drugs Cyclosporin A (CsA) and FK506 were used to inhibit CaN function (Cyert, 1993). Calcineurin has been shown to regulate Ca^{2+} pumps and exchangers to maintain Ca^{2+} homeostasis (Stark, 1996). Calcineurin is also known to regulate adaptation to high salt stress (Hirata *et al.*, 1995). However, in higher animals, it is better known to regulate the transcription of the T-cell growth factor, interleukin-2 (Schreiber and Crabtree, 1992). Dephosphorylation of the transcription factor NF-ATp (nuclear factor-activated T cells) by CaN is required for the translocation of NF-AT from the cytoplasm to the nucleus, in response to an increased intracellular Ca^{2+} level. Calcineurin also plays a role in programmed cell death (Shibasaki and McKeon, 1995) and in hippocampal long-term

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depression (Mulkey *et al.*, 1994). Furthermore, studies reveal that CaN plays a critical role in the pathogenesis of hypertrophic cardiomyopathy (Molkentin, 1998). Thus, calcineurin as a key signaling molecule has been shown to be involved in diverse types of physiological processes.

Caenorhabditis elegans has been an ideal model to study gene functions especially at the organism level using a genetic approach. Moreover, *C. elegans* has been useful for identifying interactions between molecules in biochemical signaling pathways that are associated with a certain behavior or phenotype. In this study, we have identified and characterized the *C. elegans* homologue of calcineurin B, which binds calcium and functions together with calcineurin A as a heterodimeric protein phosphatase. Null mutants of calcineurin B showed multiple adverse phenotypes including defects in locomotion and egg laying. Interestingly, these phenotypes are quite similar to those observed in gain-of-function mutants of *unc-43*, which encodes the Ca^{2+} -calmodulin-dependent protein kinase CaMKII. Recently, a G-protein signaling pathway regulated by *unc-43* has been found to be involved in locomotory and egg-laying functions (Robatzek and Thomas, 2000). Our results describing the relationship between the *cnb-1* null mutant and mutants of *unc-43* indicate that calcineurin and CaMKII may have opposing and complementary functions in this G-protein signaling pathway in *C. elegans*.

MATERIALS AND METHODS

C. elegans Strains

The following strains were obtained from the *Caenorhabditis* Genetics Center (CGC) at the University of Minnesota, Duluth, MN: Bristol N2, CB224 *dpy-11(e224)V*, CB1482 *sma-6(e1482)II*, MT1092 *unc-43(n498)IV*, *unc-43(n1186)IV*, and CB1282 *dpy-20(e1282)IV*. KJ300 *cnb-1(jh103)* was isolated by reverse genetics method (Park *et al.*, 2001b). *cnb-1(ok276)* was isolated by the *C. elegans* Knockout Consortium (G. Molder, Oklahoma). Worm breeding and handling were conducted as described (Brenner, 1974).

Cloning of *C. elegans* Calcineurin A and Calcineurin B cDNAs and Northern Analysis

To obtain full-length cDNA clones of *C. elegans* calcineurin A (*cna-1*) or calcineurin B (*cnb-1*), cDNA library screening was conducted following plaque hybridization procedure (Sambrook *et al.*, 1989). A mixed-stage worm cDNA library (kindly provided by P. Okkema and A. Fire) was probed with the partial cDNA clones, yk375h10 or yk496e12 (obtained from Y. Kohara). The largest inserts of 2869 base pairs and 943 base pairs for *cna-1* and *cnb-1*, respectively, were sequenced and confirmed by Northern analysis. For Northern blotting, total RNA was prepared from staged animals and transferred to a Zeta probe membrane (Bio-Rad, Hercules, CA) as described (Krause, 1995; Cho *et al.*, 2000). The membrane was hybridized in hybridization buffer (0.25 M Na_2HPO_4 , pH 7.2), 0.25 M NaCl, 1 mM EDTA, 7% SDS, 50% Formamide, 5% dextran sulfate, and 100 $\mu\text{g}/\text{ml}$ denatured salmon sperm DNA at 42°C with random-primed ^{32}P -labeled probes. Exposure of the blots after high stringent washing in 0.5× SSC and 0.1% SDS was performed on x-ray films (Fuji) overnight at −80°C.

Expression and Purification of GST-CNA-1 and GST-CNB-1, Ca^{2+} -binding, and Yeast Two-hybrid Assays

Complementary DNAs encoding the entire open reading frames of *cna-1* or *cnb-1* were subcloned in pGEX4T-3 or pGEX4T-1 (Pharma-

cia, Piscataway, NJ), encoding GST-CNA-1 or GST-CNB-1, respectively. Syntheses of GST-fused proteins were carried in *Escherichia coli* strains (BL21) at 30°C for CNA-1 and at 37°C for CNB-1 in the presence of 1 mM IPTG. Cells were harvested and sonicated according to the methods described (Zhao *et al.*, 1993).

Ca^{2+} -binding assay for CNB-1 was performed as described earlier (Maruyama *et al.*, 1984). Purified GST-CNB-1 (see above) was resolved by 12% SDS-PAGE, transferred to PVDF membrane, and then probed with $^{45}\text{Ca}^{2+}$ (Cho *et al.*, 2000). Purified GST, bovine calmodulin (CaM; Sigma), and *C. elegans* calsequestrin (CSQ-1) were used as controls.

Wild-type *cna-1* and *cnb-1* cDNAs, covering the entire open reading frames, were fused in-frame to the GAL4 DNA-binding domain and GAL4 activation domain of the yeast vectors pAS2-1 and pACT2 (Clontech, Palo Alto, CA) to produce the plasmids CJ1 and CJ2, respectively. Plasmids were transformed into the yeast strain AH109 according to the manufacturer's protocol (Clontech). Transformants were plated on synthetic dropout (SD) media lacking Trp and Leu. Interaction assays were conducted on plates containing 5 mM 3-aminotriazole (3-AT) in SD without Trp, Leu, His, and Ade in absence or presence of 2 mM CaCl_2 .

In Vitro Phosphatase Assay

Equimolar concentrations (0.016 nmoles each) of purified GST-CNA-1 and GST-CNB-1 (see above) were used to conduct phosphatase assay (Promega) using a phosphopeptide (100 μM) as a substrate. To test the Ca^{2+} dependency of calcineurin, 0.2 mM EGTA (Ca^{2+} chelator) was used in the reactions. Inhibition of phosphatase activity by the immunosuppressant drug, cyclosporin A, CsA (Calbiochem, La Jolla, CA), was tested by preincubating GST-CNA-1 and GST-CNB-1 together with 1 μM each of CsA and bovine cyclophilin (Sigma, St. Louis, MO) at 4°C for 1 h. The dephosphorylation of the phosphopeptide was determined spectrophotometrically at 595 nm. The optical density was further converted to pmol phosphate release/min/ μg protein using appropriate standards supplied with the kit.

Construction and Expression of *gfp* Fusion Constructs

Two cosmid clones C02F4 and F55C10 were obtained from A. Coulson (The Sanger Center, UK). To check the reporter gene (green fluorescent protein, *gfp*) expression, promoterless GFP vectors, pPD95.70 containing the nuclear localization signal (NLS), pPD95.79 and pPD95.75 (vectors provided by A. Fire) were used in the present study. All constructs (pJJ001–pJJ004) were generated as translational fusions with *gfp*. Microinjection using pRF4 (dominant *rol-6*) as a transformation marker was performed as described by Mello and Fire (1995).

Immunofluorescence and Immunogold Microscopy

Wild-type *C. elegans* was immunostained as described (Miller and Shakes, 1995; Cho *et al.*, 2000). For gonad immunostaining, gonads were extruded by decapitating adult *C. elegans* and fixed with 3% formaldehyde, 0.1 M K_2HPO_4 (pH 7.2), for 1 h and postfixed with cold (−20°C) 100% methanol for 5 min. Antibody incubations and washes were performed as described (Jones *et al.*, 1996). Anti-CNA-1 mouse monoclonal and anti-CNB-1 rabbit polyclonal antibodies were used as primary antibodies. TRITC-conjugated anti-mouse (Sigma) and FITC-conjugated anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) were used as secondary antibodies. Sperm immunostaining was conducted according to the methods described previously (Arduengo *et al.*, 1998).

Immunogold staining with anti-CNB-1 antibody for N2 worms was carried out as described previously (Park *et al.*, 2001a). Samples were examined under a transmission electron microscope (Jeol 1200 EXII, Peabody, MA).

A. LGV **B.** **C.**

Figure 1. Genomic organization of calcineurin genes in *C. elegans*. (A) Genetic and physical maps of the *cnb-1* region. The *cnb-1* locus relative to the nearby genetic markers on the gene cluster region of LG V is shown. *cnb-1*, which is mapped to the cosmid F55C10 (Accession No. Z74036), and the neighboring cosmids are indicated. The predicted F55C10.1 gene (*cnb-1*) structure has been revised and is presently shown to contain six exons (black boxes). (B) Northern blot analysis detects a 1-kb band in RNA samples of wild-type N2 worms. (C) Amino acid sequence alignment for calcineurin B of *C. elegans* (CECNB-1), *Drosophila* (DMCNB-1, Accession No. P48451), human (HSCNB-1, Accession No. NP000936), and yeast (SCCNB-1, Accession No. NP_012731). Regions of identity among calcineurin B homologues are boxed. Four Ca^{2+} -binding sites are shown (I–IV). The myristoylation signature is indicated by a black bar.

Isolation of a *cnb-1* Deletion Mutant from a Mutagenized DNA Library

TMP (Trimethylpsoralen)/UV method was used to generate *C. elegans* deletion mutant. Screening of mutants from the mutagenized DNA library was carried out by a nested PCR-based method and subsequent sib selections as described (Barstead, 1999). Primers were designed based on the predicted sequences spanning the full genomic DNA of *cnb-1*: outer upstream primer (5'-ACA TTC TAC TAC ATT CTG GCT GTG TGA TCC-3') and downstream primer (5'-ATG AGC ATC ATT TAT TTG GCG GAC C-3'), inner upstream primer (5'-AAG CCC TCT GCT GGA CTG CTG TCC ACC-3'), and downstream primer (5'-AAT GCG AGG AAA CGC TTC CCA ATT GGC-3'). A homozygous line of animals with a 950-base pair deletion relative to the wild-type was isolated. This animal was outcrossed six times to wild-type animals to establish the strain KJ300 *cnb-1(jh103)* and was used in subsequent analysis. Deletion region for the *cnb-1* hermaphrodites was determined by nested PCR followed by sequencing the PCR products.

Construction of Double Mutant Strain and Phenotype Analysis

Double mutants of *unc-43(n498);cnb-1(jh103)*, *unc-43(n1186);cnb-1(jh103)*, and *goa-1(n1134);cnb-1(jh103)* were constructed by stan-

dard genetic methods. *dpy-20(e1282)* was used as a genetic marker. PCR was used to detect the *cnb-1* mutant.

The brood size of N2, *cnb-1*, *unc-43*, and *unc-43(n498);cnb-1(jh103)* hermaphrodites was determined by placing individual worms on seeded plates and allowing self-fertilization at the indicated temperature. The P0 mother was then transferred to a fresh plate at 24-h intervals for each of the next 4 days. Total F1 progeny on the plates were counted. The brood size of crossed progeny was determined by placing a single N2 or *cnb-1* hermaphrodite with three wild-type males on seeded plates and allowing crossing at 20°C for 2 d. Total F1 progeny and the number of male progeny on the plates were counted.

Body length, body width, and uterine embryos were carefully examined under a dissecting microscope. L4-stage larvae were transferred to a new plate and allowed to grow for 36 h. The resulting 1-d adult worms were then examined for phenotypes. Movement of animals was examined by placing five adult hermaphrodites on a bacterial lawn, and after 5 min the tracks made by the worms were photographed. Serotonin-mediated egg-laying phenotypes were examined as previously described (Trent *et al.*, 1983). Levamisole-mediated egg-laying phenotypes were examined in the same way at concentrations ranging from 25 to 100 μM .

Sperm morphology was analyzed in hermaphrodites as previously described (L'Hernault and Roberts, 1995) by hand-dissecting hermaphrodite spermatheca in sperm medium (50 mM NaCl, 25 mM KCl, 5 mM CaCl_2 , 1 mM MgSO_4 , 5 mM HEPES, 10 mM dextrose, 4 mM levamisole, pH 7.8). The spermatheca was observed with a Zeiss Universal microscope equipped with Nomarski optics.

Transformation Rescue

To test for rescue of *cnb-1(jh103)* homozygotes, *cnb-1* genomic DNA cloned in pGEM-T Easy vector (Promega) and *csq-1::gfp* fusion construct as a transformation marker (Cho *et al.*, 2000) at final concentrations of 50 and 50 $\mu\text{g}/\text{ml}$, respectively, were coinjected into *cnb-1* mutant animals using standard methods (Mello and Fire, 1995). The injected parents were allowed to self-fertilize. Individual GFP-expressed progeny were then picked and examined for phenotypes.

RESULTS

Characterization of the *C. elegans* Calcineurin B (*cnb-1*) Gene

C. elegans calcineurin B-like genes have been physically mapped to the cosmid F55C10 and F30A10, respectively. However, alignment studies and molecular cloning (this study) have revealed that F55C10.1 is the putative calcineurin B (*cnb-1*), the regulatory subunit of the protein phosphatase 2B with four EF-hand motifs for Ca^{2+} binding (see below). The *cnb-1* locus is physically mapped to the gene cluster region of chromosome V (LGV) on the cosmid F55C10 that corresponds to the region between *unc-42* and *sma-1* loci on the genetic map (Figure 1A). Northern blot analysis confirmed a single 1.0-kb mRNA transcript of *cnb-1* (Figure 1B).

C. elegans calcineurin B, as in other species, is also a small protein of 171 amino acids exhibiting high sequence homology with other organisms (80% identity with human and *Drosophila* calcineurin B and 58% with the yeast; Figure 1C). A putative initiation Met (ATG) codon is located at nucleotide position 51, which is surrounded by a relatively good consensus sequence for translation (Kozak, 1991) also found in other *C. elegans* genes (Krause, 1995). Additionally, the cDNA appears to contain a partial SL1 leader sequence at the 5' end. The *C. elegans* calcineurin A (*cna-1*) gene was recently identified by Kuhara *et al.* (2002). The *tax-6* locus was shown to encode calcineurin A, and our results confirmed the *cna-1/tax-6* sequence and gene structure from previously obtained results (Kuhara *et al.*, 2002).

CNB-1 Is a Ca^{2+} -binding Protein and Enhances CNA-1 Phosphatase Activity

It is well known that CaN binds Ca^{2+} and calmodulin (CaM), as originally shown by Klee *et al.* (1979). Calcineurin B, the regulatory subunit of CaN binds 4 molecules of Ca^{2+} with high affinity ($K_d \leq 10^{-6}$ M), and has sequence homology with CaM and troponin C, two other calcineurin binding proteins (Aitken *et al.*, 1982). Recombinant GST-CNB-1 had strong Ca^{2+} -binding activity (Figure 2A, lane 4) whereas GST alone showed no Ca^{2+} -binding activity (Figure 2A, lane 1). Thus, Ca^{2+} overlay experiments confirmed that CNB-1 has high affinity for Ca^{2+} as in other Ca^{2+} -binding proteins such as *C. elegans* calsequestrin CSQ-1 (Cho

et al., 2000) and bovine CaM (Figure 2A, lane 2 and lane 3, respectively).

Calcineurin is a tight heterodimer composed of the catalytic A subunit and the regulatory B subunit (Klee *et al.*, 1988; Perrino *et al.*, 1995). This interaction is necessary for the function and stabilization of the enzyme. We utilized the yeast two-hybrid system to check if CNA-1 interacted with CNB-1. As shown in Figure 2B, the subunits interacted with each other in the presence of Ca^{2+} as indicated by the growth of colonies in the selective media (compare panels 2 and 3).

To further test if *C. elegans* calcineurin exhibits phosphatase activity in vitro, a serine/threonine phosphatase assay was conducted with purified full-length GST-CNA-1 and GST-CNB-1 expressed in *E. coli*. It was previously demonstrated that GST does not interfere with or possess phosphatase activity (Chin-Sang and Spence, 1996). We carried out experiments with or without GST-CNB-1 to check whether the rate of dephosphorylation can be regulated in the absence or presence of the regulatory subunit, and at the same time tested whether there was basal activity of GST-CNA-1. As shown in Figure 2C, GST-CNA-1 alone showed a dephosphorylation activity (~ 10 pmol phosphate/min/ μg protein), whereas addition of GST-CNB-1 elevated the activity by ~ 2 -fold indicating that the full activity of the enzyme requires both subunits of calcineurin. Addition of EGTA (a Ca^{2+} -chelator) abolished the phosphatase activity to control levels indicating that the phosphatase required Ca^{2+} for its activity (Figure 2C). Furthermore, we tested if the immunosuppressant, cyclosporin A (CsA), affects the phosphatase activity. Cyclophilin-CsA complex has long been known to block the phosphatase activity of calcineurin (Liu *et al.*, 1991). Previously, several cyclophilin (*cyp*) genes had also been cloned and characterized in *C. elegans* indicating once again a well-conserved role(s) for cyclophilin in signal transduction and protein folding (Page *et al.*, 1996). In the present study, we used bovine cyclophilin with CsA to test if *C. elegans* calcineurin activity is affected. As expected, the phosphatase activity of CNA-1 was completely inhibited (Figure 2C) in the presence of cyclophilin-CsA implicating a conserved phenomenon of phosphatase inhibition by the immunosuppressant drug.

Expression and Localization of Calcineurin in *C. elegans*

We examined the temporal and spatial pattern of *cna-1* and *cnb-1* expression using the *gfp* reporter driven by *cna-1* or *cnb-1* 5'-upstream regulatory sequences. Both *cna-1* and *cnb-1* reporter transgenes expressed in diverse tissues. Expression was detected at all stages of development starting from early comma stage embryos to adult stages. Calcineurin is expressed in vulval muscle, body-wall muscle, spermatheca (Figure 3, A–C), and in a majority of neuronal cell bodies in the head and tail similar to previously obtained results (Kuhara *et al.*, 2002).

Polyclonal antibodies against CNA-1 and CNB-1 were generated, and western blots with total protein extracts from wild-type worms detected a single band around 60 kDa for CNA-1 and 16 kDa for CNB-1, respectively (Figure 2D). Immunostaining performed with these antibodies for both subunits of calcineurin showed similar localization to GFP expression patterns and additionally showed localization in

Figure 2. Biochemical characterization of *C. elegans* calcineurin. (A) *cnb-1* is a Ca^{2+} -binding protein. Ca^{2+} -binding assay was performed with GST (glutathione-S-transferase) (lane 1), *C. elegans* calsequestrin, GST-CSQ-1 (lane 2), bovine CaM (lane 3), and GST-CNB-1 (lane 4). $^{45}\text{Ca}^{2+}$ -labeled proteins are shown by the dark signals on the x-ray film. GST (lane 1) is used as a negative control. A corresponding gel shown in the right panel is stained with Coomassie Blue. Molecular weight markers (in kDa) are denoted by arrows. (B) Interaction between CNA-1 and CNB-1 in the yeast two-hybrid system. Growth of yeast transformants on SD –Trp –His –Leu –Ade with 5 mM 3-AT (panel 2) and 5 mM 3-AT + 2 mM CaCl_2 (panel 3) are shown. Plates supplemented with 5 mM 3-AT + 2 mM CaCl_2 select for a positive interaction between the fusion proteins. Positive interaction between AD/T-antigen and DNA-BD/p53 fusion proteins and the negative or no interaction between AD/T-antigen and DNA-BD/lamin C were used as positive (+ control), and negative controls (– control), respectively. The array of the yeasts containing the different constructs is indicated in the schemes shown in panel 1. (C) Serine/threonine phosphatase activity of CNA-1. Dephosphorylation of the phosphopeptide by GST, GST-CNA-1 (CnA), and GST-CNA-1 + GST-CNB-1 (CnAB) are shown. Ca^{2+} -dependence of the phosphatase activity (– Ca^{2+}) was determined by adding 0.2 mM EGTA (Ca^{2+} -chelator) during the CnAB reaction. Inhibition of phosphatase activity was determined by adding 1 μM each of CsA and bovine cyclophilin (+CsA) in the CnAB reaction mixture. Assays were performed in triplicate with equimolar concentrations of CNA-1 and CNB-1. (D) Western blot analysis with anti-CNA-1 and anti-CNB-1 antibodies. Worm protein extracts prepared from N2 wild-type worms or *cnb-1(jh103)* mutants (CNB, lane 2). The band is absent in the *cnb-1(jh103)* deletion worms indicating they are functionally null mutants.

hypodermal seam cells (Figure 3D). We localized CNB-1 at the subcellular level in specific tissues of wild-type animals by immunogold electron microscopy (EM). Signals of CNB-1 were observed in the seam cells of the lateral hypodermis of wild-type hermaphrodites consistent with our immunostaining data (Figure 3, D–F). Additionally, the male gonad also expressed CNB-1. This was evident from the scattered and distinct cytoplasmic signals of CNB-1 surrounding the cellularized spermatids (Figure 3G).

Based on these observations we further examined wild-type male sperm and immunostained with anti-CNA-1 or anti-CNB-1 antibodies. As expected, we observed robust staining in the wild-type sperm for both proteins and the staining was distinctly cytoplasmic (Figure 4A). We also confirmed CNA-1 and CNB-1 localization in the spermatheca by immunostaining isolated gonads (Figure 4B).

Hence, electron microscopy and immunostaining data reveal that calcineurin is expressed in the *C. elegans* male germline, and therefore may have possible roles in germline development.

cnb-1 Is Involved in Normal Cuticle Formation, Sperm Morphology, and Brood Size

We have isolated *cnb-1(jh103)* deletion mutants by PCR-based TMP/UV mutagenesis method. The *cnb-1(jh103)* null mutants are viable but exhibit multiple phenotypes. A second deletion mutant strain, *cnb-1(ok276)*, was also isolated (kindly donated by the *C. elegans* Knockout Consortium, Oklahoma) and exhibited identical phenotypes to *cnb-1(jh103)*. We further checked the protein profile of the *cnb-1(jh103)* deletion mutants and found no protein band on

Figure 3. Expression and localization of calcineurin. Images show only representative expression. CNA-1::GFP expressed in (A) ventral nerve cord (arrow) and vulva muscles (arrowhead) and (B) body-wall muscles of the midbody region. CNB-1::GFP expresses in spermatheca (C; arrowhead) and intestine (C; arrow). Nerve cord expression can also be observed in this image. (D) Immunostaining of wild-type worms with anti-CNB-1 antibodies detected CNB-1 in hypodermal seam cells (arrow). Bar, 20 μ m. (E–G) Immunogold electron micrograph (EM) showing the subcellular localization of CNB-1. (E) Transverse section through the midbody of a hermaphrodite showing CNB-1 localization as indicated by the signals of gold particles in the seam cells of the lateral hypodermis (LH) closely apposed to the cuticle (arrowhead). (F) Another region of seam cell showing signals. Lateral hypodermis (LH), cuticle (arrowhead), and excretory canal (arrow) are labeled. (G) Localization of CNB-1 in a wild-type male gonad. Distinct and scattered signals of CNB-1 are observed mainly in the cytoplasmic regions of the cellularized spermatids. Bar, 1 μ m.

western blots confirming that the deletion mutants are functionally null (Figure 2D, lane 2).

The *cnb-1(jh103)* mutants were examined for phenotypic defects compared with the wild-type worms. At the surface of the worm, the cuticle of *cnb-1* mutants appears to thin out, resulting in the animals having a more transparent appearance. This phenotype seems consistent with a loss of calcineurin in hypodermal tissue in *cnb-1* mutant worms. *cnb-1* mutants also have a significantly decreased brood size compared with wild-type (Table 1). This observation taken together with the immunostaining data led us to believe that there may be defects in *cnb-1* sperm. Indeed, under close inspection, *cnb-1* sperm morphology was different from wild-type sperm. The defective sperm were smaller and smoother than wild-type sperm (Figure 4C) and displayed smaller pseudopods. Moreover, sperm, which normally gather in high numbers in the spermatheca, were scarcely found in mutant worms. In addition, these *cnb-1*-deficient spermatheca were filled with oocyte debris indicative of a possible endomitotic oocyte (*emo*)-like defect caused by spermatheca defects. To distinguish whether the small brood size phenotype observed in the mutant was a direct cause of defective sperm or spermatheca defects, we mated N2 male worms with *cnb-1* hermaphrodites. As Figure 4D shows,

wild-type sperm only partially rescues the low brood size, and the percentage of outcrossed progeny of *cnb-1* hermaphrodites is almost the same as outcrossed of N2 hermaphrodites. This suggests that altered sperm alone cannot account for the decreased fertility in *cnb-1* mutants, and that defective spermatheca and/or oocytes may also play a role in this phenotype.

Defects in *cnb-1* Mutants Resemble Defects Observed in *unc-43* Mutants

Along with its small brood size, *cnb-1* shows several other characteristic phenotypes. Firstly, mutant worms have small and slender bodies compared with wild-type animals. *cnb-1* worms also retained embryos in the uterus varying from early to very late in development, such as threefold stage embryos, which was not seen in the wild-type worms. *cnb-1* mutations also cause the worms to be uncoordinated (*unc*), or lethargic in their movement. Generally wild-type worms move rapidly in a sinusoidal pattern as evidenced from the tracks made by the worms (Figure 5A), but *cnb-1* mutants moved slowly and with decreased amplitude of tracks. (Figure 5B). Each of these phenotypes was not constant over the lifetime of the worm, but rather all became more severe as

Figure 4. Calcineurin in sperm and the spermatheca. (A) Immunostaining of wild-type sperm with anti-CNA-1 (top left panel) and anti-CNB-1 (bottom left panel) antibodies and the respective nuclei are shown by DAPI staining (right panels). Bar, 2 μ m. (B) Immunostaining of wild-type and *cnb-1* mutant spermatheca with calcineurin antibodies. Anti-CNA-1 (top left panel) and anti-CNB-1 (top middle panel) antibodies stain wild-type spermatheca. Spermatheca of *cnb-1* mutant worms do not show any anti-CNB-1 staining (top right panel). DAPI staining of spermatheca is shown in the bottom panels. Bar, 20 μ m. (C) Sperm and spermatheca defects. Compared with wild-type (left panel) *cnb-1* mutant worms display defects in sperm and the spermatheca. *cnb-1* sperm (middle and right panel). Arrows point to *cnb-1* sperm, which are fewer in number and show a smoother appearance compared with N2 sperm (left panel). The surrounding *cnb-1* spermatheca appears to contain debris. (D) Mating of N2 and *cnb-1* hermaphrodites with N2 males. Three N2 males were mated with one N2 or one *cnb-1* hermaphrodite for 2 days. Total progeny and outcrossed progeny are indicated by white and black bars, respectively.

the worm grew older. Brood size, body size, and embryo retention phenotypes are quantified and summarized in Table 1. We then sought to determine whether *cnb-1* genomic DNA could rescue the *cnb-1(jh103)* phenotypes by microinjection. Movement of these transgenic animals appeared to be fully rescued (Figure 5C) and there was a significant recovery in brood size (146 ± 27 progeny; Table 1). The small body size and retention of late-stage embryos in the uteri were almost completely restored to normal state (Table 1) indicating that these pleiotropic phenotypes observed in the mutant were specifically caused by the loss of *cnb-1* gene.

Although the phenotypes of *cnb-1* mutants appeared to be pleiotropic, it is interesting to note that similar phenotypes were observed in other particular mutants. Mutants of the *unc-43* gene, which encodes the Ca^{2+} /calmodulin-dependent protein kinase CaMKII also show defects in brood size and progressive defects in body size, egg lay-

ing, and movement (Reiner *et al.*, 1999). We compared phenotypes of gain-of-function and loss-of-function mutants of *unc-43* with those of the *cnb-1* null mutant and observed some interesting relationships. *cnb-1* mutants and *unc-43(n498)*[gain-of-function (*gf*)] mutants were quite similar in phenotype, with both mutants showing much lower brood sizes, delayed egg laying, and smaller body size (Table 1). In addition, both worms displayed severe uncoordinated movement (Unc) phenotypes (Figure 5, B and E). Conversely, *unc-43(n1186)* [loss-of-function (*lf*)] mutants showed phenotypes mostly opposite to that of *unc-43(gf)* and *cnb-1* mutants, displaying hyperactive movement (Figure 5D), a significantly higher brood size, and earlier egg-laying compared with that of *cnb-1*. On the other hand, *sma-6* mutants, which also have a small body size phenotype, do not show other defects present in *cnb-1* mutants suggesting that small body size

Table 1. Brood size, body size, and egg-laying phenotypes

	Brood size (self progeny at 20°C)	Body length (mm)	Body width (μm)	Late stage embryos in uterus	
				< comma ^a	≥ comma ^b
N2 (WT)	270 ± 45 (10)	1.12 ± 0.05 (17)	78.53 ± 2.35 (17)	13 ± 1.8	0 (17)
<i>cnb-1</i>	106 ± 17 (15)	0.79 ± 0.03 (20)	55.13 ± 2.22 (20)	6 ± 0.9	2 ± 0.9 (20)
<i>cnb-1;Ex[cnb-1]</i>	146 ± 27 (8)	1.04 ± 0.04 (17)	68.38 ± 3.05 (17)	13 ± 3.3	0 (17)
<i>sma-6</i>	123 ± 17 (10)	0.80 ± 0.04 (20)	69.88 ± 2.75 (20)	10 ± 2.1	0 (20)
<i>unc-43(gf)</i>	61 ± 12 (15)	0.96 ± 0.04 (19)	71.18 ± 2.81 (19)	10 ± 1.7	12 ± 1.9 (19)
<i>unc-43(null)</i>	173 ± 29 (15)	1.01 ± 0.05 (19)	70.53 ± 2.29 (19)	6 ± 2.8	0 (19)
<i>unc-43(gf);cnb-1</i>	8 ± 7 (16)	0.62 ± 0.03 (16)	56.41 ± 3.16 (16)	4 ± 1.3	3 ± 1.6 (16)

Values are expressed as means ± SD; number in parentheses indicates the sample sizes for worms.

^a Number of embryos prior to comma stage inside a 1-d-old adult.

^b Number of embryos from comma stage onwards (comma–3-fold stage) inside a 1-d-old adult.

alone does not directly affect these other phenotypes and that *sma-6* and *cnb-1* likely function in different pathways.

Phenotypes Related to G-protein–coupled Phosphorylation Signaling Pathways in *C. elegans*

It was shown that UNC-43/CaMKII regulates a G-protein pathway involving the G_o-protein α-subunit, *goa-1*, in locomotory and egg-laying behavior (Robatzek and Thomas, 2000). A transgenic gain-of-function mutant of *goa-1*, *syIs9[goa-1(gf)]*, displayed lethargic movement and egg retention behavior similar to phenotypes seen in *unc-43(gf)* mutants (Mendel *et al.*, 1995). Conversely, loss-of-function mutants of *goa-1* showed hyperactive movement and premature egg laying (Mendel *et al.*, 1995). Thus, mutations in

goa-1 result in similar phenotypes to those of *unc-43* mutants, and, consequently, opposite phenotypes to that of *cnb-1* mutants. We decided to further test whether *cnb-1* could be operating in similar G-protein pathways. Along with the phenotypes tested in Table 1, *goa-1* also had defects in serotonin-regulated egg laying (Mendel *et al.*, 1995). Thus, we tested whether *cnb-1* and *unc-43* mutants had defects in this egg-laying behavior as well. Exogenous serotonin and imipramine were shown to stimulate egg laying in wild-type worms (Trent *et al.*, 1983). However, both *cnb-1* and *unc-43(gf)* mutants failed to respond to exogenous serotonin, whereas *unc-43(lf)* mutants were hypersensitive to serotonin treatment by laying even more eggs than wild-type (Table 2). *cnb-1* and *unc-43(gf)* mutants also show a decreased sen-

Figure 5. Locomotion defects in mutants of *cnb-1* and *unc-43*. Five animals were placed in the center of a bacterial lawn and photographed 5 min later. (A) Tracks of movement by wild-type animals; (B) *cnb-1* mutants; (C) rescued *cnb-1* mutants; (D) *unc-43(lf)* mutants; (E) *unc-43(gf)* mutants; (F) *cnb-1; unc-43(gf)* double mutants. Bar, 1 mm.

Table 2. Characterization of serotonin-mediated egg-laying phenotypes

Genotype	No. of animals laying the indicated number of eggs after treatment with:											
	M9 buffer				5-HT				Imipramine			
	>7	4-7	1-3	0	>7	4-7	1-3	0	>7	4-7	1-3	0
N2 (WT)	0	0	2	46	19	6	20	3	20	18	6	4
<i>cnb-1</i>	0	0	1	47	0	0	8	40	0	5	21	22
<i>cnb-1</i> ;Ex[<i>cnb-1</i>]	0	0	3	45	6	4	21	17	22	19	7	0
<i>unc-43</i> (null)	0	0	2	46	26	18	4	0	3	15	26	4
<i>unc-43</i> (gf)	0	1	11	36	1	6	19	3	4	18	18	8
<i>unc-43</i> (gf); <i>cnb-1</i>	0	0	10	38	0	0	12	36	0	0	14	34

Worms were treated with either exogenous 5-HT, imipramine, or control M9 buffer for 90 min after which laid eggs were counted.

sitivity to imipramine, an agent that induces endogenous release of serotonin from stores in the presynaptic HSN neuron, compared with wild-type animals. When compared with the egg-laying defects observed in serotonin-treated *syIs9[goa-1(gf)]* mutants reported elsewhere (Mendel *et al.*, 1995) *cnb-1* and *unc-43(gf)* mutants show slightly more severe phenotypes in response to the treatment.

We next tested whether the serotonin-mediated egg-laying phenotypes observed in *cnb-1* and *unc-43* mutants were concentration-dependent. Unlike wild-type animals, which show increased egg laying in response to elevated concentrations of serotonin (Figure 6A), all three mutants displayed resistance to serotonin even at high concentrations (Figure 6, B and C). We also tested the egg-laying response to another exogenous agent, levamisole. Levamisole is an agonist of the UNC-29 nicotinic acetylcholine receptor localized in postsynaptic muscle, and levamisole treatment to wild-type *C. elegans* results in muscle hyper-contraction and subsequent egg laying (Figure 7A). In contrast, *cnb-1* mutants and both *unc-43(gf)* and *unc-43(lf)* mutants are resistant to levamisole at all concentrations (Figure 7, B, C, and D). Fi-

nally, *goa-1(n1134)* loss-of-function mutants also show concentration-dependent resistance to levamisole (Figure 7E). This further suggests that *cnb-1*, like *unc-43* and *goa-1*, are involved in similar aspects in the regulation of egg laying.

To further validate the possibility that calcineurin, UNC-43, and GOA-1 may be functioning together to regulate these various functions, we attempted to generate double mutants between *cnb-1* and *unc-43* and *goa-1* mutants and assess possible genetic relationships. Although we generated both *unc-43(lf);cnb-1* and *goa-1(n1134);cnb-1* double mutants, the resulting animals were arrested in early larval stages with severe morphological defects. Thus, epistatic phenotypes could not be assessed in these worms. On the other hand, *unc-43(gf);cnb-1* double mutants were viable and developed to adulthood. Since *unc-43(gf)* encodes a protein kinase, mutants should have hyperactive phosphorylation activity in pathways where *unc-43* may be involved in. Likewise, *cnb-1*, which encodes a protein phosphatase that was shown to have enzyme activity, should produce null mutants that have a loss of dephosphorylation activity in pathways which calcineurin may function in. If these two proteins function

Figure 6. Dose-dependent serotonin-mediated egg laying. Color-coded area in bars represent percentage of worms from the total number tested. Respective number of eggs were laid after serotonin treatment at the given concentrations. In each case N = 24. (A) N2 worms show serotonin-mediated egg laying in a dose-dependent manner, whereas (B) *cnb-1* and (C) *unc-43(gf)* mutants and (D) *unc-43(gf);cnb-1* double mutants were mostly resistant even at high concentration of serotonin.

Figure 7. Dose-dependent levamisole-mediated egg laying. Color-coded area in bars represent percentage of worms from the total number tested. Respective number of eggs were laid after levamisole treatment at the given concentrations. In each case $N = 24$. (A) N2 worms show levamisole-mediated egg laying in a dose-dependent manner, whereas (B) *cnb-1*, (C) *unc-43(gf)*, (D) *unc-43(lf)*, (E) *goa-1(n1134)* mutants, and (F) *unc-43; cnb-1* double mutants were mostly resistant even at high concentrations of levamisole.

within a G-protein-coupled phosphorylation pathway in an opposing manner, then a double mutant of *cnb-1* and *unc-43(gf)* would have phosphorylation pathways that are hyperpolarized without any opposing dephosphorylation activity, and, thus, should display more severe phenotypes than animals with single mutations alone. As we had predicted, this double mutant had even more severe defects than the individual mutants by themselves. The worms displayed extremely lethargic movement (Figure 5F), an average body size of less than half that of normal worms, synergistically small brood sizes with most worms being sterile, and severe egg retention leading to internally hatched young and eventual death of the parent worm (Table 1). *unc-43(gf);cnb-1* double mutants also result in increased resistance to serotonin, imipramine, and levamisole treatment compared with the single mutants alone (Table 2; Figures 6D and 7F). In comparison to single mutants, synergistic effects in the double mutants can be clearly seen in fertility and movement phenotypes, and possibly egg-laying phenotypes as well. Therefore, our results suggest that calcineurin can regulate multiple functions in *C. elegans* that both *unc-43* and *goa-1* are known to be involved in, and a role for calcineurin phosphatase activity in a CaMKII-dependent G-protein coupled phosphorylation signaling pathway in *C. elegans* is a distinct possibility.

DISCUSSION

Calcineurin, a serine/threonine protein phosphatase, plays important roles in the transduction of Ca^{2+} signals to regulate various cellular processes (Klee *et al.*, 1979; Stewart *et al.*, 1982; Klee *et al.*, 1988). In this study, we identified and characterized calcineurin from *C. elegans*, both at its molecular and cellular levels. In contrast to higher animals where calcineurin subunits are encoded by more than one gene, *C. elegans* calcineurin subunits are each encoded by a single gene. Results reported here also show that the homologues of the mammalian and *Drosophila* calcineurin subunits exist

in *C. elegans*, and thus, they represent a conserved branch of the PP2B family of protein phosphatases having important roles in normal physiology (Crabtree, 2001).

The subunits of *C. elegans* calcineurin were shown to interact with one another, and this interaction conferred strong phosphatase activity to the heterodimer, which is consistent with studies on other conserved forms of calcineurin (Klee *et al.*, 1979; Stewart *et al.*, 1982; Klee *et al.*, 1988). In addition, Ca^{2+} -dependent phosphatase activity of CNA-1 was potently inhibited by the immunosuppressant CsA (Figure 2C) in agreement with previous reports (Liu *et al.*, 1991). Sequence homology and Ca^{2+} overlay experiments verified that CNB-1 has strong calcium binding affinity. Thus, we believe that the biochemical function of calcineurin as a calcium-binding heterodimeric protein phosphatase is conserved in *C. elegans*.

A deletion null mutant of *cnb-1* was isolated by target-selected mutagenesis. *cnb-1(jh103)* mutants are viable although loss of calcineurin activity leads to pleiotropic defects for the worms. It was shown that transgenic mice expressing a mutated form of CnB that cannot bind the CnA subunit will die early in embryo development (Graef *et al.*, 2001). These mice exhibit vascular developmental abnormalities that stem from its inability to dephosphorylate and subsequently translocate the transcription factor NFAT from the cytoplasm into the nucleus. Thus, the dephosphorylation activity of calcineurin is dependent on both CnA and CnB subunits in vivo. This is also observed in *S. cerevisiae* as mutations in either CnA or CnB lead to a complete loss of calcineurin phosphatase activity and function in yeast (Cyert and Thorner, 1992). Finally, a recent study showed that a chemotaxis-defective mutant *tax-6(p675)*, which displays thermotaxis, body size, and growth defects as well, carries a mutation in the *cna-1* locus (Kuhara *et al.*, 2002). Thus, a viable mutant of *cnb-1* in a multicellular organism like *C. elegans*, although quite surprising, is important for further studies in the delineation of calcineurin's diverse functions in vivo.

Mutants of *cnb-1* showed pleiotropic phenotypes including cuticle defects, small body size, decreased brood size, and locomotory and egg-laying defects. These defects were consistent with the loss of calcineurin function in tissues that normally express the protein phosphatase; these include hypodermal tissue, spermatheca, sperm, body-wall muscle, and vulva muscle. Most of the phenotypes that we observed in *cnb-1* mutants appeared to be very similar to and characteristic of those observed in *unc-43(gf)* yet opposite to those in *unc-43(lf)* mutants. A double mutant between this *cnb-1* null mutant and an *unc-43(gf)* mutant causes a synergistic effect of movement, fertility, and possibly egg-laying phenotypes as well, which may suggest a complementary relationship between the Ca^{2+} -calmodulin-dependent protein kinase CaMKII and the Ca^{2+} -calmodulin-dependent protein phosphatase calcineurin. Opposing functions of calcineurin and CaMKII have also been observed biochemically in integrin signaling, in the receptor associated protein RAP, in skeletal muscle dystrophin, and in T-cell signaling (Ngheim *et al.*, 1994; Walsh *et al.*, 1995; Peterson *et al.*, 1996; Bouvard *et al.*, 1998). Thus, this kind of biochemical role for calcineurin in *C. elegans* is a reasonable and plausible function.

It was previously shown that mutations in *goa-1*, the homologue of the $G_o\alpha$ -subunit of the G_o protein, can suppress locomotory defects associated with *unc-43(n498)* gain-of-function mutants (Robatzek and Thomas, 2000). Besides an uncoordinated movement phenotype, loss-of-function mutants of *goa-1* also exhibited defects in egg laying and reduced brood size (Mendel *et al.*, 1995) similar to those seen in *unc-43(lf)* mutants and opposite to those observed in our *cnb-1* mutant. We tested to see if *cnb-1* was also defective in another *goa-1* related phenotype, serotonin-mediated egg laying (Mendel *et al.*, 1995). *cnb-1* mutants exhibited defects in serotonin-induced egg-laying similar to those seen in *unc-43(gf)* mutant and a transgenic *goa-1* gain-of-function mutant *syIs9[goa-1(gf)]*. In addition, the dose-dependent serotonin-mediated and levamisole-mediated egg-laying curves confirmed that calcineurin is involved in similar aspects of *C. elegans* egg laying as *unc-43* and *goa-1* are involved in. From these data we suggest that calcineurin may be involved in G-protein-coupled phosphorylation pathways in locomotion, egg laying, and brood size in *C. elegans*.

The involvement of calcineurin in G-protein-mediated signaling has been observed in many different pathways including regulation of NFAT in cardiac myocytes, T helper cell immunity, and cardiac hypertrophy (Mende *et al.*, 1998; Bikah *et al.*, 2000; Ichida and Finkel, 2001). In addition, phosphatase activity as an inhibitor of G-protein phosphorylation signaling is a common method of pathway regulation (Xiao *et al.*, 1999) so it is likely that calcineurin may be fulfilling this role in *C. elegans*. Finally, calcium oscillations evoked by G-protein coupled receptors and stimulated by regulators of G-protein signaling (RGS) proteins could initiate Ca^{2+} /calmodulin-dependent calcineurin activity in *C. elegans* which has two homologous RGS proteins, EGL-10 and EAT-16, directly involved in G-protein pathways (Wilkie, 2000).

Although movement and egg-laying phenotypes are known to be involved in a G-protein-mediated pathway, other phenotypes observed in *cnb-1* mutants such as body size and fertility were not yet determined to be involved in

this specific pathway. Body-size phenotypes are not likely regulated by this specific pathway since small body size in *unc-43* mutants is caused by tonically contracted body-wall muscle (Reiner *et al.*, 1999), which we did not observe in *cnb-1* mutants. On the other hand, the relationship of the brood size phenotype to the *unc-43*-regulated G-protein signaling pathway may be more difficult to determine. We showed that calcineurin expresses in the spermatheca and sperm, and loss of calcineurin function results in burst oocytes derived from defects in the spermatheca and sperm morphology defects. Attempted rescue of this phenotype by mating *cnb-1* hermaphrodites with wild-type males (Figure 4) showed that the cause of this defect may be from multiple factors. To assess whether this phenotype is involved in this same pathway similar experiments need to be performed on mutant *goa-1* and *unc-43* worms.

Just as calcineurin is involved in many types of signaling, CaMKII and $G_o\alpha$ are also general signaling molecules and have diverse functions. *unc-43* mutants are not only involved in defective locomotion, egg laying, and brood size, but also show abnormal defecation behavior (Reiner *et al.*, 1999). We also assessed the defecation cycle in *cnb-1* mutants but worms showed normal defecation behavior. In the same way, *goa-1* mutants also show other defective behaviors. Among these behaviors, *goa-1* males have problems executing the "turning" behavior of male mating, which may be a result of defective diagonal muscles (Loer and Kenyon, 1993; Mendel *et al.*, 1995). We also tested whether calcineurin RNAi-affected male worms show defects in turning behavior, but no significant defects nor any expression of calcineurin in diagonal muscle tissue could be detected. The common defects observed in all *cnb-1*, *unc-43*, and *goa-1* mutants suggest that locomotion and egg-laying defects may be specific to a CNB-1/UNC-43 G-protein-coupled phosphorylation pathway in *C. elegans*.

Calcineurin may function upstream or downstream of *unc-43*, which is known specifically to regulate locomotion via the G_o/G_q signaling network (Robatzek and Thomas, 2000). If a double mutant of *cnb-1* and either *unc-43(lf)* or *goa-1(lf)* was generated, epistatic phenotypes between these functionally antagonistic proteins would distinguish where calcineurin may function in relation to UNC-43 or GOA-1. Unfortunately, both *unc-43(lf);cnb-1* and *goa-1(n1134);cnb-1* resulted in worms that were arrested in the L1 larval stage with severe morphology defects, indicating that both UNC-43 and GOA-1 in association with calcineurin are essential for developmental signaling pathways distinct from the G-protein pathway described above. Although egg-laying phenotypes could not be assessed in these double mutants, we attempted to observe locomotory behavior. Although *goa-1(n1134);cnb-1* movement phenotypes were not assessed, *cnb-1;unc-43(lf)* double mutants exhibited hyperactive movement similar to *unc-43(lf)* mutants, although it was difficult to quantify this behavior due to the small size of the worm and its severe defects. Nevertheless, our observations indicate that *unc-43* might be epistatic to *cnb-1*, which could further verify the role of calcineurin in a G-protein-mediated signaling pathway.

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REFERENCES

- Aitken, A., Cohen, P., Santikarn, S., Williams, D.H., Calder, A.G., Smith, A., and Klee, C.B. (1982). Identification of the NH₂-terminal locking group of calcineurin B as myristic acid. *FEBS Lett.* 150, 314–318.
- Arduengo, P.M., Appleberry, O.K., Chuang, P., and L'Hernault, S.W. (1998). The presenilin protein family member SPE-4 localizes to an ER/Golgi derived organelle and is required for proper cytoplasmic partitioning during *Caenorhabditis elegans* spermatogenesis. *J. Cell Sci.* 111, 3645–3654.
- Barstead, R.J. (1999). Reverse genetics. In: *C. elegans: A Practical Approach*, ed. I.A. Hope, Oxford, UK: Oxford University Press Inc., 97–118.
- Bikah, G., Pogue-Caley, R.R., McHeyzer-Williams, L.J., and McHeyzer-Williams, M.G. (2000). Regulating T helper cell immunity through antigen responsiveness and calcium entry. *Nat. Immunol.* 1, 402–412.
- Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. *Genetics* 77, 71–94.
- Bouvard, D., Molla, A., and Block, M.R. (1998). Calcium/calmodulin-dependent protein kinase II controls $\alpha 5\beta 1$ integrin-mediated inside-out signaling. *J. Cell Sci.* 111, 657–665.
- Chin-Sang, I.D., and Spence, A.M. (1996). *Caenorhabditis elegans* sex-determining protein FEM-2 is a protein phosphatase that promotes male development and interacts directly with FEM-3. *Genes Dev.* 10, 2314–2325.
- Cho, J.H. *et al.* (2000). Calsequestrin, a calcium sequestering protein localized at the sarcoplasmic reticulum, is not essential for body-wall muscle function in *Caenorhabditis elegans*. *J. Cell Sci.* 113, 3947–3958.
- Cohen, P. (1989). The structure and regulation of protein phosphatases. *Annu. Rev. Biochem.* 58, 453–508.
- Crabtree, G.R. (1999). Generic signals and specific outcomes: signaling through Ca²⁺, calcineurin, and NF-AT. *Cell* 96, 611–614.
- Crabtree, G.R. (2001). Calcium, calcineurin and the control of transcription. *J. Biol. Chem.* 276, 2313–2316.
- Cyert, M.S. (1993). The function of Ca²⁺/calmodulin-regulated phosphatase in yeast. *Adv. Protein Phosphatases* 7, 429–443.
- Cyert, M.S., and Thorner, J. (1992). Regulatory subunit (CNB1 gene product) of yeast Ca²⁺/calmodulin-dependent phosphoprotein phosphatases is required for adaptation to pheromone. *Mol. Cell Biol.* 12, 3460–3469.
- Graef, I.A., Chen, F., Chen, L., Kuo, A., and Crabtree, G.R. (2001). Signals transduced by Ca²⁺/calcineurin and NFATc3/c4 pattern the developing vasculature. *Cell* 105, 863–875.
- Guerini, D., and Klee, C.B. (1991). Structural diversity of calcineurin, a Ca²⁺ and calmodulin-stimulated protein phosphatase. *Adv. Protein Phosphatases* 6, 391–410.
- Hirata, D., Harada, S.-I., Namba, H., and Miyakawa, T. (1995). Adaptation to high salt stress in *Saccharomyces cerevisiae* is regulated by Ca²⁺/calmodulin-dependent phosphoprotein phosphatase (calcineurin) and cAMP-dependent protein kinase. *Mol. Gen. Genet.* 249, 257–264.
- Ichida, M., and Finkel, T. (2001). Ras regulates NFAT3 activity in cardiac myocytes. *J. Biol. Chem.* 276, 3524–3530.
- Jones, A.R., Francis, R., and Schedl, T. (1996). GLD-1, a cytoplasmic protein essential for oocyte differentiation shows stage- and sex-specific expression during *Caenorhabditis elegans* germline development. *Dev. Biol.* 180, 165–183.
- Kincaid, R.L. (1993). Calmodulin-dependent protein phosphatases from microorganisms to man: a study in structural convertism and biological diversity. *Adv. Second Messenger Phosphoprotein Res.* 27, 1–23.
- Klee, C.B., Crouch, T.H., and Krinks, M.H. (1979). Calcineurin: a calcium- and calmodulin-binding protein of the nervous system. *Proc. Natl. Acad. Sci. USA* 76, 6270–6273.
- Klee, C.B., Ren, H., and Wang, X. (1998). Regulation of the calmodulin-stimulated protein phosphatase, calcineurin. *J. Biol. Chem.* 273, 13367–13370.
- Klee, C.B., Draetta, G.F., and Hubbard, M.J. (1988). Calcineurin. *Adv. Enzymol. Relat. Areas Mol. Biol.* 61, 149–200.
- Kozak, M. (1991). Structural features in eukaryotic mRNAs that modulate the initiation of translation. *J. Biol. Chem.* 266, 19867–19870.
- Krause, M. (1995). Transcription and translation. In: *Methods in Cell Biology*, vol. 48, ed. H.F. Epstein and D.C. Shakes, San Diego, CA: Academic Press, 513–529.
- Kuhara, A., Inada, H., Katsura, I., and Mori, I. (2002). Negative regulation and gain control of sensory neurons by the *C. elegans* calcineurin TAX-6. *Neuron* 33, 751–763.
- Liu, J., Farmer, J.D., Lane, W.S., Friedman, J., Weissman, I., and Schreiber, S.L. (1991). Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. *Cell* 66, 807–815.
- Loer, C.M., and Kenyon, C.J. (1993). Serotonin-deficient mutants and male mating behavior in the nematode *Caenorhabditis elegans*. *J. Neurosci.* 13, 5407–5417.
- L'Hernault, S.W., and Roberts, T.M. (1995). Cell biology of nematode sperm. In: *Methods in Cell Biology*, vol. 48, ed. H.F. Epstein and D.C. Shakes, San Diego, CA: Academic Press, 273–301.
- Maruyama, K., Mikawa, T., and Ebashi, S. (1984). Detection of calcium binding proteins by ⁴⁵Ca autoradiography on nitrocellulose membrane after sodium dodecyl sulfate gel electrophoresis. *J. Biochem.* 95, 511–519.
- Mello, C., and Fire, A. (1995). DNA transformation. *Methods in cell biology*. In: *Methods in Cell Biology*, vol. 48, ed. H.F. Epstein and D.C. Shakes, San Diego, CA: Academic Press, 452–480.
- Mende, U., Kagen, A., Cohen, A., Aramburu, J., Schoen, F.J., and Neer, E.J. (1998). Transient cardiac expression of constitutively active G α_q leads to hypertrophy and dilated cardiomyopathy by calcineurin-dependent and independent pathways. *Proc. Natl. Acad. Sci. USA* 95, 13893–13898.
- Mendel, J.E., Korswagen, H.C., Liu, K.S., Hajdu-Cronin, Y.M., Simon, M.I., Plasterk, R.H.A., and Sternberg, P.W. (1995). Participation of the protein G α_o in multiple aspects of behavior in *C. elegans*. *Science* 267, 1652–1655.
- Miller, D.M., and Shakes, D.C. (1995). Immunofluorescence microscopy. In: *Methods in Cell Biology*, vol. 48, ed. H.F. Epstein and D.C. Shakes, San Diego, CA: Academic Press, 365–394.
- Molkentin, J.D. (1998). Calcineurin inhibition and cardiac hypertrophy. *Science* 282, 1007a.
- Molkentin, J.D., and Dorn, G.W. (2001). Cytoplasmic signaling pathways that regulate cardiac hypertrophy. *Annu. Rev. Physiol.* 63, 391–426.

- Mulkey, R.M., Endo, S., Shenolikar, S., and Malenka, R.C. (1994). Involvement of a calcineurin/inhibitor-1 phosphatase cascade in hippocampal long-term depression. *Nature* 369, 486–488.
- Ngheim, P., Ollick, T., Gardner, P., and Schulman, H. (1994). Interleukin-2 transcriptional block by multifunctional Ca^{2+} /calmodulin kinase. *Nature* 371, 347–350.
- Page, A.P., MacNiven, K., and Hengartner, M.O. (1996). Cloning and biochemical characterization of the cyclophilin homologues from the free-living nematode *Caenorhabditis elegans*. *Biochem. J.* 317, 179–185.
- Park, B.J. *et al.* (2001a). Calreticulin, a calcium-binding molecular chaperone, is required for stress response and fertility in *C. elegans*. *Mol. Biol. Cell* 12, 2835–2845.
- Park, B.J., Lee, J.I., Lee, J., Kim, S., Choi, K.Y., Park, C.S., and Ahnn, J. (2001b). Isolation of deletion mutants by reverse genetics in *Caenorhabditis elegans*. *Korean J. Biol. Sci.* 5, 65–69.
- Perrino, B.A., Ng, L.Y., and Soderling, T.R. (1995). Calcium regulation of calcineurin phosphatase activity by its B subunit and calmodulin. *J. Biol. Chem.* 270, 340–346.
- Peterson, C.M. *et al.* (1996). The receptor-associated protein (RAP) binds calmodulin and is phosphorylated by calmodulin-dependent kinase II. *EMBO J.* 15, 4165–4173.
- Robatzek, M., and Thomas, J.H. (2000). Calcium/calmodulin-dependent protein kinase II regulates *C. elegans* in concert with a Go/Gq signaling network. *Genetics* 156, 1069–1082.
- Reiner, D.J., Newton, E.M., Tian, H., and Thomas, J.H. (1999). Diverse behavioral defects caused by mutations in *Caenorhabditis elegans unc-43* CaM Kinase II. *Nature* 402, 199–203.
- Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*. 2nd ed., Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- Schreiber, S.L., and Crabtree, G.R. (1992). The mechanism of action of cyclosporin A and FK506. *Immunol. Today* 13, 136–142.
- Shibasaki, F., and McKeon, F. (1995). Calcineurin functions in Ca^{2+} activated cell death in mammalian cells. *J. Cell Biol.* 131, 735–743.
- Stark, M.J.R. (1996). Yeast protein serine/threonine phosphatases: multiple roles and diverse regulation. *Yeast* 12, 1647–1675.
- Stewart, A.A., Ingebritsen, T.S., Manalan, A., Klee, C.B., and Cohen, P. (1982). Discovery of a Ca^{2+} - and calmodulin-dependent protein phosphatase: probable identity with calcineurin (CaM-BP₈₀). *FEBS Lett.* 137, 80–84.
- Trent, C., Tsung, N., and Horvitz, H.R. (1983). Egg-laying defective mutants of the nematode *C. elegans*. *Genetics* 104, 619–647.
- Walsh, M.P., Busaan, J.L., Fraser, E.D., Fu, S.Y., Pato, M.D., and Michalak, M. (1995). Characterization of the recombinant C-terminal domain of dystrophin: phosphorylation by calmodulin-dependent protein kinase II and dephosphorylation by type 2B protein phosphatase. *Biochemistry* 34, 5561–5568.
- Wilkie, T.M. (2000). G-protein signaling: satisfying the basic necessities of life. *Curr. Biol.* 10, 853–856.
- Xiao, R., Cheng, H., Zhou, Y., Kuschel, M., and Lakatta, E.G. (1999). Recent advances in cardiac β 2-adrenergic signal transduction. *Circ. Res.* 85, 1092–1100.
- Zhao, S., Bouchard, P., Diltz, C.D., Shen, S.H., and Fischer, E.H. (1993). Purification and characterization of a protein tyrosine phosphatase containing SH2 domains. *J. Biol. Chem.* 268, 2816–2820.