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Molecules and Cells

Calcineurin Regulates Coelomocyte Endocytosis via DYN-1 and CUP-4 in *Caenorhabditis elegans*

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C. elegans **coelomocytes are macrophage-like scavenger cells that provide an excellent *in vivo* system for the study of clathrin-mediated endocytosis. Using this *in vivo* system, several genes involved in coelomocyte endocytosis have been identified previously. However, the detailed mechanism of endocytic pathway is still unknown. Here, we report a new function of calcineurin, an evolutionarily conserved Ca^{2+}/calmodulin-dependent Ser/Thr protein phosphatase, in coelomocyte endocytosis. We found that calcineurin mutants show defective coelomocyte endocytosis. Genetic analysis suggests that calcineurin and a GTPase, dynamin (DYN-1), may function upstream of an orphan receptor, CUP-4, to regulate endocytosis. Therefore, we propose a model in which calcineurin may regulate coelomocyte endocytosis via DYN-1 and CUP-4 in *C. elegans*.**

INTRODUCTION

Calcineurin belongs to the family of Ca^{2+}/calmodulin-dependent serine/threonine protein phosphatase (Crabtree, 1999; Klee et al., 1979; 1998; Stewart et al., 1982) and consists of two subunits, catalytic subunit A and regulatory subunit B (Klee et al., 1988). Calcineurin is abundantly expressed in brain as well as other tissues (Kincaid, 1993) and its function has been well studied in a variety of organisms, from yeast to human. In the yeast, *Saccharomyces cerevisiae*, calcineurin has been implicated to maintain Ca^{2+} homeostasis via the regulation of transcription factors for Ca^{2+} pumps and exchangers (Stark, 1996). In higher animals, calcineurin dephosphorylates the NF-AT transcription factor which translocates from the cytosol to the nucleus (Garcia-Cozar et al., 1998; Im and Rao, 2004; Jain et al., 1993; Loh et al., 1996; Luo et al., 1996), where it activates several downstream genes such as interleukin-2, which is required for T cell growth (Schreiber and Crabtree, 1992).

Caenorhabditis elegans calcineurin binds calcium and functions as a heterodimeric protein phosphatase in a biochemically conserved fashion (Bandyopadhyay et al., 2002). We have previously reported that calcineurin is extensively expressed in neurons as well as in hypodermal seam cells, body-wall muscle, vulva muscle, sperm and the spermatheca (Bandyopadhyay et al., 2002; Kuhara et al., 2002; Lee et al., 2004). In addition, loss of function and gain of function mutants of calcineurin A, *tax-6(p675)* and *tax-6(jh107)* as well as the null mutant of calcineurin B, *cnb-1(jh103)* have been isolated and characterized (Bandyopadhyay et al., 2002; Kuhara et al., 2002; Lee et al., 2004). Previous *C. elegans* mutant have revealed that calcineurin regulates Ca^{2+}-dependent signaling in various processes including thermotaxis, chemotaxis, locomotion, egg-laying behavior and defecation.

In this study, we further investigated a novel calcineruin function in *C. elegans* endocytosis. Endocytosis is a crucial cellular mechanism required to internalize fluid from the extracellular medium, intake nutrients, and recycle membrane components (Mukherjee et al., 1997). Clathrin-mediated endocytosis is the best-studied endocytic pathway in worms and mammals (Brodsky et al., 2001; Grant and Sato, 2006), and many of its components including receptors, adaptors, and regulators have been isolated and extensively studied. The large GTPase dynamin is one of these components, involved in pinching off the coated pit from the plasma membrane to form a clathrin-coated vesicle (Grant and Sato, 2006). Dynamin contains a GTPase domain, PH domain, GED and PRD (Hinshaw, 2000). Previously, dynamin was reported to be a physiological substrate of calcineurin, which has relatively narrow substrate specificity compared to those of other phosphatases (Klee et al., 1988; Morioka et al., 1999). Furthermore, the Ca^{2+}-dependent interactions between calcineurin and dynamin 1 and subsequent function in clathrin-mediated synaptic vesicle recycling have been reported in mammalian systems (Lai et al., 1999). Especially, the dynamin 1 phosphorylation site and its phosphorylation-dependent binding partners have been extensively studied in mammals *in vitro* and *in vivo* (Anggono et al., 2006; Clayton et al., 2009; Graham et al., 2007; Tan et al., 2003).

Here, we investigated the function of calcineurin in coelomo-

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cyte endocytosis using *C. elegans* as an *in vivo* organism-level model. Our results indicate that calcineurin specifically functions in coelomocyte endocytosis probably through the dephosphorylation of dynamin. In addition, CUP-4 may function downstream as a receptor for foreign materials, regulating coelomocyte endocytosis. So far, only a few identified genes, including *cup-4*, have been cloned and studied in coelomocyte endocytosis. Therefore, our results may help to reveal the exact molecular pathway that regulates coelomocyte endocytosis.

MATERIALS AND METHODS

C. elegans strains and maintenance

The following strains were obtained from the *Caenorhabditis* Genetics Center (CGC): Bristol N2, RB950 *cup-4(ok837)* III, NP147 *cdIs10[pcc1::CUP-4::GFP+pRF4(rol-6(su1006))]*, and CX51 *dyn-1(ky51)* X. GS1912 *arIs37[pmyo-3::ssGFP]* I;*dpy-20(e1282)* IV was kindly provided by Dr. Hyeon-Sook Koo. KJ300 *cnb-1(jh103)* V and KJ306 *tax-6(jh107)* IV were previously isolated (Bandyopadhyay et al., 2002; Lee et al., 2004). KJ326 *dyn-1(jh133)* X and KJ327 *dyn-1(jh134)* X were isolated using reverse genetic methods (Park, 2001) and were outcrossed eight times with N2 wild-type worms in order to remove other accompanying mutations. Worm breeding and handling were conducted as previously described (Brenner, 1974).

Plasmid construction

To construct DYN-1/PRD::GST, the C-terminal 948 bp of *dyn-1* cDNA, which covers the proline-rich domain, was amplified from a cDNA libray and a yk clone (yk44d2.5) with the following primers: outer upstream primer (5′-GAT TGA TCC ACA ACT TGA GAG ACA-3′) and outer downstream primer (5′-GGG CAT TGT ACA ATT ACT ATG GG-3′), inner upstream primer (5′-CCG TAA TTT GGT TGG ATC CTA CA-3′) and inner downstream primer (5′-ATG ACA AGT GAG CTA GAA TTC AGA TGT-3′). The amplified *dyn-1* fragment was then subcloned into pGEX4T-2 (Pharmacia) using *Bam*HI and *Eco*RI restriction enzyme sites (pAN393).

Preparation of DYN-1/PRD::GST protein, worm protein extract and GST pull-down assay

The GST-fused recombinant protein was over-expressed in BL21 DE3 *E. coli* overnight at 18°C in the presence of 0.5 mM IPTG (after the culture reached an OD_{600} of 0.5). The fusion protein was affinity purified using a glutathione sepharose (Pharmacia) column and eluted. Wild-type (N2) worms were washed several times with M9 buffer (3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl and 1 ml of 1 M $MgSO_4$) and collected in five volumes of lysis buffer (25 mM Tris pH 8, 8 mM EDTA, 1% Triton X-100, 1 mM PMSF and proteinase inhibitor cocktail tablets (Roche-Applied Science). The worms were sonicated and the lysate was centrifuged at 4°C at 12,000 rpm for 15 min. The supernatant was collected and the protein concentration was determined using the Bradford method (Bio-Rad). 20 μg purified DYN-1/PRD::GST proteins and 10 μg GST proteins were incubated in 40 μl glutathione sepharose slurry overnight at 4°C. The mix was then incubated with 500 μg wild-type (N2) worm lysate in binding buffer (50 mM Tris-Cl pH 7.4, 100 mM NaCl, 2 mM $MgCl_2$, 0.2% Triton X-100, 0.5 mg/ml Bovine Serum Albumin, 0.5 mM β-mercaptoethanol) for 4 h. The samples were then washed three times in binding buffer and run on a SDS-PAGE gel. Western blot was performed using anti-TAX-6 polyclonal antibody. The Ca^{2+} concentration was determined as previously described (Lee et al., 2003).

Isolation of a *dyn-1* deletion mutant from a mutagenized DNA libray

The TMP (Trimethylpsoralen)/UV method was used to generate *dyn-1* deletion mutants. The mutagenized *C. elegans* deletion mutant library was screened using a nested PCR-based method and subsequent sib selections as previously described (Park, 2001). Primers were designed based on the predicted sequences spanning the specific genomic DNA of *dyn-1 (C02C6.1)*. For primers for *dyn-1(jh133)* were as follow: outer upstream primer (5′-CTT CTC AAT GCT CTT CCT CAT ATC A-3′) and outer downstream primer (5′-CTT GGA ATG TTG AAA TGG CAA T-3′), inner upstream primer (5′-AGT TCA GCG TAT AAC CAC CAG G-3′) and inner downstream primer (5′-CCA CCC TCA ATA TCC TTC AGC-3′). To confirm the homozygous deletion, the deletion downstream primer (5′-ACG CAT CCG AAG TGG CGA GAT C-3′) was paired with the inner upstream primer. The primers used for *dyn-1(jh134)* were: outer upstream primer (5'-TTA GTG CTG AAG CAA AAG CCT C-3′) and outer downstream primer (5'-TGA CAA GTG AGC TAG GAT GGA GA-3′), inner upstream primer (5′-ATT GCC ATT TCA ACA TTC CAA G-3′) and inner downstream primer (5′-GCT AGG ATG GAG ATG TAA AAG CAT-3′). To confirm the homozygous deletion, the deletion upstream primer (5′-GAT TGA TCC ACA ACT TGA GAG ACA-3′) was paired with the inner downstream primer.

Each line of homozygous animals with an 807 bp- (*jh133*) and a 996 bp-deletion (*jh134*) was isolated. These lines were outcrossed eight times into wild-type animals to establish the KJ326 *dyn-1(jh133)* and KJ327 *dyn-1(jh134)* strain that were used in subsequent analyses. Deletion regions for *dyn-1(jh133)* and *dyn-1(jh134)* hermaphrodites were determined using nested PCR and sequencing.

Phenotype analysis

L4-stage larvae were transferred to a new plate and allowed to grow for 24 h. The resulting one-day-old adult worms were then examined for phenotypes. For the *dyn-1(ky51)* mutant, L4-stage larvae were incubated at 20°C for 20 h and then shifted to 25°C. Young adult worms were incubated for at least three more hours at 25°C to induce temperature-sensitive phenotypes.

To check for defects in ssGFP uptake by coelomocytes, all mutant strains were crossed into *arIs37[pmyo-3::ssGFP];dpy-20(e1282)* or *cdIs10[pcc1::CUP-4::GFP+pRF4]*. The resulting ssGFP-expressing animals were maintained and used for subsequent analysis. To quantitate the phenotypes, we calculated the percentage of total coelomocytes labeled with GFP.

Microscopy

Worms were mounted onto a 2% agarose pad in 10 mM levamisole (Sigma) and photographed using a Carl Zeiss (Axio Imager, A1).

RESULTS

Calcineurin functions in coelomocyte endocytosis

Previous work has demonstrated multiple functions for the phosphatase calcineurin in many behaviors and developmental processes important to *C. elegans*. We sought to determine whether calcineurin also plays a role in regulating *in vivo* subcellular processes in the nematode. Coelomocyte endocytosis is one of the major clathrin-mediated endocytoses in *C. elegans*. Even though *c*oelomocyte *up*take defective (*cup*) mutants have been isolated from genetic screens (Fares and Greenwald, 2001a), the precise *in vivo* mechanism of coelomocyte endocy-

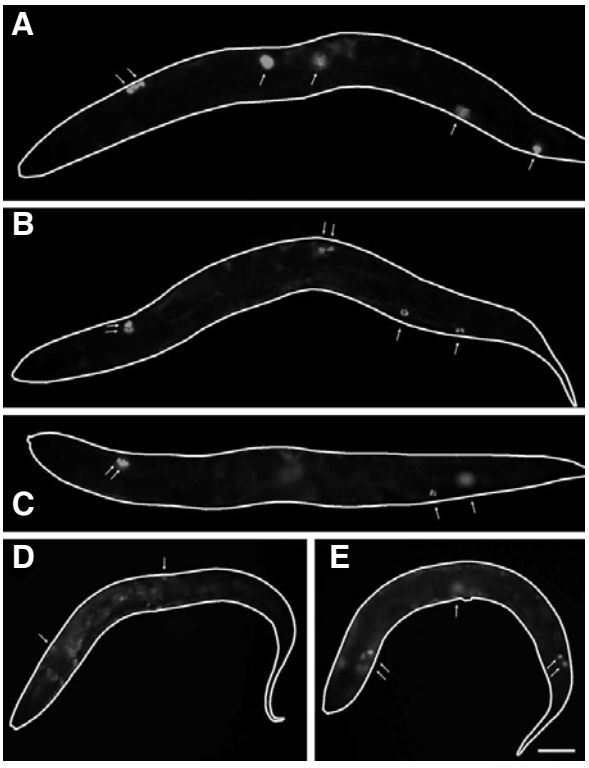

Fig. 1. Calcineurin mutants display defects in coelomocyte endocytosis. ssGFP was monitored for its expression and secretion in wild-type and mutant animals. Coelomocytes are indicated by arrows. Some coelomocytes are out of focus. (A) Coelomocytes labeled with ssGFP are shown in wild-type animals [*arls37;dpy-20(e1282)*]. Each pair of ventral anterior, ventral posterior and dorsal coelomocytes are shown along the worm body (left to right order). (B, C) *arls37;tax-6(jh107)(gf) dpy-20(e1282).* Some *tax-6(jh107)(gf)* mutant animals showed relatively normal ssGFP uptake by coelomocytes (B), whereas others showed defective uptake. Ventral posterior coeolomocytes were not clear (C). (D) *arls37;tax-6(p675) (lf) dpy-20(e1282). tax-6(p675)(lf)* mutant animals showed defective ssGFP uptake into coelomocytes. ssGFP was mainly accumulated in the pseudocoelom (body cavity). Only two coelomocytes are clear. (E) *arls37;dpy-20(e1282);cnb-1(jh103). cnb-1(jh103)* mutant animals also failed to uptake ssGFP into coelomocytes. Coelomocytes are clearly recognizable but ssGFP still remained in the body cavity. Scale bar, 50 μm.

tosis is still unknown.

We examined whether calcineurin mutants have specific defects in coelomocyte endocytosis. In order to observe specific endocytosis in coelomocytes, we used a very simple but powerful assay developed by Fares and Greenwald (Fares and Greenwald, 2001a). The animal used in this assay expresses a chromosomally-integrated signal sequence (ss)-GFP fusion protein in body wall muscles (*pmyo-3::ssGFP*). The *arls37 [pmyo-3::ssGFP] I;dpy-20(e1282) IV* starin continuously secretes ssGFP from the body wall muscle into the body cavity (pseudocoelom). Six coelomocyte cells take up molecules from the pseudocoelom via endocytosis and show strong ssGFP labeling in this strain (Fig. 1A). We observed every single coelomocyte to determine whether it showed normal or defective endocytosis according to qualitative criteria (Supplementary Fig. S1). Using this assay, we found that 25% and 60% of *tax-6(jh107)(gf)* and *cnb-1(jh103)* mutant animals, respectively, displayed defective coelomocyte endocytosis (Figs. 1B, 1C, and 1E; Table 1). In calcineurin mutants, ssGFP was accumulated in the pseudocoelom. Coelomocytes were not clearly distinguishable compared to wild type animals (Fig. 1A), suggesting decreased and/or defective coelomocyte endocytosis. We further tested coelomocyte endocytosis in *tax-6(p675)* loss-of-function mutants. As shown in Fig. 1D and Table 1, *tax-6(p675)(lf)* mutant animals also showed defective coelomocyte endocytosis. These results suggest that calcineurin functions in endocytotic proesses in *C. elegans*.

Table 1. Coelomocyte endocytosis in calcineurin mutants

Genotypes	Normal CUP-4[a]	CUP-4[-][b]	CUP-4[+][c]
	Cup %[d]	Cup %[d]	Cup %[d]
Wild-type	5 (n = 270)	98 (n = 120)	6 (n = 120)
dyn-1(ky51)	90 (n = 120)	98 (n = 114)	18 (n = 120)
dyn-1(jh133)	99 (n = 160)	ND[e]	ND[e]
dyn-1(jh134)	99 (n = 160)	ND[e]	ND[e]
tax-6(jh107)(gf)	25 (n = 132)	96 (n = 204)	4 (n = 120)
tax-6(p675)(lf)	64 (n = 150)	86 (n = 174)	16 (n = 150)
cnb-1(jh103)	60 (n = 330)	95 (n = 222)	9 (n = 108)
tax-6(jh107);dyn-1(ky51)	90 (n = 96)	ND[e]	ND[e]
tax-6(p675);dyn-1(ky51)	87 (n = 126)	ND[e]	ND[e]
cnb-1(jh103);dyn-1(ky51)	91 (n = 90)	ND[e]	ND[e]

The number of coelomocytes examined is indicated in parentheses.
[a]Wild-type background [*arls37;dpy-20(e1282)*]
[b]*cup-4* mutant background [*arls37;cup-4(ok837);dpy-20(e1282)*]
[c]*cup-4* over-expression background [*arls37;dpy-20(e1282);cdIs10*]
[d]Percentages of total coelomocytes that showed qualitative defective in GFP uptake.
[e]Not determined

CUP-4, a putative ligand-gated ion channel, acts downstream of calcineurin

In order to determine how calcineurin may regulate coelomocyte endocytosis, we sought to understand the relationship between calcineurin and previously isolated *cup* mutants (Fares and Greenwald, 2001a). Specifically, we studied *cup-4* which encodes a putative orphan receptor highly similar to the nicotinic acetylcholine receptor in *C. elegans* (Patton et al., 2005). *cup-4(ok837)* mutants display severe defects in coelomocyte endocytosis (Fig. 2B) compared to wild-type animals (Fig. 2A). We then analyzed the genetic interaction between calcineurin and *cup-4*. Interestingly, all three calcineurin and *cup-4* double mutants displayed defective endocytosis, very similar to that in *cup-4* single mutants (Figs. 2C-2E and Table 1). This suggests that *cup-4* may function downstream of calcineurin.

To clarify whether calcineurin and *cup-4* act in the same pathway, we tested whether over-expression of *cup-4* in coelomocytes rescues the defects in calcineurin mutants. We hypothesized that if calcineurin acts at the upstream of CUP-4, the over-expression of CUP-4 could bypass the endocytic defects in calcineurin mutants. Coelomocyte-specific *cup-4* expression reportedly rescued the Cup phenotype in *cup-4* mutants (Patton et al., 2005). Therefore, we used transgenic animals expressing extra-copies of *cup-4* under the coelomocyte-specific promoter as an over-expression line of *cup-4* in this study.

We crossed the *cup-4* over-expression line (*cdIs10*) into *arls37;dpy-20(e1282);calcineurin* mutants. Interestingly, the

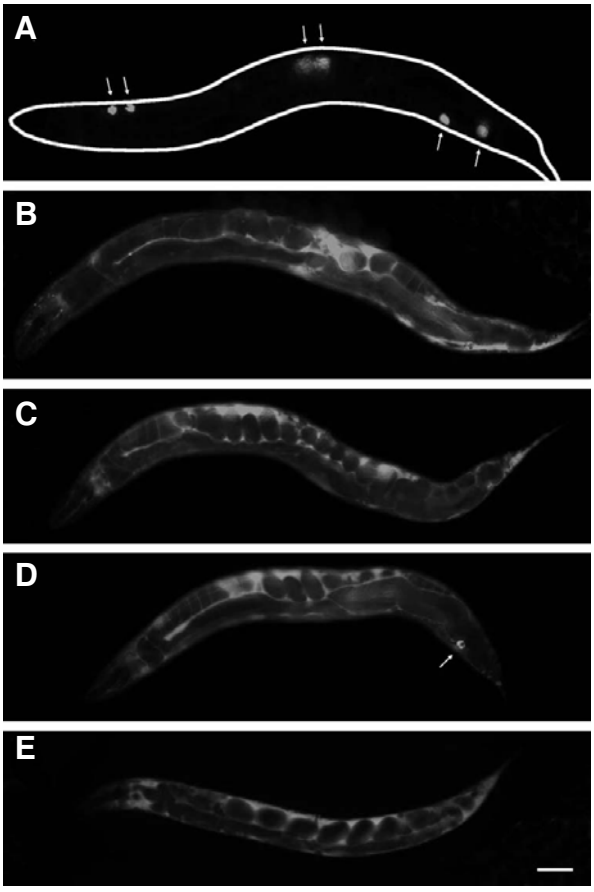

Fig. 2. *cup-4* may act downstream of *tax-6/cnb-1*. *cup-4* and calcineurin double mutants were observed for coelomocyte endocytosis defects. (A) Wild-type [*arls37;dpy-20(e1282)*] animals displayed normal coelomocyte endocytosis. ssGFP is accumulated in coelomocytes (indicated by arrows). (B) *arls37;cup-4(ok837);dpy-20 (e1282)* animals displayed severe defects in coelmocyte endocytosis as described. (C) *arls37;cup-4(ok837);tax-6(jh107) dpy-20(e1282)* and (D) *arls37;cup-4(ok837);tax-6(p675) dpy-20(e1282)* (E) *arls37; cup-4(ok837);dpy-20(e1282);cnb-1(jh103)* animals showed defects similar to those of *cup-4(ok837)* mutants. Arrow (D) indicates a dorsal coelomocyte. Scale bar, 50 μm.

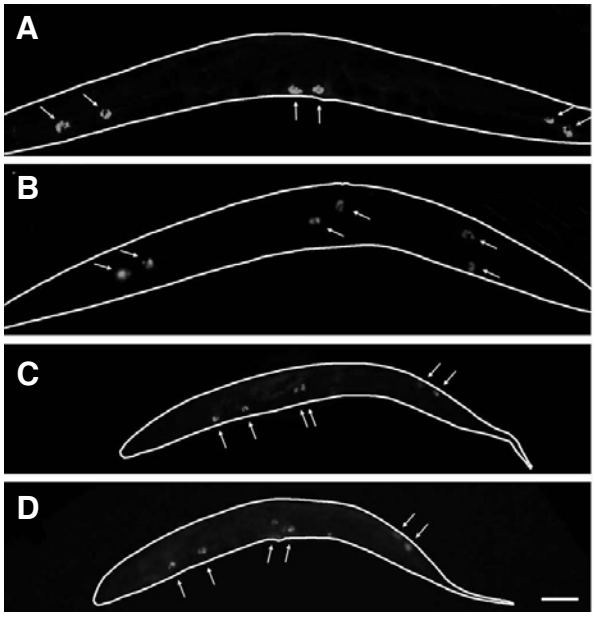

Fig. 3. CUP-4 over-expression rescues Cup phenotypes in calcineurin mutants. A *cup-4* over-expressing line (*cdls10*) was crossed with calcineurin mutants to confirm their genetic interaction. Arrows indicate coelomocytes. (A) *arls37;dpy-20(e1282);cdls10* animals showed six distinct coelomocytes labeled with ssGFP in (B) *arls37;tax-6(jh107) dpy-20(e1282);cdls10*, (C) *arls37;tax-6(p675) dpy-20(e1282);cdls10* and (D) *arls37;dpy-20(e1282);cnb-1(jh103); cdls10* animals. ssGFP was not detected in the body cavities of animals in (B-D). Scale bar, 50 μm.

cup-4 over-expression almost completely rescued endocytosis defects in all three calcineurin mutants (Figs. 3B-3D and Table 1). Therefore, we suggest that CUP-4 may act downstream of calcineurin to regulate coelomocyte endocytosis.

Calcineurin interacts with dynamin

We wanted to understand how a phosphatase might be involved in this endocytic process. Previous studies have shown that dynamin, a GTPase, known for its essential role in many endocytotic processes, is a calcineurin substrate (Klee et al., 1998; Liu et al., 1994; Morioka et al., 1999). We tested whether this biochemical relationship is conserved in *C. elegans* by confirming the interaction between calcineurin and dynamin. A GST pull-down assay showed that DYN-1/PRD interacted with endogenous TAX-6 (Fig. 4A). In addition, the interaction between TAX-6 and DYN-1 was enhanced by increasing the Ca^{2+} concentration. Thus, DYN-1 physically interacts with endogenous calcineurin in Ca^{2+}-dependent manner similar to mammalian dynamin.

Isolation of *dyn-1* deletion mutants

In addition to a previously isolated temperature-sensitive dynamin mutant, *dyn-1(ky51)*, we isolated two deletion alleles of *dyn-1* by screening a *C. elegans* deletion library. We selectively targeted two regions of the *dyn-1* gene: the N-terminal region encoding the GTPase domain, a main functional domain (van der Bliek, 1999; Warnock and Schmid, 1996) and the C-terminal PRD, which is known to interact with calcineurin (Lai, Hong et al., 1999). We successfully recovered both *dyn-1* alleles (*jh133* and *jh134*). The *dyn-1(jh133)* mutant has an 807 bp deletion that covers most of the GTPase domain and *dyn-1(jh134)* mutant has a 996 bp deletion that covers most of the PRD (Fig. 4B). A homozygous *dyn-1(jh33 or 134)* deletion mutant was confirmed using either nested or internal PCR primer sets (Figs. 4B-4F). Homozygotes of both *dyn-1* deletion alleles showed 100% larva lethality. Development was arrested at the first larva stage (L1) and they eventually died confirming that *dyn-1* is indeed essential for *C. elegans* development (Table 2).

Dynamin, like calcineurin, acts upstream of CUP-4 in coelomocyte endocytosis

Becasue dynamin is involved in various endocytic pathways (Chen et al., 1991; Fares and Greenwald, 2001a; Grant and Hirsh, 1999; van der Bliek and Meyerowitz, 1991), and is a calcineurin substrate (Klee et al., 1998; Liu et al., 1994), and has been well established to act in cooperation with calcineurin in mammals (Graham et al., 2007; Marks and McMahon, 1998; Tan et al., 2003), we observed endocytic defects in coelomocytes of dynamin mutants.

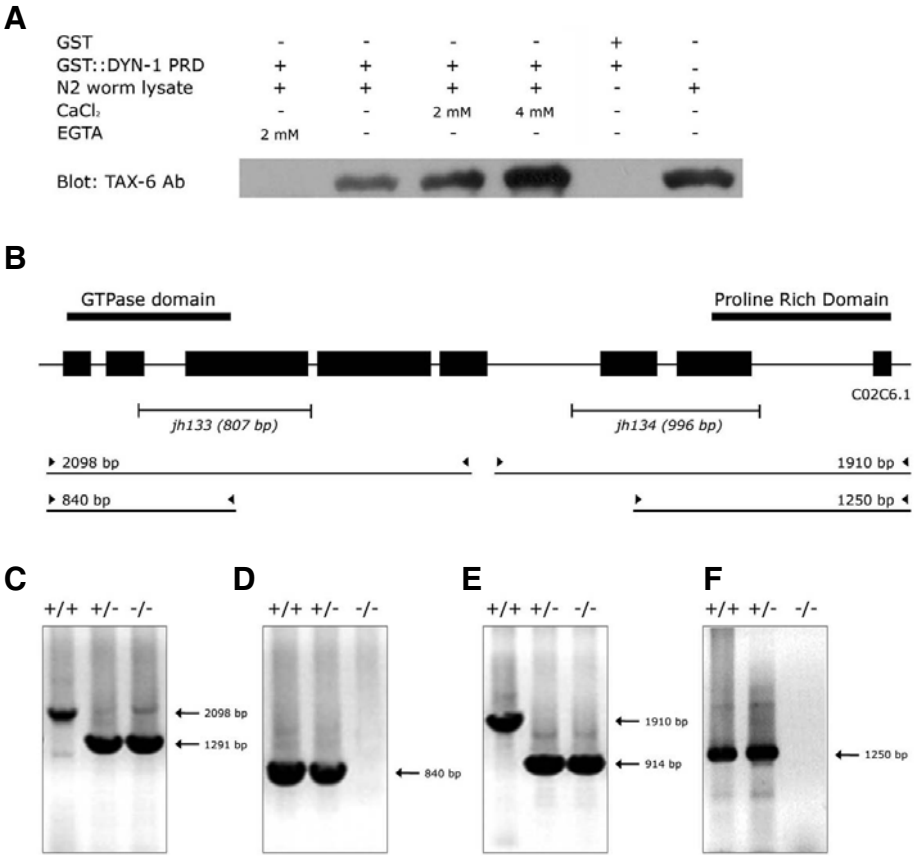

Fig. 4. Physical interaction between calcineurin and dynamin and isolation of *dyn-1* deletion mutants. (A) GST pull-down assay. Purified GST and DYN-1/PRD::GST were incubated with wild-type worm lysate and resolved using SDS-PAGE. Immunoblotting using anti-TAX-6 antibody showed that DYN-1 (second lane) pulled down TAX-6 proteins. Increased Ca^{2+} concentration induced stronger binding between TAX-6 and DYN-1 proteins (third and forth lanes). Purified GST bound to DYN-1/PRD::GST to detect non-specific interactions (fifth lane). Calcineurin in worm lysate was confirmed (sixth lane). (B) The *dyn-1* gene is physically mapped onto the cosmid C02C6.1 and consists of eight exons (indicated by boxes). The upper horizontal bars indicate the GTPase domain and the Proline Rich Domain. The lower horizontal bars show the deleted regions, which were removed from the ends of exon 2 through exon 3 (*jh133*) and from the fifth intron through exon 7 (*jh134*), respectively. Arrowheads show the primer sets used for initial sib selection. (C) Nested and (D) internal PCR bands were obtained from single worm PCR of wild-type (+/+), heterozygous (+/-), and homozygous (-/-) *dyn-1(jh133)*. (E) Nested and (F) internal PCR bands was obtained from single worm PCR of wild-type (+/+), heterozygous (+/-), and homozygotus (-/-) *dyn-1(jh134)*.

Table 2. The lethalities of *dyn-1* deletion mutants

Genotype of P_0	Lethality (%)[a]	n[b]
unc-3(e151)/dyn-1(jh133)	24.3 ± 3.3	3518
unc-3(e151)/dyn-1(jh134)	22.6 ± 2.7	1666

[a]The percentages of L1 arrested and dead worms among the total progeny
[b]The number of progeny examined

As previously described (Fares and Greenwald, 2001a), *dyn-1 (ky51)* mutant animals failed to show clear and distinguishable coelomocytes, and much of the ssGFP signal remained in the pseudocoelom at the restrictive temperature, indicating defective coelomocyte endocytosis (Fig. 5B and Table 1). We also tested coelomocyte endocytosis in *dyn-1* deletion mutants (*jh133* and *jh134*). Since both alleles of *dyn-1* deletion mutant animal are larva lethal, we observed the phenotype at the L1 larval stage before they arrested development. We first generated double mutants between the transgenic animal [*arls37[pmyo-3::ssGFP];dpy-20(e1282)*] and trans-heterozygous *dyn-1* mutants [*unc-3(e151)/dyn-1(deletion)*]. We selected L1 arrested progenies from the double mutants [*arls37;dpy-20(e1282);dyn-1(deletion+/-)*] and photographed them before they died. Expectedly, both *dyn-1* deletion mutants showed very severe defects in coelomocyte endocytosis (Figs. 5F and 5G; Table 1) compared to those of wild-type worms (Fig. 5E). We could observe only four ventral coelomocytes at this stage (Fig. 5E) because only four of the six coelomocytes are present at hatching.

Although dynamin function has been elucidated in coelomocyte endocytosis, whether it functioned through the CUP-4 receptor or calcineurin was unknown. Interestingly, *cup-4(ok837)* mutants have significantly reduced phosphatidylinositol 4,5-bisphosphate ($PI_{4,5}P_2$) in the plasma membrane (Patton et al., 2005), which has been shown to be important for in the stimulation of clathrin-mediated endocytosis (De Matteis and Godi, 2004; Haucke, 2005). Since dynamin has a PH (Pleckstrin Homology) domain that can bind $PI_{4,5}P_2$ of plasma membrane (Salim et al., 1996), we hypothesized that DYN-1 may function with CUP-4. To test this possibility, we analyzed the genetic interaction between *dyn-1* and *cup-4*. We found that *cup-4* and *dyn-1* double mutant displayed severe defects in coelomocyte endocytosis similar to those observed in *cup-4* single mutant (Fig. 5C and Table 1). Again, it was difficult to verify a genetic interaction simply by observing defective endocytosis in the double mutant, because both single mutants have severe defects in coelomocyte endocytosis. Thus we further accessed their genetic interaction by analyzing *cup-4* over-expression in dynamin mutants. We found that the defective endocytosis of dynamin mutants was completely rescued by *cup-4* over-expression (Fig. 5D and Table 1). In addition, we analyzed the genetic interaction between calcineurin and *dyn-1* in the regulation of coelomocyte endocytosis. As expected, *calcineurin;dyn-1(ky51)* double mutants displayed defects similar to those of the *dyn-1(ky51)* single mutant (Table 1). We, therefore, suggest that *cup-4* may be functioning downstream of both dynamin and calcineurin to regulate coelomocyte endo-

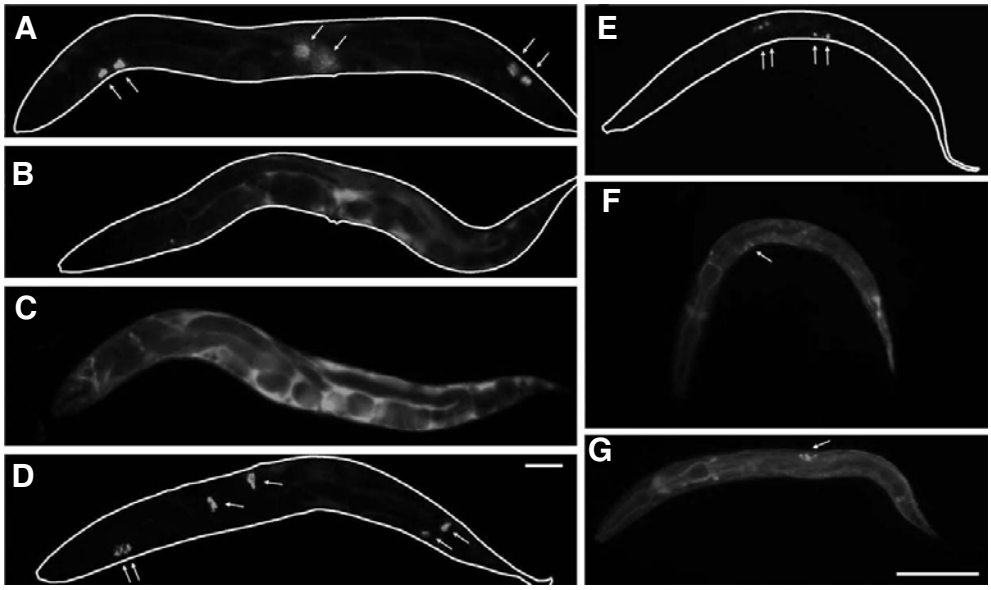

Fig. 5. *cup-4* may function downstream of *dyn-1* to regulate coelomocyte endocytosis. (A) Wild-type [*arls37;dpy-20(e1282)*] animals showed normal endocytosis in coelomocytes (indicated by arrows). (B) *dyn-1(ky51)* mutant animals displayed defective uptake in coelomocytes at 25°C as described. The accumulation of ssGFP signal was observed in the body cavity. (C) *cup-4* may act downstream of *dyn-1*. *cup-4* and *dyn-1* double mutant was observed for coelomocyte endocytosis defects. *arls37;cup-4(ok837);dpy-20(e1282);dyn-1(ky51)* double mutants showed defects similar to those of the *cup-4(ok837)* single mutant (Fig. 2B). The ssGFP signal was strong in the body cavity. (D) CUP-4 over-expression rescued the Cup phenotype of *dyn-1(ky51)* mutant animals. The CUP-4 over-expression line (*cdIs10*) was crossed with the *dyn-1* mutant to assess their genetic interaction. Strong ssGFP was detected in six coelomocytes (indicated by arrows) in *arls37;dpy-20(e1282);dyn-1(ky51);cdIs10* animals. (E-G) ssGFP was monitored in the L1 larva stage of wild-type and *dyn-1* deletion mutant animals. Arrows indicate coelomocytes. Both alleles of *dyn-1* deletion mutants exhibited the most severe defects in uptake (F, *jh133* and G, *jh134*) compared to those of wild-type animals (E). Scale bars, 50 μm.

cytosis. However, we cannot rule out the possibility that *cup-4* may act in parallel with calcineurin and dynamin.

DISCUSSION

Here, we have shown that calcineurin functions in coelomocyte endocytosis in *C. elegans*. The calcineurin A gain-of-function mutant, *tax-6(jh107)*, the calcineurin A loss-of-function mutants, *tax-6(p675)* and calcineurin B null mutant, *cnb-1(jh103)* showed approximately 25%, 64% and 60% defective endocytoses in coelomocytes, respectively. Although the calcineurin mutants have relatively mild defects compared to those of dynamin and *cup-4* mutants, they are specific and significant, indicating that calcineurin is involved in coelomocyte endocytosis in *C. elegans* (Fig. 1 and Table 1). In light of the calcium-dependent and functional interaction between calcineurin and dynamin, we suggest that DYN-1 might be calcineurin target in *C. elegans* coelomocyte endocytosis. Calcineurin likely regulates dynamin function by dephosphorylating and targeting it to the plasma membrane, as has been proposed in mammals (Cousin and Robinson, 2001; Liu et al., 1994; Marks and McMahon, 1998). In fact, dynamin I is phosphorylated *in vivo* at Ser774, Ser778, Ser822, Ser851 and Ser857, located in the PRD (Graham et al., 2007; Tan et al., 2003; Tomizawa et al., 2003). Ser774 and Ser778 are the major phosphorylation sites for cyclin-dependent kinase 5 (cdk5) (Graham et al., 2007). The amino acid sequence of *C. elegans* DYN-1 is 61% identical to that of human dynamin 1 and has well conserved functional domains including the PRD (Clark et al., 1997). In addition, amino acid sequence analysis has revealed that *C. elegans* DYN-1 has potential PRD phosphorylation sites similar to those of rat dynamin I. Therefore, we speculate that calcineurin dephosphorylates DYN-1 to regulate coelomocyte endocytosis.

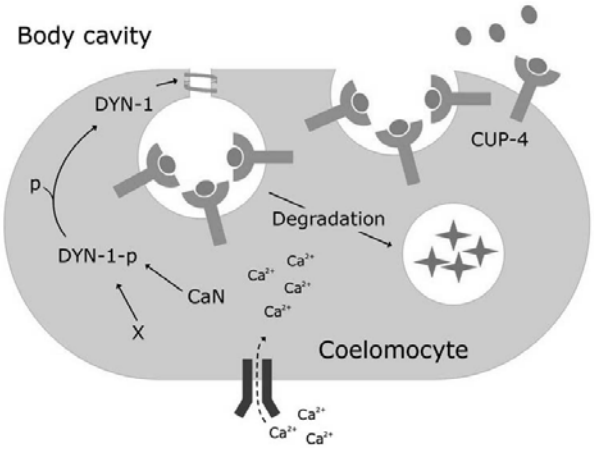

Fig. 6. A proposed model for coelomocyte endocytosis in *C. elegans*. CUP-4, a receptor for foreign molecules, binds to ligand. A rise in intracellular Ca^{2+} concentration activates calcineurin (CaN) to dephosphorylate DYN-1. Dephosphorylated DYN-1 translocates to the plasma membrane to cleave off endocytosed vesicles.

Based on the genetic interactions among the three genes, we propose a working model of coelomocyte endocytosis that consists of CUP-4 as a receptor for foreign molecules, DYN-1 as a vesicle pinching GTPase and TAX-6/CNB-1 as a DYN-1 regulator (Fig. 6). In this model, calcineurin may dephosphorylate

DYN-1 to translocate it from the cytosol to the plasma membrane in order to initiate membrane vesicle budding. The model also includes, the possibility of another DYN-1 regulator(s) (indicated by 'X' in Figure) because calcineurin mutants displayed a relatively mild defect. This might be a reason why calcineurin was not identified in Fare and Greenwald's original *cup* mutant screening.

In this study, both gain-of-function and null calcineurin mutants showed defective coelomocyte endocytosis, although there was a difference in phenotypic penetrance. This difference suggests that endocytosis might be tightly regulated by gain control and feedback mechanisms involving protein interactions and signal transduction including phosphorylationn and dephosphorylation, controlled in turn by intracellular Ca^{2+} levels. At the nerve terminal, many proteins are rapidly dephosphorylated upon Ca^{2+} influx (Cousin and Robinson, 2001). Calcineurin has been proposed to dephosphorylate proteins including dynamin, amphiphysin and synaptojanin (Bauerfeind et al., 1997; Lai et al., 1999). After stimulus-dependent dephosphorylation, these proteins are rapidly rephosphorylated by protein kinases such as cdk5 (Evans and Cousin, 2007; Tan et al., 2003) in order to retrieve synaptic vesicles. Both gain-of-function and loss-of-function calcineurin mutants may cause endocytic defects since both vesicle formation and vesicle retrieval are necessary for proper endocytosis. Additional evidence of this is shown through the inhibition of Cdk5, which depleted synaptic vesicles in the synaptosome (Tan et al., 2003). Blocking endocytosis at different steps may also account for different phenotypic penetrances in calcineurin *gf* and *null* mutants.

Since a Ca^{2+} influx triggers dynamin dephosphorylation via calcineurin (Bauerfeind et al., 1997; Marks and McMahon, 1998), the Ca^{2+} concentration in coelomocytes might be another key regulator of endocytosis process. Previous and recent studies suggest that Ca^{2+} concentration is also tightly controlled for proper endocytosis. A high Ca^{2+} concentration could inhibit dynamin function in synaptic vesicle endocytosis (Cousin and Robinson, 2000; Liu et al., 1996) or deplete $PI_{4,5}P_2$ (Thyagarajan et al., 2008), which is required to form clathrin coated vesicles at the plasma membrane (Haucke, 2005; Martin, 2001). These suggest that coelomocyte endocytosis may require a tightly balanced signaling, including Ca^{2+}-dependent phosphorylation/dephosphory-lation.

Coelomocytes are scavenger cells located in the body cavity (pseudocoelom) that actively and nonspecifically endocytose soluble materials from the pseudocoelom. Although coelomocyte endocytosis is not essential for the survival of animals, three out of eleven recently screened *cup* (coelomocyte uptake defective) genes (Fares and Greenwald, 2001a) encode *C. elegans* homologs of human disease genes, such as mucolipidosis type IV, X-linked myotubular myopathy and Charcot-Marie-Tooth type 4B1/4B2 (Dang et al., 2004; Fares and Greenwald, 2001b). These genes hint at the importance of coelomocyte endocytosis and its exact control mechanism. In this study, we identified a novel calcineurin function in the regulation of coelomocyte endocytosis in *C. elegans*. We also revealed a plausible genetic pathway in which calcineurin controls coelomocyte endocytosis via dynamin. In addition, we suggest, for the first time, a possibility that CUP-4, coelomocyte-specific receptor, may act downstream of dynamin and calcineurin. Most genes involved in coelomocyte endocytosis are still under investigation. Therefore, our results may prompt further studies on coelomocyte endocytosis.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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