BMB
reports

Calcineurin may regulate multiple endocytic processes in *C. elegans*

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Calcineurin is a serine/threonine protein phosphatase controlled by Ca^{2+} and calmodulin that has been implicated in various signaling pathways. Previously, we reported that calcineurin regulates coelomocyte endocytosis in *Caenorhabditis elegans*. So far, simple and powerful *in vivo* approaches have been developed to study various endocytic processes in *C. elegans*. Using these *in vivo* assays, we further analyzed the endocytic phenotypes of calcineurin mutants. We observed that the calcineurin mutants were defective in apical endocytosis in the intestine as well as synaptic vesicle recycling in the nerve cord. However, we found that calcineurin mutants displayed normal receptor-mediated endocytosis in oocytes. Therefore, our results suggest that calcineurin may regulate specific sets of endocytic processes in nematode. [BMB reports 2011; 44(2): 96-101]

INTRODUCTION

Calcineurin is a Ca^{2+}/calmodulin-dependent serine/threonine protein phosphatase (1-4) that exists as a heterodimer of catalytic subunit A and regulatory subunit B (5). Moreover, it has been well studied from yeast to human. Calcineurin has been shown to function in maintaining Ca^{2+} homeostasis in yeast (6), and it is known to be expressed in the brain as well as other tissues in mammals where it regulates T-cell growth, programmed cell death, hippocampal long-term depression, and pathogenesis of hypertropic cardiomyopathy (7-10).

Caenorhabditis elegans calcineurin possesses well conserved calcium binding ability and functions (11). It is extensively expressed in neurons as well as other tissues, including hypodermal seam cells, body-wall muscle, vulva muscle, sperm, and the spermatheca (11-13). Studies have revealed that calcineurin functions in regulating various Ca^{2+}-dependent signaling pathways, including thermotaxis, chemotaxis, locomotion, egg-laying behavior, and defecation in *C. elegans* (11-13). In addition, we have previously shown that calcineurin regulates endocytosis in coelomocytes, which are scavenger cells located in the body cavity of *C. elegans* (14). In that study, we demonstrated that calcineurin may regulate coelomocyte endocytosis in *C. elegans* via a GTPase, dynamin, as well as an orphan receptor, CUP-4.

In this study, we further analyzed the function of calcineurin in various endocytic pathways such as yolk endocytosis in oocytes, apical endocytosis in the intestine, and synaptic vesicle recycling in the nerve cord using *in vivo* assay systems featuring *C. elegans*. We observed defects in each type of endocytosis in three calcineurin mutants: loss-of-function(*lf*) and gain-of-function(*gf*) mutants of calcineurin A, *tax-6(p675)* and *tax-6 (jh107)*, as well as the null mutant of calcineurin B, *cnb-1 (jh103)*. Our results indicate that calcineurin specifically functions in intestinal endocytosis and synaptic vesicle recycling but not in receptor-mediated endocytosis in oocytes. Further genetic and molecular analysis of calcineurin may determine the exact role of calcineurin in each specific endocytic pathway and further identify other components involved in endocytosis.

RESULTS

Calcineurin mutants display normal endocytosis of yolk protein

Previously, we showed that calcineurin mutants display defects in coelomocyte endocytosis (14), which is one of the major clathrin-mediated endocytic processes in *C. elegans* (15). To investigate whether or not calcineurin plays a general role in endocytosis or more specific roles in subsets of endocytic processes in nematode, we looked at *in vivo* cellular processes involving endocytosis. Yolk uptake observed in *C. elegans* oocytes is another dramatic example of receptor-mediated endocytosis (16, 17). Yolk proteins, which are synthesized in the intestine and secreted into the body cavity, are taken up by oocytes in the germline. Grant and Hirsh previously created an *in vivo* monitoring system using transgenic animal expressing a yolk protein (YP170)::GFP reporter to visualize yolk endocytosis in *C. elegans* (17). Using this assay, they found that many

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DOI 10.5483/BMBRep.2011.44.2.96

Received 1 November 2010, Accepted 8 December 2010

Keywords: Calcineurin, *C. elegans*, Endocytosis, Intestine, Synaptic vesicle recycling

components are required for yolk protein endocytosis. Therefore, we used the same assay system to examine yolk protein endocytosis in calcineurin mutants.

We found that the calcineurin mutants did not show any defects in yolk protein uptake (Fig. 1). Yolk protein was exclusively accumulated in oocytes and embryos of wild-type animals. *tax-6(p675)(lf)* and *tax-6(jh107)(gf)* mutant animals exhibited accumulation of yolk protein in oocytes and embryos, similar to wild-type animals. In addition, *cnb-1(jh103)* mutant animals also displayed normal yolk protein endocytosis, similar to *tax-6* mutant animals. On the other hand, the positive control, *dyn-1(ky51)* mutant animals, showed high levels of accumulation of yolk protein in the pseudocoelom at the non-permissive temperature of 25°C, as previously described (17).

It has been described that many components, such as *dyn-1* as well as *rme* (receptor-mediated endocytosis) genes, are shared in oocytes and coelomocyte endocytosis (15, 18-20). Surprisingly, our results show that calcineurin mutants displayed normal RME in oocytes, suggesting that calcineurin may not regulate yolk endocytosis by oocytes or may have a minor role in RME.

Calcineurin mutants exhibit altered apical trafficking in the intestine

The intestine of *C. elegans* performs various important functions, including uptake and transport of nutrients, production and secretion of yolk proteins, and the rhythmic oscillation control of the defecation cycle (21-24). Recently, genetic *in vivo* approaches have been developed to study endocytic trafficking in the intestine (20, 25). Since we have shown that calcineurin is expressed in the intestine (11), we analyzed intestinal trafficking in calcineurin mutants using a lipid marker, FM4-64 (18). It was previously shown that FM4-64 accumulates in gut granules upon administration to the apical surface of the intestine. Thus, we analyzed FM4-64 uptake from apical (lumenal) membranes by feeding the marker to calcineurin mutants.

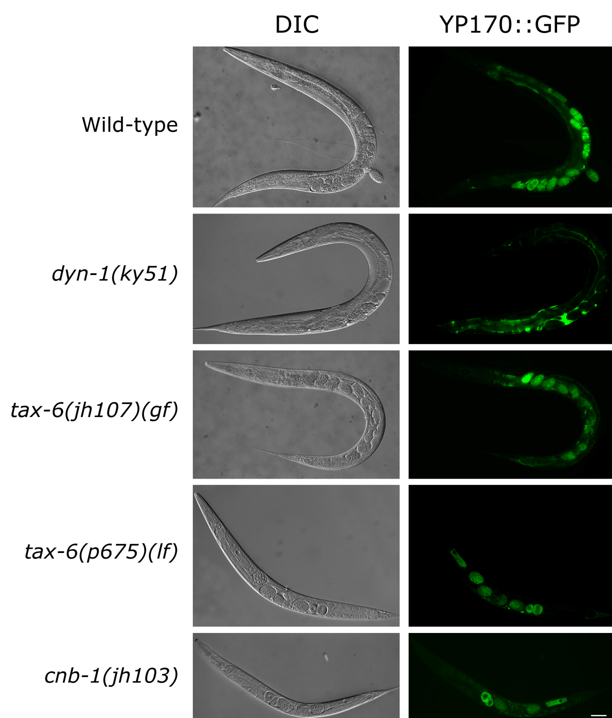

Fig. 1. Calcineurin mutants exhibit normal endocytosis of YP170::GFP in oocytes. DIC (left panel) and fluorescence micrographs (right panel) are shown. YP170::GFP was normally endocytosed into oocytes and embryos of wild-type animals (n = 20). *dyn-1(ky51)* mutant animals showed YP170::GFP in the body cavity. YP170::GFP remained in the body cavity but not in oocytes and embryos, which suggests failure of endocytosis (n = 23). *tax-6(jh107)(gf)*, *tax-6(p675)(lf)*, and *cnb-1(jh103)* mutant animals displayed YP170::GFP in oocytes and embryos, similar to wild-type animals (n = 15, n = 23, and n = 50, respectively). Scale bar, 50 μm.

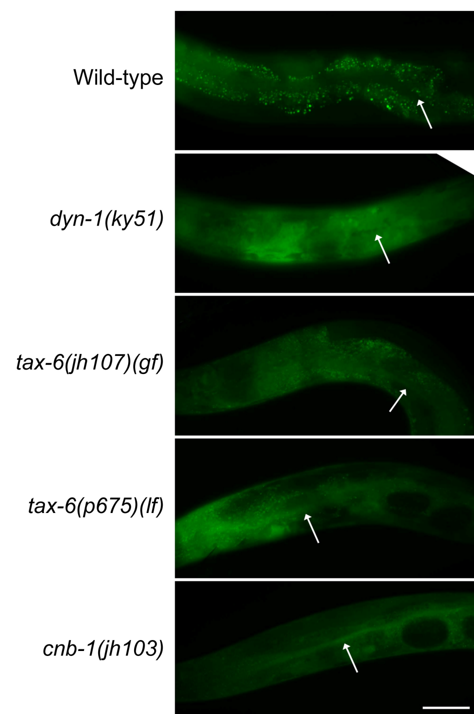

Fig. 2. Calcineurin mutants show defective apical endocytosis in the intestine. FM4-64 dye was fed to wild-type and mutant animals. FM4-64 dye normally accumulates in gut granules in wild-type animal. *dyn-1(ky51)* mutant showed defective endocytosis in apical membrane of the intestine. The dye was not accumulated in gut granules, and the intestinal lumen was filled with dye, suggesting failed uptake from the apical membrane of the intestine. *tax-6(jh107)(gf)* mutant, *tax-6(p675)(lf)* mutant, and *cnb-1(jh103)* mutant displayed similar defects to that of *dyn-1(ky51)* mutant. Arrows indicate the position of intestinal lumen. Scale bar, 50 μm.

As shown in Fig. 2, wild-type animals showed normal uptake of dye from the luminal intestine membrane; the intestinal lumen indicated by an arrow is empty. However, *dyn-1(ky51)* mutant displayed a severe defect in dye uptake from the intestinal lumen, as expected. It was hard to distinguish between the intestinal lumen (indicated by arrow) and gut granules in *dyn-1(ky51)* mutant, suggesting that *dyn-1* is responsible for intestinal endocytosis. Interestingly, all three calcineurin mutants exhibited similar phenotypes to that of *dyn-1(ky51)* mutant. FM4-64 staining in gut granules was extremely reduced, and the intestinal lumen was still filled with dye in all three calcineurin mutants. These results suggest that calcineurin may function in apical endocytosis in the intestine of *C. elegans*.

Calcineurin mutants show defective recycling of synaptic vesicles

Calcineurin has been implicated in synaptic vesicle recycling via dynamin at the cellular level (26, 27), and we previously showed that calcineurin is highly expressed in the nervous system of *C. elegans* (11). Thus, we examined whether or not calcineurin can mediate synaptic vesicle recycling *in vivo* by using the synaptic protein synaptogyrin (SNG-1). SNG-1::GFP fusion protein has been developed for visualizing presynaptic nerve terminals *in vivo* and is used as a synaptic marker to examine synaptic vesicle recycling (28, 29). This fusion protein can be used to target synaptic vesicle proteins to synaptic vesicles along the nerve cord in living animals without any extracellular stimulation. Using this *in vivo* synaptic marker, we were able to observe whether or not recycling of synaptic vesicle-associated SNG-1 protein occurs normally in calcineurin mutants.

As shown in Fig. 3, SNG-1::GFP was localized to synaptic varicosities (arrows), as evidenced by a punctuated staining pattern in wild-type animals indicative of normal synaptic vesicle recycling. These puncta represent pools of synaptic vesicles (28, 29). However, as expected, SNG-1::GFP was mostly diffused at synapses in *dyn-1(ky51)* mutant animals rather than being punctate as in the wild-type, suggesting failure of synaptic vesicle recycling. We quantified the number of endogenous SNG-1::GFP puncta in animals (See Materials and Methods). The numbers of SNG-1::GFP puncta observed were 33.4 ± 1.0 in wild-type animals (n = 19) and 11.5 ± 0.8 in *dyn-1 (ky51)* mutant animals (n = 15). Interestingly, *tax-6(jh107)(gf)* mutant animals showed a distinct punctate expression pattern of SNG-1::GFP, which was similar to wild-type. However, the quantitative assay revealed that *tax-6(jh107)(gf)* mutant animals had fewer SNG-1::GFP puncta (24.8, n = 19, SE = 0.7) compared to wild-type animals (33.4, n = 19, SE = 1.0), suggesting insufficient recycling of synaptic vesicles. SNG-1::GFP in both *tax-6(p675)(lf)* and *cnb-1(jh103)* mutant animals was even more diffused at synapses, similar to that in *dyn-1(ky51)* mutant animals. The numbers of SNG-1::GFP puncta observed were 16.5 ± 0.8 (n = 11) and 14.9 ± 0.8 (n = 14) in *tax-6(p675)(lf)* and *cnb-1(jh103)* mutant animals, respectively.

Although these abnormalities in SNG-1::GFP staining suggest a defect in synaptic vesicle recycling by endocytosis, they could also be caused by non-specific defects in the development of the nervous system. To rule out this possibility, we observed the structure of the GABAergic and cholinergic nervous system in mutant animals using specific reporter constructs. We investigated the architecture of the GABAegic nervous system in transgenic animals expressing GFP in all GABAergic neurons using the *unc-47p::GFP* reporter construct (30). We photographed all animals in L1 stage to show for certain the structure of GABAergic neurons. We could see 6-DD (Dorsal D-type), RME, RIS, and AVL neurons at this stage (31). As shown in Fig. 4, wild-type animals showed normal development of GABAergic neurons. *dyn-1(ky51)* mutant, which has displayed severe defects in synaptic vesicle recycling, also displayed normal development of GABAergic neurons. Similar to

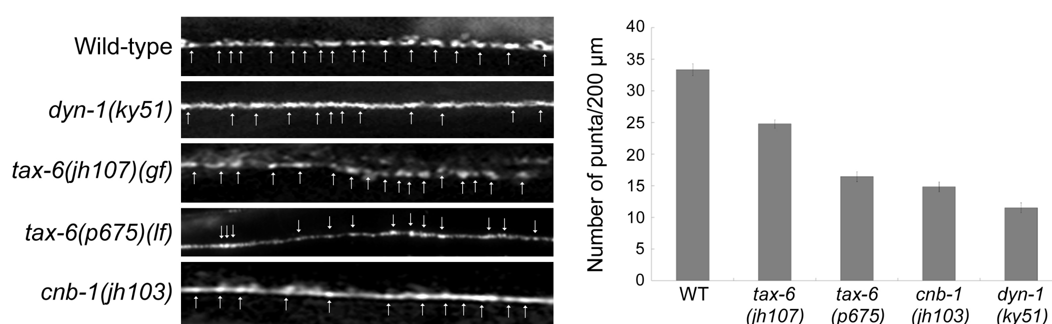

Fig. 3. Calcineurin mutants display defective recycling of synaptic vesicles at synapses. Synaptic varicosities along the nerve cord are shown by arrows. The staining pattern of SNG-1::GFP was observed at the dorso-ventral nerve cord of wild-type and mutant adult animals. Wild-type animals showed a punctuated staining pattern for SNG-1::GFP. In contrast, *dyn-1(ky51)* mutant animals displayed a diffused staining pattern for SNG-1::GFP. *tax-6(jh107)(gf)* mutant animals exhibited a similar punctuated staining pattern, as seen in wild-type animals. *tax-6(p675)(lf)* and *cnb-1(jh103)* mutant animals showed a similarly diffused staining pattern for SNG-1::GFP to that of *dyn-1(ky51)* mutant animals. The number of SNG-1::GFP puncta was counted in 200 μm of the dorsal nerve cord. *tax-6(jh107)(gf)* mutant animals exhibited significantly reduced SNG-1::GFP puncta compared to wild-type animals.

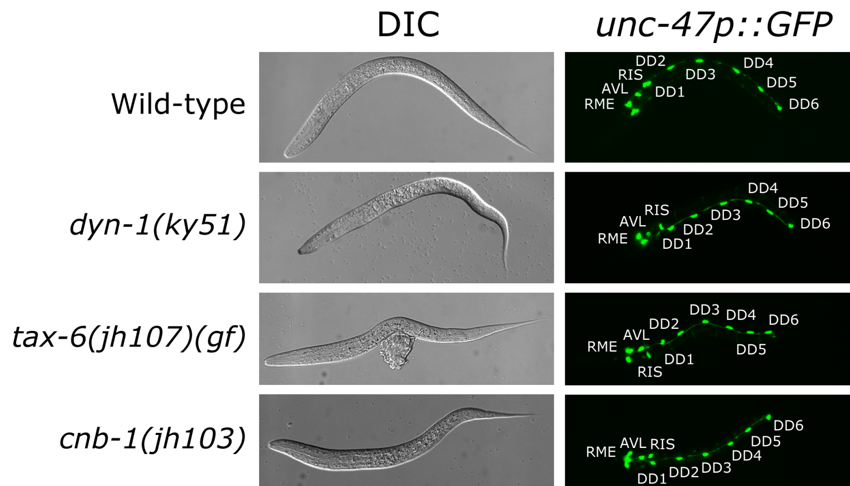

Fig. 4. GABA nervous system develops normally in *dyn-1* and calcineurin mutants. Development of GABAergic neurons was observed using *unc-47p::GFP* reporter construct in wild-type and mutant L1 larva. DIC (left panel) and fluorescence micrographs (right panel) are shown. Six DD neurons and interneurons were well organized in all animals. Scale bar, 50 μm.

wild-type animal, calcineurin mutants, *tax-6(jh107)(gf)* and *cnb-1 (jh103)*, showed normal GABA neuronal development. To observe the development of cholinergic neurons, we used transgenic animals expressing GFP in all DA (Dorsal A-type) and DB (Dorsal B-type) motor neurons following treatment with *unc-129p::GFP* reporter construct (32). Using this transgenic animal, we found that calcineurin mutants exhibited normal development of cholinergic neurons (data not shown). Taken together, these results suggest that the nervous system develops normally in calcineurin mutant animals and that the aberrant SNG-1::GFP staining patterns are likely caused by defects in the endocytosis of synaptic vesicles in these animals.

DISCUSSION

In this study, we further showed that calcineurin functions in various endocytic processes, such as endocytosis in the intestine and synaptic vesicle recycling, in *C. elegans*. Similar to our previous studies on coelomocyte endocytosis (14), we found that both gain-of-function(*gf*) and loss-of-function(*lf*) mutants of calcineurin showed defective endocytosis in the intestine as well as synaptic vesicle recycling with different phenotypic frequencies. As previously suggested, we expect that endocytosis in the intestine or nervous system might be regulated by a tightly balanced signal transduction event such as phosphorylation/dephosphorylation controlled by intracellular Ca^{2+}.

Defective endocytosis in the intestines of calcineurin mutants may provide a plausible clue to understanding the smaller body size of calcinuerin loss-of-function(*lf*) and null mutants. Impaired feeding behavior was shown to result in smaller body size in *C. elegans* (33). It has also been shown that abnormal food uptake caused by aberrant pharyngeal pumping results in smaller body size in feeding-defective mutants (33). Recently, it was shown that *tax-6(p675)(lf)* mutant displays a slightly but significantly reduced pharyngeal pumping rate, as does *cnb-1(jh103)* mutant (34). A defect in apical food uptake from the intestine as well as feeding defect may result in caloric restriction in calcineurin mutants, and this may result in smaller body size in calcineurin loss-of-function(*lf*) and null mutants. Further studies may be needed to examine this possibility.

Consistent with previous reports in cellular systems (26, 27), we have shown that calcineurin functions in synaptic vesicle recycling *in vivo*. At the cellular level, calcineurin is known to regulate synaptic vesicle recycling via dynamin, a large GTPase (26, 27). Thus, further study could reveal the genetic relationship between calcineurin and dynamin or other components in the regulation of synaptic vesicle recycling. Our study also provides evidence that calcineurin may function in various endocytic processes. The exact mechanism for the regulation of endocytosis in tissues is still unknown, even though many genes have been identified as being involved. Therefore, our results may help the understanding of the mechanisms of various endocytic pathways.

MATERIALS AND METHODS

C. elegans strains and maintenance

The following strains were obtained from the *Caenorhabditis* Genetics Center (CGC): Bristol N2, NM1233 *jsIs219[pSY3[sng-1::GFP]+pRF4(rol-6(su1006))]* II, PR675 *tax-6(p675) IV, RT130 pwIs23[vit-2::GFP], CX51 dyn-1(ky51)* X and EG1285 *lin-15B (n765) oxIs12[Punc-47::GFP+lin-15(+)]* X. KJ300 *cnb-1(jh103)* V and KJ306 *tax-6(jh107)* IV were previously isolated (11, 13, 14). Strain *evIs82[punc-129::GFP]* was kindly provided from the laboratory of Dr. Hwai-Jong Cheng. Worm breeding and handling were conducted as previously described (35).

Endocytosis assay

One-day-old adult worms grown from L4-stage larvae were ex-

amined for phenotypes. For *dyn-1(ky51)* mutant, L4-stage larvae were incubated at 20°C for 20 hours and then shifted to 25°C. Shifted young adult worms were incubated for at least three more hours at the same temperature to induce temperature-sensitive phenotypes.

To check yolk uptake by oocytes, we crossed all of the mutant strains with *pwIs23[vit-2::GFP]*, and then the resulting crossed mutant animals were visually checked by fluorescence microscopy for uptake of yolk.

To check apical endocytosis in the intestine, animals were soaked in 30 mM FM4-64 solution (Molecular Probe). After 1.5 hours, animals were recovered for 30 min on OP50-seeded plates and visualized by fluorescence microscopy.

To check for defects in synaptic vesicle recycling, all of the mutant animals were crossed with *jsIs219[pSY3[sng-1::GFP]+pRF4]*. Dorso-ventral nerve cord of the resulting double mutant animals were photographed by fluorescence microscopy. For *in vivo* quantification of the SNG-1::GFP puncta, 200 μm of the dorsal nerve cord from the mid body of the animals was observed, and the number of puncta was measured.

Microscopy

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

Acknowledgements

The authors gratefully acknowledge CGC for the nematode strains. We also thank Dr. Hyeon-SooK Koo and Hwai-Jong Cheng for sharing the strains. This work was supported by the Mid-career Researcher Program through a National Research Foundation of Korea grant funded by the Ministry of Education, Science, and Technology (No. 2010-0000051).

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