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Novel calcineurin interacting protein-2: the functional characterization of CNP-2 in *Caenorhabditis elegans*

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Calcineurin (Cn) is a serine/threonine phosphatase implicated in a wide variety of biological responses. To identify proteins that mediate Cn signaling pathway effects, we used yeast two-hybrid assays to screen for Cn interacting proteins, discovering a protein encoded by the gene, *cnp-2* (Y46G5A.10). Utilizing serially deleted forms of Cn as baits, we demonstrated that the catalytic domain of Cn (TAX-6) binds with CNP-2, and this physical interaction was able to be reconstituted *in vitro*, supporting our yeast two-hybrid results. *cnp-2* is a nematode-specific novel gene found in *C. elegans* as well as its closest relative, *C. briggsae*. CNP-2 was strongly expressed in the intestine of *C. elegans*. To study the function of *cnp-2*, we performed *cnp-2* RNAi knock-down and characterized phenotypes associated with Cn mutants. However, no gross defects were revealed in these RNAi experiments. CNP-2 was proven to be a Cn binding protein; however, its role remains to be elucidated. [BMB reports 2008; 41(6): 455-460]

INTRODUCTION

Calcineurin (Cn) is a Ca^{2+}/calmodulin activated Ser/Thr protein phosphatase (1-3) that is evolutionarily well conserved from yeast to human. It is composed of a catalytic subunit (CnA) and a regulatory subunit (CnB) (4). The catalytic CnA subunit contains a catalytic domain and regulatory domain, and the CnA regulatory domain contains the CnB binding domain, CaM-binding domain, and auto-inhibitory domain (AI) (5, 6). The CnB subunit, as well as CaM, bind to unique sites on CnA and are necessary for Cn to be fully active. In the absence of Ca^{2+}/CaM, the autoinhibitory (AI) domain occupies the active site and calcineurin enzyme activity is inhibited.

In *C. elegans*, single homologs of calcineurin A and calcineurin B exist encoded by *tax-6* and *cnb-1*, respectively. Cn is expressed in hypodermal seam cells, body-wall muscle, vulva muscle, neuronal cells, sperm, and the spermatheca of *C. elegans* (7-9). Mutants with loss of Cn function exhibit pleiotropic defects such as a small body size, decreased brood size, abnormal thermotaxis, slow movement, and egg laying defects (7, 9).

Cn functions in diverse tissues, including T-cells, muscle, and neurons (to regulate T cell development) (10); it also functions in muscle remodeling (11) and the modulation of neuronal excitability (12). Cn accomplishes multiple functions through a variety of mechanisms such as dephosphorylation of its substrates (NFAT, cdk-4, GABA) (13-15) and binding with its activators (Hsp 70) (16) and inhibitors (RCAN-1, cain/cabin1) (17-19). Many of the proteins that physically bind with Cn are shown to be important for maintaining normal homeostasis and are associated with disease upon dysfunction (20-25). Thus, understanding the novel proteins that physically interact with Cn would allow for the identification of additional signaling pathways in which Cn directly participates, aiding in the identification of potential drug targets. As a first step towards achieving this goal, we used the yeast two-hybrid method to identify proteins that physically interact with *C. elegans* calcineurin. One of these interacting proteins was identified as Y46G5A.10. We named this gene *cnp-2*, where '*cnp*' denotes calcineurin binding protein and the succeeding numeral indicates the order of its discovery.

In this paper, we show the physical interaction between TAX-6 and CNP-2, the spatial and temporal expression patterns of *cnp-2*, and the characterization of *cnp-2* knock-down phenotypes in a variety of mutant backgrounds.

RESULTS AND DISCUSSION

TAX-6 physically interacts with CNP-2

In order to understand how calcineurin regulates various biological processes, a yeast two-hybrid system was used to screen for proteins that interact with TAX-6. From the *C. elegans* cDNA library, which was cloned into a prey vector, we screened a total of 4.5×10^6 transformants and identified Y46G5A.10 as a calcineurin binding partner. We named this gene *cnp-2* with regard to calcineurin binding protein. In order to delineate the region of TAX-6 participating in CNP-2 inter-

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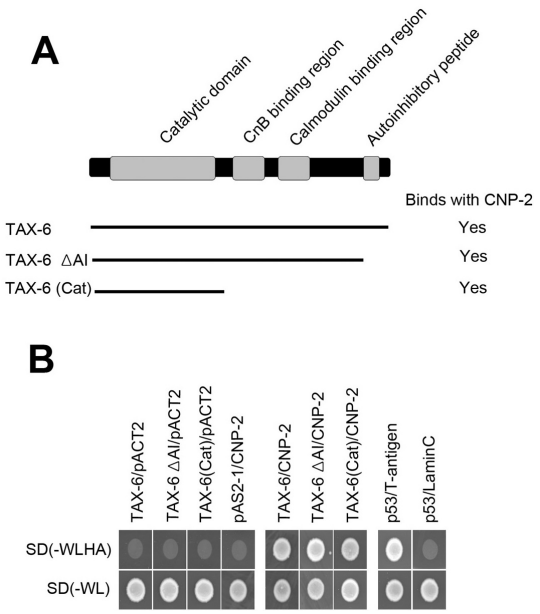

Fig. 1. Interaction between TAX-6 and CNP-2 in the yeast two-hybrid system. (A) Schematic diagram showing the major features of TAX-6. The regions of TAX-6 covered by the bait vectors are indicated as solid lines. (B) Yeast two-hybrid analysis of the physical interaction between TAX-6 and CNP-2. The identity of the bait vector and prey vector co-transformed into yeast is indicated as 'bait/prey' above each lane. The lower lanes indicate the ability of the co-transformants to grow on synthetic dropout (SD) media lacking Trp (W) and Leu (L). The upper lanes indicate the ability or inability of the transformants to activate two independent reporter genes, as assayed by the presence or absence of growth on SD media lacking Trp (W), Leu (L), His (H), and Ade (A). T-antigen/p53 interaction was used as a positive control and Lamin C/p53 interaction was used as a negative control.

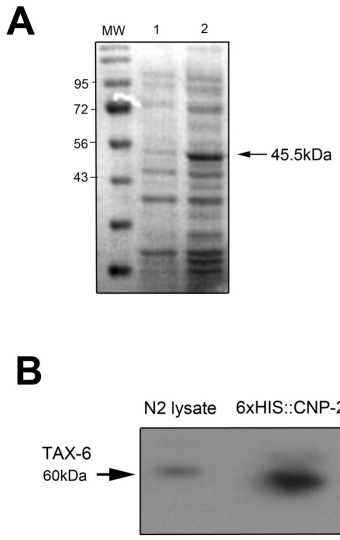

Fig. 2. (A) Expression and solubilization of 6xHis::CNP-2. The 6xHis::CNP-2 protein was expressed at 37°C in *Escherichia coli* strain BL21 (DE3). After harvesting, the cells were lysed by sonication and both the soluble and insoluble fractions were analyzed by 10~20% SDS-PAGE. Lane 1 shows the soluble fraction in PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) and lane 2 shows the soluble fraction in the screened buffer (100 mM Tris, 50 mM NaCl, 100 mM urea, pH 8.2). The arrow indicates the over-expressed 6xHis::CNP-2 protein of 45.5 kDa. The proteins were Coomassie-blue stained. (B) *In vitro* analysis of the physical interaction between TAX-6 and CNP-2. 6xHis::CNP-2 or 6xHIS was immobilized on a Ni^{2+} column. The soluble fraction of N2 lysate protein was washed with washing buffer and the sample was subjected to Western blot analysis with anti-TAX-6 antibody as a probe. *C. elegans* lysate was used as a positive control.

action, we used two additional bait constructs and tested them in the yeast two-hybrid system (Fig. 1A). One of the baits, TAX-6 ΔAI, is a C-terminal truncated form lacking the autoinhibitory domain. The other bait construct, TAX-6 (Cat), contains the catalytic domain of Cn. When transformed along with *cnp-2*, all three baits activated the reporter genes (Fig. 1B), as evidenced by their ability to grow on synthetic dropout media lacking Trp, Leu, His, and Ade. Our results indicate that TAX-6 physically interacts with CNP-2, and the catalytic domain of TAX-6 is sufficient for such interaction.

N-terminal of CNP-2 binds with TAX-6

The CNP-2 N-terminal (coded by exon 1 to exon 6) was sub-cloned into pRSET vector (Invitrogen) in such a way that the resultant construct expressed the 6xHis fused N-terminal segment of CNP-2, presenting the successful induction of the fusion protein after adding IPTG. Although expressed, all fusion proteins went to the pellet fraction during our preliminary purification attempts using PBS buffer. In order to solubilize the protein, we screened a series of buffers varying in composition and pH and found that one buffer (100 mM Tris, 50 mM NaCl, 100 mM urea, pH 8.2) partially solubilized 6xHis::CNP-2 protein (Fig. 2A). To validate our yeast two-hybrid result, we purified 6xHis::CNP-2 protein and performed a His-tag pull-down assay with *C. elegans* lysates. Anti-TAX-6 antibody was used to probe the proteins bound with immobilized CNP-2. As shown in Fig. 2B, 6xHis::CNP-2 displayed binding with endogenous TAX-6, whereas the control 6xHIS failed to bind with TAX-6. This result indicates that CNP-2 does physically interact with TAX-6, consistent with our yeast two-hybrid data. Currently, we do not know the significance of the physical interaction between TAX-6 and CNP-2. The physical interaction of CNP-2 with the catalytic domain of TAX-6 raises the possibility that CNP-2 could be a substrate. Consistent with this possibility, we found several putative Ser/Thr phosphorylation sites in CNP-2, including CK2 phospho sites and PKC phospho sites. However, further experiments are needed to test this hypothesis.

cnp-2 encodes a novel nematode-specific protein in *C. elegans*

The *cnp-2* gene of *C. elegans* was physically mapped to chromosome II, and corresponds to the region between the *rol-1* and *sup-6* loci on the genetic map (Fig. 3A). The genomic DNA of *cnp-2* is 6422 bp in size and composed of twenty exons and nineteen introns.

A standard BLASTp search of the CNP-2 sequence against the entire database of non-redundant proteins deposited in NCBI - using moderately stringent (BLOSUM 62) as well as less stringent (BLOSUM 45) substitution matrices - resulted in the retrieval of only one ortholog from another organism, *C. briggsae*, which is a close relative of *C. elegans*. This result suggests that CNP-2 is probably confined to nematodes. Interestingly, there is a low degree of relatedness between *C. elegans* CNP-2 and *C. briggssae* CNP-2 (CBG07907); they are 54% identical and 64% similar to each other (Fig. 3B). Considering the fact that the median percent identity between *C. elagans* protein and its counterpart in *C. briggsae* is very high (80%) (26), the observed low percentage of identity suggests that CNP-2 has either been rapidly evolving ever since the divergence of the two species, or there was reduced selection pressure for CNP-2 during the course of evolution.

Spatial and temporal expression patterns of CNP-2 in *C. elegans*

Next, we examined the spatial and temporal expression patterns of CNP-2 by cloning the near 1.8 kb upstream region of *cnp-2*, followed by the first exon, first intron, and part of the second exon, into pPD95.75 (Fig. 4A). Under the control of *cnp-2* regulatory elements, the resultant construct drove expression of a translational fusion of the N-terminal portion of CNP-2 with the GFP reporter. Worms injected with this construct emitted fluorescence at all stages of development (Fig. 4B-I), suggesting that CNP-2 may be required at all developmental stages of *C. elegans*. CNP-2 was mainly expressed in the intestine and other unidentified cells (Fig. 4G-I). As suggested by the relatively more prominent expression of CNP-2 in the intestine, CNP-2 might function in intestinal development and/or maintenance. Further experiments are needed to verify this hypothesis.

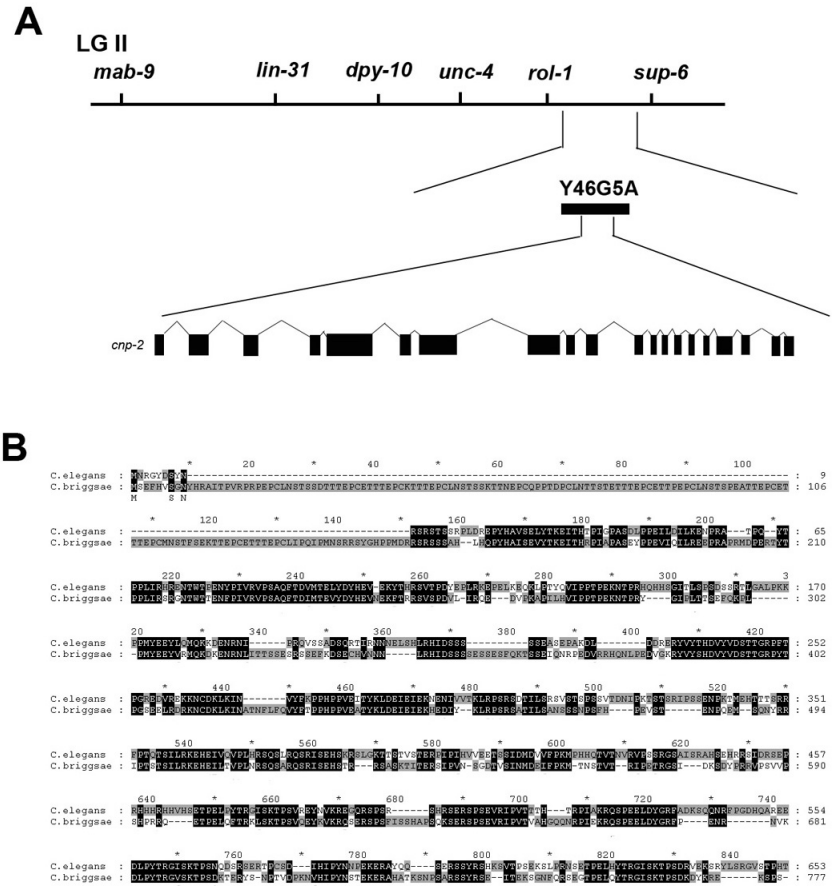

Fig. 3. (A) Genomic organization of the *cnp-2* gene in *C. elegans*. The *cnp-2* gene was physically mapped to chromosome II and corresponds to the region between the *rol-1* and *sup-6* loci. The *cnp-2* genomic DNA is 6422 bp in size, and has twenty exons and nineteen introns. (B) Amino acid sequence alignment of CNP-2. Pairwise global alignments between *C. elegans* CNP-2 with a *C. briggsae* ortholog (CBG07907) are shown.

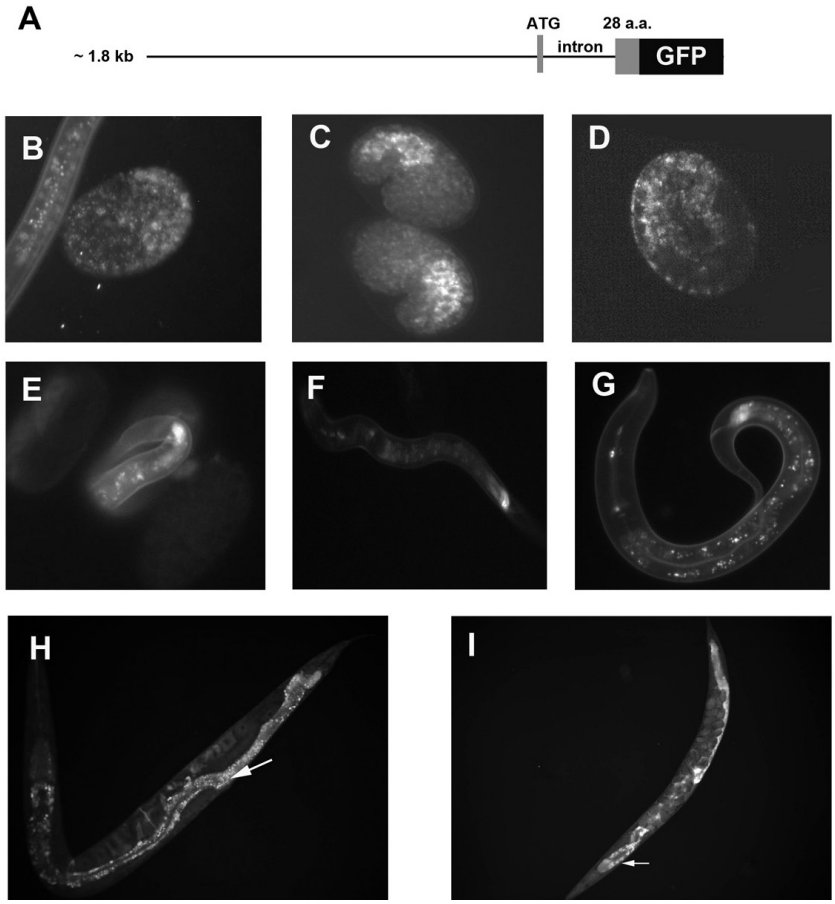

Fig. 4. Spatial and temporal expression patterns of CNP-2. (A) Schematic diagram showing the 5' regulatory regions (~1.8 kb) of *cnp-2* fused with the reporter GFP for CNP-2 expression pattern analysis. ATG denotes the *cnp-2* start codon. The first intron and part of the second exon encoding the initial 28 amino acids are also included in the construct. (B-I) GFP was expressed throughout development. Gastrula stage (B), comma stage (C), 2-fold stage (D), 3-fold stage (E), L1-L2 stage (F), L3-L4 stage (G), adult stage (G-I). CNP-2:: GFP was expressed in the intestine (H-I, arrow) and some unidentified cells (G).

Knock-down of *cnp-2* in different mutant backgrounds

We performed *cnp-2* knock-down by allowing the worms to feed on bacteria expressing *cnp-2* dsRNA and then examined some of the general phenotypes, which included embryonic development, brood size, and gross morphology. The *cnp-2* RNAi worms were indistinguishable from the mock RNAi treated worms (data not shown) raising two possibilities: either *cnp-2* is a non-essential gene in *C. elegans* or *cnp-2* knock-down was not effective in our experiment. In order to rule out the latter possibility, we performed the knock-down experiment in two RNAi hypersensitive mutants, *rrf-3* and *lin-35* (27, 28). Consistent with our previous experiment, no obvious phenotypic abnormalities were found in the mutants when compared to the control strains (data not shown), suggesting it is unlikely that the initial *cnp-2* knock-down experiment was ineffective. However, there is the possibility that the residual amount of transcript present after *cnp-2* RNAi is sufficient enough to carry out its function in *C. elegans*. Our results are consistent with reports by three separate research groups who performed *cnp-2* RNAi and found no evidence of abnormalities upon examining other phenotypes such as lethality, body morphology, maternal sterility, larval lethality, and larval arrest (29-31).

Since CNP-2 physically interacted with Cn, we reasoned that analyzing Cn loss-of-function phenotype abnormalities may provide insight into the role of CNP-2 in *C. elegans*. So we examined embryonic lethality, movement, egg laying, and body morphology. However, no changes were found in the *cnp-2* RNAi worms relative to control worms, suggesting that *cnp-2* does not play a role in any of the observed Cn pathways. Alternatively, it is possible that *cnp-2* knock-down might modulate the phenotype(s) of Cn mutants analogous to the role of *cnp-3* (T23C6.3) as an enhancer of Cn loss-of-function phenotypes (unpublished data). In order to test this possibility, we performed *cnp-2* knock-down in *tax-6(lf)* and *tax-6(gf)* mutants and examined embryonic lethality, movement, egg laying, and body morphology. The phenotypic characteristics of the *tax-6(lf)* and *tax-6(gf)* mutants were neither enhanced nor suppressed, indicating that *cnp-2* does not modulate any of the aforementioned phenotypes in Cn mutants. Ideally, the ambiguity of the *cnp-2* knock-down phenotypes could be resolved by analyzing a *cnp-2* null mutant. In order

to elucidate the functions of *cnp-2* relative to calcineurin in *C. elegans*, isolation and further characterization of a *cnp-2* mutant are needed.

MATERIALS AND METHODS

C. elegans strains and maintenance

The following strains were obtained from the *Caenorhabditis elegans* Genetics Center (CGC) at the University of Minnesota, USA: Bristol N2, *rrf-3(pk1426), lin-35(n745),* and *tax-6(p675)*. *tax-6(jh107)* was isolated in our laboratory by the reverse genetics method (32). Worm breeding and handling were conducted as previously described (33).

Yeast two-hybrid assays

Yeast two-hybrid screening was conducted using the full length of *tax-6* cloned into pAS2-1 as bait, and the *C. elegans* cDNA library cloned into pACT2 as a prey vector. The screening was performed according to the manufacturer's protocol (Clontech).

Protein expression, purification, and *in vitro* binding assay

The partial Y46G5A.10 (*cnp-2*) cDNA, which contains the exons 1 to 6, was subcloned into pRSET B vector using *BamH*I and *Pst*I restriction enzyme sites. The 6xHis::CNP-2 protein was expressed at 37°C in *Escherichia coli* strain BL21 (DE3), and affinity-purified using a Ni^{2+} affinity column (34). The 6xHis::CNP-2 protein was resuspended in the binding buffer (20 mM sodium phosphate, 500 mM NaCl, pH 7.8) and incubated with wild-type (N2) *C. elegans* protein extract at 4°C for 4 hours. The sample was then washed three times with binding buffer and subjected to SDS-PAGE. Western blot analysis was performed using *C. elegans* anti-TAX-6 antibody as the primary antibody.

GFP fusion construct and microinjection

To construct fusion plasmid (*cnp-2::gfp*), a genomic fragment containing the 1.8 kb 5' upstream *cnp-2* gene sequence, as well as exon1, intron1, and partial exon 2, was fused to a promoterless GFP vector, pPD95.75 (kindly provided by A. Fire), using *Hind*III and *BamH*I restriction enzyme sites. A mixture of plasmids (100 ng of *cnp-2::gfp* fusion construct and 100 ng of pRF4 as a transformation marker) were microinjected into the gonads of young adults as previously described (35).

RNA interference

To perform bacteria-mediated RNAi, an *Nco*I -*Pst*I fragment of ~1.2 kb Y46G5A.10 (*cnp-2*) cDNA was cloned into an L4440 vector. The bacteria-mediated RNAi experiment was conducted as previously described (36). Briefly, bacteria producing double-stranded RNA (dsRNA) were grown in LB with 50 μg/ml ampicillin overnight at 37°C and then seeded onto NGM agar plates containing 1 mM IPTG and 50 μg/ml ampicillin. Six to eight hermaphrodites at the L4 stage [*rrf-3, lin-35, tax-6(lf), tax-6(gf)*] were transferred onto the aforementioned plates and incubated for ~40 h at 20°C. Then, worms were replica-plated onto plates seeded with the same bacteria and allowed to lay eggs for 24 h before being removed. Finally, the progenies were scored for RNAi phenotypes.

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