Calcineurin homologous proteins regulate the membrane localization and activity of sodium/proton exchangers in *C. elegans*

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Allman E, Wang Q, Walker RL, Austen M, Peters MA, Nehrke K. Calcineurin homologous proteins regulate the membrane localization and activity of sodium/proton exchangers in *C. elegans*. Am J Physiol Cell Physiol 310: C233–C242, 2016. First published November 11, 2015; doi:10.1152/ajpcell.00291.2015.—Calcineurin B homologous proteins (CHP) are N-myristoylated, EF-hand Ca$^{2+}$-binding proteins that bind to and regulate Na$^{+}$/H$^{+}$ exchangers, which occurs through a variety of mechanisms whose relative significance is incompletely understood. Like mammals, *Caenorhabditis elegans* has three CHP paralogs, but unlike mammals, worms can survive CHP loss-of-function. However, mutants for the CHP ortholog *pbo-1* are unfit, and *pbo-1* has been shown to be required for proton signaling by the basolateral Na$^{+}$/H$^{+}$ exchanger NHX-7 and for proton-coupled intestinal nutrient uptake by the apical Na$^{+}$/H$^{+}$ exchanger NHX-2. Here, we have used this genetic model organism to interrogate PBO-1’s mechanism of action. Using fluorescent tags to monitor Na$^{+}$/H$^{+}$ exchanger trafficking and localization, we found that loss of either PBO-1 binding or activity caused NHX-7 to accumulate in late endosomes/lysosomes. In contrast, NHX-2 was stabilized at the apical membrane by a nonfunctional PBO-1 protein and was only internalized following its complete loss. Additionally, two *pbo-1* paralogs were identified, and their expression patterns were analyzed. One of these contributed to the function of the excretory cell, which acts like a kidney in worms, establishing an alternative model for testing the role of this protein in membrane transporter trafficking and regulation. These results lead us to conclude that the role of CHP in Na$^{+}$/H$^{+}$ exchanger regulation differs between apical and basolateral transporters. This further emphasizes the importance of proper targeting of Na$^{+}$/H$^{+}$ exchangers and the critical role of CHP family proteins in this process. The Department of Biology, Oberlin College, Oberlin, Ohio; and Department of Biochemistry and Molecular Biology, Pennsylvania State University, University Park, Pennsylvania

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program, and mutations in nhx-7/pbo-4 reduce or eliminate pBoc without altering the underlying calcium signals.

Loss of PBO-1 in worms causes an amalgamation of these phenotypes, resulting in cellular acidosis, slow growth, reduced fat stores, and a weak PBO muscle contraction without significantly altering oscillations in intestinal calcium (51). Moreover, mutation of a conserved CHP-binding domain in NHX-7 suppresses its ability to complement an nhx-7/pbo-4 mutant, supporting the functional relevance of PBO-1 binding to impact NHX-7 activity (3). Coupled with the fact that rhythmic calcium oscillations occur in the intestinal cytoplasmin every ~50 s and that CHP family proteins are thought to transduce calcium signals, these observations motivated us to further explore PBO-1’s mechanism of action as related to NHE activity in the worm.

Our results support the idea that CHP family proteins stimulate trafficking, membrane retention, and activity of NHEs in a paralog-dependent manner and suggest that two new pbo-1 paralogs may contribute to cell-specific functions through similar regulatory mechanisms. Together, these results represent one of the first reports of CHP loss-of-function, resulting in an NHE trafficking defect in vivo, and suggest a broadly conserved function between worm and mammalian systems.

MATERIALS AND METHODS

Strains, alleles, and culturing techniques. Standard culture techniques were used to maintain nematodes on nematode growth medium (NGM)-agar plates seeded with OP50 bacteria (10). The wild-type strain is Bristol N2. Transgenes were introduced using microinjection with a pha-1(+)-marker in a pha-1(e2123ts)III temperature-sensitive mutant strain, and transgenic progeny were selected for and maintained at 20°C. Genetic crosses were performed using standard mating techniques. Pbo-1 mutant strains are TA105 pbo-1(sa7)III and TA111 pbo-1(tm3716)III as described previously (51). Strains containing the pha-1(e2123ts)III and calcium-kinin-like EF-hand protein family member (cfh-2)(ok2944)IV alleles as well as the green fluorescent protein (GFP) integrated localization markers were obtained from the C. elegans Genetics Center (University of Minnesota), whom we kindly acknowledge, as well as the C. elegans Gene Knockout Consortium, and were outcrossed before use. A complete list of strains used in this work is shown in the Supplemental Material (Supplemental material for this article is available online at the journal website.).

Molecular biology. pELA3 and pKT107 are as previously described (3). An ~3-kb region of genomic DNA upstream of the start codon for the cfh-2 gene was cloned into pFH661::wCherry to generate pELA37. An ~2-kb region of genomic DNA upstream of the CEP5248 operon and a small ~400-bp region of genomic DNA upstream of the start codon for the cfh-1 gene was cloned into pFH661::wCherry to generate pELA46 and pELA38, respectively.

RNA interference. Freshly transformed HT115 bacteria were grown at 37°C to midlog phase, induced with 1 mM isopropyl-β-D-thiogalactoside for 1 h, and seeded on NGM plates. Larval L3 stage worms were placed on the RNAi plates and moved to new plates at 24 h, and their progeny were screened.

Microscopy. Images of transgenic strains and immunohistochemically stained animals were acquired at the University of Rochester Confocal Core. Images were acquired at room temperature using an Olympus IX81 inverted laser-scanning confocal microscope, with ×10 (numeric aperture (NA) 0.40), ×20 oil (NA 0.85), ×40 oil (NA 1.30), ×60 oil (NA 1.42), or ×100 oil (NA 1.40) objectives. Live worms were anesthetized with a solution of 1 mg/ml tetramisole in M9 buffer on 2% agarose pads under a cover slip. Z stacks ranging from 5 to 30 slices were obtained using the optimal slice depth. Olympus Fluoview1000 software was used for image acquisition and for post hoc image processing and analysis. The same acquisition parameters were used when analyzing relative transporter membrane abundance in separate genetic backgrounds.

Osmotic stress assays. To assess the excretory cell’s ability to function following hypotonic exposure, worms were grown on NGM agar plates containing 500 mM NaCl for 24 h and after adaptation were moved back to a low-salt NGM plate. Subsequent survival was assayed after 12 h.

Generation of the anti-NHX-2 antibody and immunohistochemical detection. A custom anti-NHX-2 antibody was raised in rabbits against the peptide CNDGFENDGYESDES and was affinity purified (Invitrogen, Carlsbad, CA). Whole worm fixation was performed using a standard peroxide tube protocol. Antibody dilutions in standard detection buffer (PBS-Tween 20) were as follows: 1:250 rabbit anti-NHX-2 and 1:1,000 goat anti-rabbit Alexa555 (Molecular Probes/Invitrogen). For V5 epitope detection, a mouse anti-V5 antibody (Invitrogen) was used at 1:2,000 with secondary detection using a goat anti-mouse Alexa555 as above. Worms were mounted on cover slips in Fluoromount G (Southern Biotech), sealed, and imaged by fluorescence microscopy.

Alternatively, RT311 (GFP::RAB-11) worms were treated with pbo-1 RNAi for two generations; 10–15 adult hermaphrodites were put in 5 μl M9 solution on poly-L-lysine-coated slides. The intestines of adult hermaphrodites were gently exposed by using a 26-gauge syringe needle to pierce the cuticle, allowing the gonads and intestines to extrude from the animals. Worms were fixed by 2% formaldehyde with 50% methanol in PBS and incubated at room temperature for 30 min in a humidified chamber. After fixation and subsequent washing with PBS containing 0.1% BSA with 0.5% Triton X-100, worms were incubated with mouse anti-GFP (monoclonal antibody; Clontech) and rabbit anti-NHX-2 antibodies for 2 h at room temperature in a humidified chamber, washed again, and then incubated with secondary goat anti-mouse or goat anti-rabbit Alexa antibodies for 30 min at room temperature. Antibody dilutions were 1:250 for NHX-2, 1:1,000 for GFP, and 1:1,000 for goat anti-rabbit Alexa555 and goat anti-mouse Alexa488. The final specimens were mounted on cover slips for imaging in Fluoromount G (Southern Biotech).

RESULTS

Both basolateral and apical NHE require PBO-1 for membrane targeting or stability. The C. elegans CHP family member pbo-1 has been shown to regulate intestinal NHE activity (51), and mutation of the PBO-1-binding domain in the basolateral NHE NHX-7 suppresses its ability to function (3). To examine the underlying mechanism, an NHX-7::mCherry-tagged fusion protein was expressed via the native nhx-7 promoter in transgenic worms, and its distribution was assessed by confocal microscopy. To avoid overexpression artifacts, qRT-PCR was used to identify transgenic lines that expressed the transcript coding for the recombinant protein at close to endogenous levels (data not shown). In a wild-type genetic background, the fusion protein colocalized with a fluorescent extracellular (EC) pH sensor fused to the aquaporin 7::mCherry fusion protein instead accumulated inside of the intestinal cells (Fig. 1, A and B). However, in a pbo-1(tm3716) loss-of-function background, which is both smaller and has a morphologically distinct intestine, the NHX-7::mCherry fusion protein instead accumulated inside of the intestinal cells (Fig. 1D), even though the EC sensor was targeted to the basolateral membrane correctly (Fig. 1E). NHX-7::mCherry mistargeting was also observed in specimens subjected to pbo-1 RNAi (Fig. 1, F and G) and in a second less-affected pbo-1 mutant, the sa7 allele, which contains a substitution of E135K that reverses the charge of a highly conserved residue in the third EF hand (Fig. 3D), qRT-PCR
Although we did not examine the interaction between NHE/CHP (Fig. 1), it is possible that the adverse phenotypes resulting from loss-of-function in the NHX-7-targeting or retention, that binding of PBO-1 to NHX-7 is important for this function, and that the adverse phenotypes confirmed >90% reduction in pbo-1 transcript levels following RNAi (data not shown). Finally, both the pbo-1 RNAi worms and the pbo-1(sa7) mutants were slightly healthier than pbo-1(tm3716) mutants, consistent with the effect of knockdown or a hypomorphic allele, respectively, vs. a complete loss-of-function in the tm3716 mutant.

Mammalian CHP1 belongs to a multifunctional protein family (18) that has been suggested to contribute to protein trafficking (6, 8). Hence, it is possible that the NHX-7-targeting phenotype could arise indirectly. To circumvent this, we mutated three residues in a region of the NHX-7 coding sequence as shown in Fig. 1H (M541R/V542R/L545R) that disrupts an amphipathic alpha helix that is structurally important for the interaction between NHE/CHP (Fig. 1I). Although we did not verify biochemically that the interaction with PBO1 was disrupted in the mutant, the same mutations in mammalian NHE1, -2, and -3 by Pang et al. (43). I: HeliQuest was used to generate alpha helical wheel projections from the sequence alignment shown in the boxed region in the alignment. The internal arrow indicates the PBO-1 binding. These mutations were based upon the approach used to disrupt CHP1 binding to mammalian NHE1, -2, and -3 by Pang et al. (43). I: HeliQuest was used to generate alpha helical wheel projections from the sequence alignment shown in the boxed region in the alignment. The internal arrow indicates the PBO-1 binding. These mutations were based upon the approach used to disrupt CHP1 binding to mammalian NHE1, -2, and -3 by Pang et al. (43).

Like wild-type NHX-7 in a pbo-1(tm3716) genetic background, the mutated NHX-7(Δpbo-1):mCherry fusion protein accumulated in the cytoplasm, even in a wild-type genetic background (Fig. 1C), and our previous work showed that this mutant was unable to complement the pbo defect in an nhx-7(ok583) loss-of-function mutant (3). Unlike the pbo-1 mutant, however, these worms were otherwise healthy. Together, these results suggest that PBO-1 contributes to NHX-7 membrane targeting or retention, that binding of PBO-1 to NHX-7 is important for this function, and that the adverse phenotypes
displayed by the pbo-1(tm3716) mutant arise independent of NHX-7. In addition, we found that a V5-PBO-1 transgenic protein, which rescues the pbo mutant phenotype (data not shown), is mainly cytoplasmic but is not distributed diffusely through the cell (Fig. 3E). Instead, the punctate distribution appears consistent with it being associated with intracellular organelles. As a caveat, this distribution may reflect overexpression, but at face value provides some support for PBO-1 being important for trafficking.

NHX-2 is an apical NHE that contributes to the worm’s viability through its physiological coupling to nutrient transporters (39, 40). NHX-2 also contains a predicted binding site and applied the affinity-purified antibody to samples fixed for immunohistochemistry. The antibody bound robustly to a tar-get and applied the affinity-purified antibody to samples fixed for immunohistochemistry. The antibody bound robustly to a target in the apical membrane of the intestine in wild-type worms (Fig. 2A) but not in worms treated with nhx-2 RNAi (Fig. 2A, inset). As predicted, the pbo-1(tm3716) mutant accumulated NHX-2 in the cytoplasm rather than at the membrane (Fig. 2C), although its distribution was markedly different from the NHX-7 (Fig. 1, C and D). Moreover, RNAi of pbo-1 resulted in a similar staining pattern (data not shown). However, when the antibody was applied to samples of pbo-1(sa7) mutants, NHX-2 labeling persisted at the apical membrane, resembling its wild-type distribution (Fig. 2B). This suggested that the physical presence of the mutant PBO-1(sa7) protein is sufficient for membrane stabilization of the NHE, even if it does not appear to support robust NHX-2 activity (51). However, given the resolution of the technique, it is also possible that NHX-2 is subapical in the sa7 mutant. Finally, not all intestinal NHE proteins require PBO-1 for targeting, since an NHX-4::mCherry fusion was found to be distributed normally to the basolateral membrane following pbo-1 (RNAi) (Fig. 2, E and F), and not all apical transporters are affected either, since the apical V-ATPase subunit VHA-6 (2) was also correctly targeted following either pbo-1 (RNAi) or in a pbo-1(sa7) mutant (Fig. 2D). The NHX-4::mCherry transgene was normally distributed in the pbo-1(sa7) mutant as well (data not shown).

PBO-1 targeting of intestinal NHEs. C. elegans has been used extensively as a genetic model to study intracellular trafficking, and there are a variety of strains expressing fluorescent transgenic fusions that label individual organelles (12,

**Fig. 2.** PBO-1 regulates NHX-2 function and retention at the apical membrane through separable mechanisms. Endogenous NHX-2 was detected with a custom-generated antibody raised against the sequence NH3-CNDGFENDGYESDES-COOH in the extreme COOH-terminus of NHX-2 protein. Antibody target recognition was visualized with an Alexa 555-conjugated secondary antibody via fluorescent micrography. White arrowheads indicate the intestinal apical membrane. A: control worms, at ×400 and ×1,000 magnification, as well as negative control nhx-2(RNAi) worms, shown in the inset on right. The exposure time was increased 10-fold for the nhx-2(RNAi) worms so as not to present a blank picture; the resulting signal was limited to autofluorescence, and the lumen/apical membrane is clearly not detected. B and C: pbo-1(sa7) missense mutant worms (B) and pbo-1(tm3716) null worms (C). D: confocal fluorescent micrograph of transgenic Pvha-6::VHA-6::mCherry protein expression in a live anesthetized pbo-1(RNAi) worm. Inset shows Pvha-6::VHA-6::mCherry in the pbo-1(sa7) mutant background. E and F: confocal fluorescent micrographs of transgenic Pnhx-4::NHX-4::mCherry protein expression in live anesthetized control and pbo-1(RNAi) worms, as labeled. White arrowheads denote labeling of the intestinal basolateral and lateral membranes, with the cell junctions being most readily visible. The scale bars are as follows: 5 μM (A–C), 20 μM (D), and 20 μM (E and F). The inset in A was acquired using a higher-magnification objective, as shown.
To determine where NHX-7 was targeted in the absence of PBO-1 binding, these marker alleles were crossed into strains expressing mutant NHX-7, and their relative distribution was assessed via confocal microscopy. The NHX-7Δpbo-1::mCherry fusion protein colocalized with GFP::RAB-7 in the intestine, which was used to mark late endosomes and lysosomes (11, 34, 50) (Fig. 3, A and B), but not with AMAN-2-, RAB-5-, or RAB-10-positive vesicles, which represented endoplasmic reticulum/Golgi, early endosomes, or basolateral recycling endosomes, respectively (data not shown). The reciprocal finding that the wild-type NHX-7::mCherry fusion protein also colocalized with RAB-7::GFP vesicles in a pbo-1Δpbo-1 genetic background (Fig. 3D) confirmed that PBO-1 binding prevents default targeting of NHX-7 to late endosome/lysosomes and suggests that the lysosomal localization is not merely a secondary result of protein misfolding.

Characteristic blue intestinal autofluorescence normally found in terminal lysosomes (or “gut granules”) (14) is comprised of anthranilic acid glucosyl esters and localized with some but not all of the labeled vesicles (Fig. 3C). This suggests that NHX-7::mCherry may be present in an overlapping subset of RAB-7(+/+)anthranilic acid(+) vesicles. The appearance of both the wild-type NHX-7::mCherry and NHX-7Δpbo-1::mCherry fusion protein inside the vesicle lumen (Fig. 3, B and D) suggested that the COOH-terminal mCherry tag may have been cleaved from NHX-7, which would be expected to reside in the membrane.

In the case of NHX-2, we speculated based upon the juxtaluminal distribution observed in the pbo-1Δpbo-1 mutant and the fact that regulation of apical NHEs such as NHE3 in mammals often occurs through membrane insertion and retrieval from recycling endosomes (52) that the NHX-2Δpbo-1 labeling observed in the pbo-1Δpbo-1 strains represented apical recycling endosomes. In worms, these organelles can be defined by RAB-11::GFP labeling. Our initial observations with live transgenic worms expressing this marker indicated that the apical recycling endosomes were slightly disorganized in live animals treated with pbo-1 RNAi (Fig. 4, B and C). This is consistent with an established role of the mammalian CHP1 being a calcium-dependent signal protein mediating organelle assembly with the microtubule to affect protein trafficking (5, 8).

Unfortunately, the standard immunohistochemistry fixation protocol used in Fig. 2 to disrupt the worm’s cuticle suppressed both GFP fluorescence and detection by commercial anti-GFP antibodies (data not shown). Hence, to test whether NHX-2 colocalized with the apical recycling endosomes as predicted, a physical exposure of the intestine was accomplished by gently slicing open the worm’s cuticle. This caused part of the intestine to extrude from the body cavity, as shown in Fig. 4D. A brief fixation period was followed by antigen detection using anti-GFP and anti-NHX-2 antibodies. This method resulted in robust detection of both epitopes, with a tissue morphology that was more akin to live worms than to fixed worms and better labeling of intracellular NHX-2 itself.

Counter to our prediction, in pbo-1Δpbo-1 worms only rarely did the NHX-2Δpbo-1 organs (Fig. 4, E and H) colocalize with the GFPΔapical recycling endosomes (Fig. 4, F and J). In confocal projections of luminal cross sections (Fig. 4, E–G) or in more peripheral cytoplasmic areas of the intestine (Fig. 4, H–J), there were occasional areas of overlap between the two labels (Fig. 4, G and J), but it seems clear that the vast majority of labeled puncta are mutually exclusive. At present, the identity of the NHX-2Δpbo-1 structures is unknown.

C. elegans code for two paralogs of pbo-1. Using BLAST to search the C. elegans genome, two predicted protein coding
regions were identified with significant homology to PBO-1. These proteins also shared homology with the three mammalian CHP family members, and like those proteins exhibited some hallmark motifs conserved in key regions such as Ca\(^{2+}\)-binding EF hands, NH\(_2\)-terminal myristoylation motifs, and predicted nuclear export signals (Fig. 5). Based upon the likelihood of their interaction with other NHEs in worms, we examined their expression patterns for overlap with the nine worm NHEs (40).

The first of these was encoded by ZK856.8, heretofore known as calcineurin-like EF-hand protein family member chpf-1. Its genomic coding region is the last in an operon consisting of six genes, which also codes for a zinc finger protein, a transcription factor, an RNA pol III subunit, and two other uncharacterized gene products (Fig. 6A). Based upon the likelihood of their interaction with other NHEs in worms, we examined their expression patterns for overlap with the nine worm NHEs (40).

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The second gene was coded for by F59D6.7 and named chpf-2. It is expressed from a single promoter (Fig. 6D) expressed solely in the excretory cell of hermaphrodites (Fig. 6E–J). This long, H-shaped cell extends canal-like processes along the basolateral surface of the hypodermis and contributes to systemic osmoregulation and waste removal. In males, the chpf-2 promoter also drove expression in the ray support cells (Fig. 6F). These cells arise postembionically during morphogenesis of the male tail, which in worms is a sensory organ involved in mating behaviors. Interestingly, NHX-9 is also expressed specifically in the excretory cell (40). Hence, we tested the hypothesis that chpf-2 regulation of nhx-9 contributes to excretory cell function.

**Loss of chpf-2 impacts excretory cell function.** The excretory system in worms works much like the mammalian renal system...
in osmoregulation and waste removal. When worms are moved to high-salt plates, hypertonic shrinkage occurs but is readily compensated for by the accumulation of organic osmolytes through defined molecular signaling pathways (13, 25). Subsequently, moving the high-salt adapted worms to low-salt plates induces hypotonic stress which requires excretory cell function to mediate systemic regulatory volume decreases, and laser ablation of the excretory cell results in fluid retention and death (41).

To test the role of chpf-2 in excretory cell function, we obtained a mutation in chpf-2 that had been generated by the C. elegans Gene Knockout Consortium. The chpf-2(ok2941) allele has a 600-nucleotide deletion that removes more than one-half of the coding sequence (Fig. 6D) and likely results in a complete loss-of-function. Because the deletion boundaries are contained within the genomic coding sequence, it is unlikely to impact surrounding genes. Our results demonstrate that the ok2941 mutant exhibited ~20% reduced survival compared with control worms after recovery from high-salt exposure, suggesting a deficiency in excretory cell function (Fig. 7A). There was no deficit noted upon the initial transfer to high salt (data not shown), suggesting that this was not a general defect in osmotic adaptation. Moreover, the general morphology of the excretory cell, as judged by wCherry labeling, appeared to be normal in the ok2941 genetic background (Fig. 7B).

The NHE family member nhx-9 is expressed strongly in the excretory cell (40), and the NHX-9 protein contains a CHP family binding motif (Fig. 1H) that is predicted to form an amphipathic alpha helix (data not shown). If NHX-9 were to interact with CHPF-2 in a mechanistically similar way as PBO-1 does with the intestinal NHEs, it would be reasonable to predict that NHX-9 would be mislocalized in the ok2941 mutant.

This prediction was tested by examining the distribution of an NHX-9::GFP translational fusion protein in the excretory cell of wild-type and ok2941 mutants (Fig. 8C). While it is not currently known whether NHX-9 normally localizes to the apical or basolateral cell surface, it was immediately obvious that there were not gross differences in the distribution of NHX-9 protein in these genetic backgrounds. However, overexpression of NHX-9::GFP from the transgenic array could occlude normal regulatory mechanisms. Moreover, the excretory cell is polarized around a central lumen where the canals are quite small, and it is possible that subtle differences such as movement in apical recycling endosomes from the apical membrane would be undetected. More informative, however, was the finding that nhx-9(ok847) deletion mutants exhibited normal responses to hypotonic challenge (Fig. 7A). Hence, it is not likely that any suspected redistribution of NHX-9 protein in the CHP family ok2941 mutant would result in measurable consequences, regardless. We conclude that the ok2941 mutant likely exerts its effect through another NHE or a separate mechanism entirely.

**DISCUSSION**

CHP family proteins vary in their structural elements and expression profiles, but are similar in that they all have been shown to bind to and regulate NHEs. However, the mechanism through which these proteins exert their effect has been obscured by conflicting results. It has been suggested that CHP protein binding is necessary for NHE ion transport, biosyn-
thetic maturation, trafficking to the membrane, or membrane stability, and that these are influenced by CHPs independently, in aggregate, or not at all (for review, see Ref. 18). A recent consensus seems to be that individual functions may be cell type specific as well as specific for the individual CHP and NHE paralogs in question.

Worms have a similar genomic complexity in the NHE and CHP gene families, with nine and three paralogs each, respectively. Here, we took advantage of the genetic reagents, the limited repertoire of tissues, and stereotypical behaviors in worms to decipher how CHP function in one tissue can influence multiple NHEs. Previous work has shown that the C. elegans CHP family protein PBO-1 is expressed in the intestine and contributes to intestinal NHE activities (3, 51). Our results suggest surprising differences in how each of these NHEs reacted to the loss of PBO-1. In the case of NHX-7, of the three approaches taken (deleting the pbo-1 gene, mutating a single residue in the pbo-1 coding region, or removing the PBO-1-binding site in NHX-7 itself) all resulted in a similar outcome: NHX-7 was targeted to late endosomes/lysosomes. Hence, a physical association between NHX-7 and PBO-1 as well as functional PBO-1 calcium-binding activity are both required for proper targeting.

Fig. 6. Calcineurin-like EF-hand protein family member (chpf)-1 and chpf-2 expression profile. A: schematic of the genomic region containing the chpf-1 gene (blue arrow outlined in red). Chpf-1 is the last gene in the operon CEOP5248 (gray arrow). The other genes in the operon are depicted as blue arrows. The promoter regions used to drive wCherry expression in B and C are depicted by solid red arrows. B and C: fluorescent micrographs of transgenic worms expressing wCherry from either the upstream operon promoter, which is widely expressed throughout the body of hermaphrodites (B), or the small intercistronic promoter, which is only expressed in males (C), in unidentified cells. Scale bars are 50 μM. D: schematic of the chpf-2 gene, whose promoter and genomic coding sequences are depicted as described in A. The limits of the chpf-2(ok2941) deletion are denoted by a black brace. E and F: fluorescent and DIC images of transgenic worms expressing chpf-2 promoter::wCherry fusions. The hermaphrodite in E exhibits excretory cell-specific expression, which is a large H-shaped cell with canals that extend on either side of the body. The male in F exhibits specific expression in cells of the male ray, which is involved in mating behavior. Scale bars are 50 and 10 μM, respectively.

Fig. 7. Chpf-2 impacts excretory cell function. A: worms were allowed to acclimate to hyperosmotic conditions for 24 h before moving them back to normosmotic media. Survival was assayed following 12 h of recovery. Values are averages of 3 paired trials with a minimum of 25 worms/trial. Significance was determined using a two-sample t-test. B: wCherry labeling of the H-shaped excretory cell in chpf-2(ok2941) mutants indicates normal gross morphology. Scale bar is 50 μM. C: NHX-9::GFP expression in the excretory cell of wild-type worms (top) or chpf-2(ok2941) mutant animals (bottom). The excretory cell lumen is denoted by a white dotted line. The scale bar, which is oriented vertically, is 5 μM.

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In contrast, the distribution of NHX-2 differed dramatically in the two genetic pbo-1 mutant backgrounds. In the deletion mutant, NHX-2 was mistargeted and accumulated in the cell (Fig. 4). However, in the missense mutant, NHX-2 was found at the plasma membrane. These differences suggested that physical binding of PBO-1 to NHX-2 suffices for membrane stability. We have previously shown that sa7 is a strong loss-of-function allele and phenocopies nhx-2(lf) (51). Thus, PBO-1 calcium binding is likely necessary for robust NHE activity, if not for membrane stability. We note that mammalian NHE3 regulation by CHP1 has been proposed to increase NHE3 constitutive transport function, protein abundance, and regulation by calcium (16, 17, 19), but that the precise mechanisms underlying this regulation are complicated and may reflect interactions with adaptor proteins or specific settings. NHX-2 contains several motifs predicted to interact with adaptor proteins, which serve to direct trafficking within the endosomal and secretory pathways, one of which falls in the middle of the PBO-1-binding domain. It is possible that this motif is masked in the presence of PBO-1. Alternatively, perhaps a regulatory motif is unmasked by PBO-1 calcium binding. Within this context, it is intriguing to speculate that NHX-2’s trafficking might be coupled to calcium signaling, given that intestinal calcium oscillations occur frequently with an ~50-s period (15). It is also possible that other interactions, such as with ERM proteins as has been shown to be important for NHE3 signaling (16), may contribute to this process, and new evidence is emerging that CHPI can regulate the exchanger set point for pH (7).

In addition to PBO-1 in the intestine, we have also reported the presence of two additional CHP isoforms, chpf-1 and chpf-2. Based simply upon sequence homology and protein motif analysis, we were unable to predict which worm paralog is orthologous to a particular mammalian CHP family protein. It is interesting to note, however, that the expression of both PBO-1, the first characterized worm CHP family member (51), and chpf-2 is quite restricted compared with chpf-1. This is also true for the limited tissue expression profile of mammalian CHP2 and CHP3 compared with CHPI (18).

Our results demonstrating that chpf-2(lf) mutants were not as effective at surviving hypertonic exposure suggested a problem with water balance and a defect in excretory cell function (Fig. 7). However, a strain lacking expression of the excretory cell-specific NHX-9 responded to hypotonic stress. We have previously shown that chpf-1 is quite restricted compared with chpf-2. In contrast, the distribution of NHX-2 differed dramatically in the two genetic pbo-1 mutant backgrounds. In the deletion mutant, NHX-2 was mistargeted and accumulated in the cell (Fig. 4). However, in the missense mutant, NHX-2 was found at the plasma membrane. These differences suggested that physical binding of PBO-1 to NHX-2 suffices for membrane stability. We have previously shown that sa7 is a strong loss-of-function allele and phenocopies nhx-2(lf) (51). Thus, PBO-1 calcium binding is likely necessary for robust NHE activity, if not for membrane stability. We note that mammalian NHE3 regulation by CHPI has been proposed to increase NHE3 constitutive transport function, protein abundance, and regulation by calcium (16, 17, 19), but that the precise mechanisms underlying this regulation are complicated and may reflect interactions with adaptor proteins or specific settings. NHX-2 contains several motifs predicted to interact with adaptor proteins, which serve to direct trafficking within the endosomal and secretory pathways, one of which falls in the middle of the PBO-1-binding domain. It is possible that this motif is masked in the presence of PBO-1. Alternatively, perhaps a regulatory motif is unmasked by PBO-1 calcium binding. Within this context, it is intriguing to speculate that NHX-2’s trafficking might be coupled to calcium signaling, given that intestinal calcium oscillations occur frequently with an ~50-s period (15). It is also possible that other interactions, such as with ERM proteins as has been shown to be important for NHE3 signaling (16), may contribute to this process, and new evidence is emerging that CHPI can regulate the exchanger set point for pH (7).

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Our results demonstrating that chpf-2(lf) mutants were not as effective at surviving hypertonic exposure suggested a problem with water balance and a defect in excretory cell function (Fig. 7). However, a strain lacking expression of the excretory cell-specific NHX-9 responded to hypotonic exposure normally, suggesting that NHX-9 is an unlikely candidate to contribute to the chpf-2(lf) mutant phenotype. It is possible that CHPF-2, like CHPI in mammals, may participate more generally in organelle trafficking and that the mutant phenotype arises from this aspect of its function. Alternatively, there may be another NHE whose loss causes an excretory cell defect.

In conclusion, previous studies have suggested that CHP’s regulation of NHES is mechanistically complex, and the results presented here suggest a similar complexity is conserved in a simple genetic model organism. The availability of genetic resources, including viable loss-of-function mutants, should help to unravel this complexity.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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