Calcineurin Homologous Proteins Regulate the Membrane Localization and Activity of Sodium-Proton Exchangers in *C. elegans*

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ABSTRACT
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, C. 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 non-functional PBO-1 protein and was only internalized following its complete loss. Additionally, two pbo-1 paralogs were identified and their expression patterns 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.

INTRODUCTION
Calcineurin B Homologous Protein (CHP) belongs to the EF-hand calcium binding family and have been shown to regulate vesicle trafficking, cell proliferation, gene transcription and Na^{+}/H^{+} exchanger (NHE) activity (23, 24, 26, 29, 36, 43). There are three CHP paralogs in mammals. CHP1 is broadly expressed and was first identified by several groups working in parallel: first, through its binding to the cytoplasmic carboxy-terminal tail of the ubiquitous mammalian NHE1 (21, 28) and second, through a role in protein trafficking (8). CHP family proteins have since been suggested to be required for both basal and calcium-
stimulated NHE activity, NHE biosynthetic maturation, transport to the membrane, and stabilization of
the transporter once it’s there (16, 28, 33, 43, 53, 54). CHP1’s affinity for calcium increases greatly when
it is bound to NHE1 suggesting a possible connection between calcium signaling and stimulation of NHE
activity (42). However, given the large number of outputs credited to CHP1, it has been difficult to
ascertain what effects can be attributed to discrete functional modalities. In addition, potential CHP
binding sites are likely conserved amongst NHEs, including organelle transporters (55), and, in some
cases, CHP1 has been shown to regulate the activity of other NHEs such as NHE3 (16, 20, 31). Further
complexity arises from two additional CHP paralogs, CHP2 (4, 35, 37, 44) and CHP3 (22, 27, 32, 45),
which are less well characterized but have nonetheless been shown to interact with NHEs and regulate
their activity.

Developing a more nuanced understanding of CHP function in a complex systems environment has
been complicated by the fact that genetic ablations are apparently lethal in mammals. However, a point
mutant in CHP1 was recently identified in the vacillator (vac) mutant mouse and demonstrated quite
elegantly to cause degeneration of Purkinje cell axons (30). The authors suggested that neuronal CHP1 is
necessary for targeting of NHE1 and that effective axonal pH homeostasis supports axonal health.

We also recently showed that loss-of-function (lf) mutations in the C. elegans CHP family member
*pbo-1* are tolerated (51). *Pbo-1* is largely expressed in the nematode intestine and *pbo-1* mutants
phenocopy the loss of intestinal NHEs, suggesting an evolutionarily conserved role in regulating their
function (51). In brief, there are two particular NHEs in the nematode intestine, NHX-2 and NHX-7, that
have been shown to contribute to intestinal homeostasis and whose loss elicits overt phenotypes (38).

NHX-2 is an apical membrane transporter whose expression is restricted to the intestine and whose
function has been linked to that of the proton-dipeptide symporter OPT-2 (39, 40). NHX-2 is closely
related to mammalian NHE3, which is also found at the apical membrane of intestinal epithelia and acts
in a similar capacity (48, 49). Mutants in NHX-2 exhibit cytoplasmic acidosis, reduced dipeptide
absorption and a starvation phenotype, and RNAi has been used as a genetic dietary restriction mimetic
(39). NHX-7 (also called PBO-4) is a basolateral membrane transporter whose expression is restricted to
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the posterior most cells of the intestine and whose activity transiently acidifies the extracellular space between the intestine and adjacent body wall muscle, causing those muscle cells to contract (9, 46). Proton extrusion is directed by intestinal oscillatory calcium signaling, which paces the rhythmic (~50s period) defecation behavior in worms (15). Contraction of the posterior body wall muscles (pBoc) is the first and most visible step in the defecation motor program (DMP), 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 pBoc 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 cytoplasm every ~50s 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 into 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 *chpf-2(ok2941)*V alleles as well as the 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 prior to use. A complete list of strains used in this work is shown in the Supplement.

**Molecular Biology**—pELA3 and pKT107 are as previously described (3). A ∼3kb region of genomic DNA upstream of the start codon for the *chpf-2* gene was cloned into pFH6II::wCherry to generate pELA37. A ∼2kb region of genomic DNA upstream of the CEOP5248 operon and a small ∼400bp region of genomic DNA upstream of the start codon for the *chpf-1* gene was cloned into pFH6II::wCherry to generate pELA46 and pELA38, respectively.

**RNA Interference**—Freshly transformed HT115 bacteria were grown at 37ºC to mid-log phase, induced with 1 mM IPTG for one-hour, and seeded onto NGM plates. Larval L3 stage worms were placed onto the RNAi plates, moved to new plates at 24 hours, 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 10x (N.A. 0.40), 20x oil (N.A. 0.85), 40x oil (N.A. 1.30), 60x oil (N.A. 1.42), or 100x oil (N.A. 1.40) objectives. Live worms were anesthetized with a solution of 1 mg/ml tetramisole in M9 buffer on 2% agarose pads under a coverslip. Z stacks ranging from 5–30 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 500mM NaCl for 24 hours and after adaptation were moved back to low salt NGM plate. Subsequent survival was assayed after 12 hours.
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 into standard detection buffer (PBS-T) were as follows: 1:250 rabbit anti-NHX-2 and 1:1000 goat anti-rabbit Alexa555 (Molecular Probes/Invitrogen). For V5 epitope detection, a mouse anti-V5 antibody (Invitrogen) was used at 1:2000 with secondary detection using a goat anti-mouse Alexa555 as above. Worms were mounted on coverslips in Fluoromount G (Southern Biotech), sealed, and imaged by fluorescence microscopy.

Alternatively, RT311 (GFP::RAB-11) worms were treated with pbo-1 RNAi for 2 generations. 10-15 adult hermaphrodites were put into 5µL 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 PBSTB (PBS containing 0.1% BSA with 0.5% Triton X-100), worms were incubated with mouse anti-GFP (Clontech; monoclonal antibody) and rabbit anti-NHX-2 antibodies for 2 hours at room temperature in a humidified chamber, washed again, then incubated with secondary goat anti-mouse or goat anti-rabbit Alexa antibodies for 30 minutes at room temperature. Antibody dilutions were 1:250 for NHX-2; 1:1000 for GFP; 1:1000 for goat anti-rabbit Alexa555 and goat anti-mouse Alexa488. The final specimens were mounted on coverslips for imaging in Fluoromount G (Southern Biotech).
RESULTS

Both basolateral and apical Na⁺/H⁺ exchangers 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). In order 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 co-localized with a fluorescent extracellular pH (EC) sensor fused to the AQP5 basolateral targeting motif (3) (Figure 1A and 1B). 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 (Figure 1D), even though the EC sensor was targeted to the basolateral membrane correctly (Figure 1E). NHX-7::mCherry mistargeting was also observed in specimens subjected to *pbo-1* RNAi (Figure 1F and 1G) 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 (Figure 3D). qRT-PCR 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)* mutant worms, consistent with the effect of knockdown or a hypomorphic allele, respectively, versus 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 3 residues in a region of the *nhx-7* coding sequence as shown in Figure 1H (M541R/V542R/L545R) that disrupts an amphipathic alpha helix that is structurally important for the interaction between NHE/CHP (Figure 1I). Although we did not verify biochemically
that the interaction with PBO1 was disrupted in the mutant, the same mutations in mammalian NHE1, NHE2, and NHE3 have been shown to suppress binding to CHP1 (43).

Like wild type NHX-7 in a \textit{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 (Figure 1C), and our previous work showed that this mutant was unable to complement the \textit{pbo} defect in an \textit{nhx-7(ok583)} loss-of-function mutant (3). Unlike the \textit{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 \textit{pbo-1(tm3716)} mutant arise independent of NHX-7. In addition, we found that a V5-PBO-1 transgenic protein which rescues the \textit{pbo} mutant phenotype (data not shown) is mainly cytoplasmic, but is not distributed diffusely through the cell (Figure 3E). Instead, the punctate distribution appears consistent with its 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 physiologic coupling to nutrient transporters (39, 40). NHX-2 also contains a predicted binding site for PBO-1 (Figure 1H). The starvation phenotype of \textit{pbo-1} mutants and the physiological impact of \textit{pbo-1} loss on proton flux across the apical membrane (51) support a role for PBO-1 in regulating NHX-2 activity.

Unfortunately, transgenic lines expressing fluorescent NHX-2 fusion proteins were not sufficiently bright for our purposes. Hence, we developed a custom anti-NHX-2 antibody 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 (Figure 2A) but not in worms treated with \textit{nhx-2} RNAi (inset in panel). As predicted, the \textit{pbo-1(tm3716)} mutant accumulated NHX-2 in the cytoplasm rather than at the membrane (Figure 2C), though its distribution was markedly different from the NHX-7 (Figure 1C and 1D). Moreover, RNAi of \textit{pbo-1} resulted in a similar staining pattern (data not shown). However, when the antibody was applied to samples of \textit{pbo-1(sa7)} mutants, NHX-2 labeling persisted at
the apical membrane, resembling its wild type distribution (Figure 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 sub-apical in the sa7 mutant. Finally, not all intestinal NHE proteins require PBO-1 for targeting, as an NHX-4::mCherry fusion was found to be distributed normally to the basolateral membrane following pbo-1 (RNAi) (Figure 2E and 2F), and not all apical transporters are affected either, as the apical V-ATPase subunit VHA-6 (2) was also correctly targeted following either pbo-1 (RNAi) or in a pbo-1(sa7) mutant (Figure 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, 47). 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-7(Δpbo-1)::mCherry fusion protein co-localized with GFP::RAB-7 in the intestine, which was used to mark late endosomes and lysosomes (11, 34, 50) (Figure 3A and 3B), but not with AMAN-2, RAB-5, or RAB-10 positive vesicles, which represented ER/Golgi, early endosomes, or basolateral recycling endosomes, respectively (data not shown). The reciprocal finding that the wild type NHX-7::mCherry fusion protein also co-localized with RAB-7::GFP vesicles in a pbo-1(lf) genetic background (Figure 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 auto-fluorescence 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 (Figure 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 wildtype NHX-7::mCherry
and NHX-7(Δpbo-1)::mCherry fusion protein inside the vesicle lumen (Figure 3B and 3D) suggested that the C-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 juxta-luminal distribution observed in the pbo-1(tm3716) 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-2+ labeling observed in the pbo(-) 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 (Figure 4B and 4C). 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 IHC fixation protocol used in Figure 2 to disrupt the worm’s cuticle suppressed both GFP fluorescence and detection by commercial anti-GFP antibodies (data not shown). Hence, in order to test whether NHX-2 co-localized 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 Figure 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-1(RNAi) worms only rarely did the NHX-2+ organelles (Figure 4E and 4H) co-localize with the GFP+ apical recycling endosomes (Figure 4F and I). In confocal projections of luminal cross sections (Figure 4E-G) or in more peripheral cytoplasmic areas of the intestine (Figure 4J-H) there were occasional areas of overlap between the two labels (Figure 4G and 4H), but it seems clear that the vast majority of labeled puncta are mutually exclusive. At present, the identity of the NHX-2+ 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, N-terminal myristoylation motifs, and predicted nuclear export signals (Figure 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 2 other uncharacterized gene products (Figure 6A). Approximately ~17% of the genes in C. elegans are contained in operons (1). Trans-splicing of one of two short leader RNAs, SL1 or SL2, occurs at the 5′ ends of pre-mRNAs of many C. elegans genes, with SL2 leaders characteristic of mRNAs expressed from operons. In the case of chpf-1, both SL1 and SL2 leaders were identified on the mature mRNA. This suggested that chpf-1 expression can be driven by both the operon promoter as well as the intercistronic region just upstream of its coding sequence. Interrogating the individual expression patterns of each of these promoter regions suggested a mutually exclusive distribution, with the operon promoter widely expressed in hermaphrodites, particularly in muscle cells (Figure 6B), and the small intercistronic promoter expressed solely in males in unidentified cells (Figure 6C). These results should be interpreted with the caveat that transgenic expression patterns do not necessarily reflect the endogenous distribution of native genes and contain neither the genomic coding sequence nor 3′ UTR. However, the widespread expression of chpf-1 from the operon promoter is consistent with a housekeeping role.

The second gene was coded for by F59D6.7 and named chpf-2. It is expressed from a single promoter (Figure 6D) expressed solely in the excretory cell of hermaphrodites (Figure 6E). 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 (Figure 6F). These cells arise post-embryonically 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 and 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 half of the coding sequence (Figure 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 to control worms after recovery from high salt exposure, suggesting a deficiency in excretory cell function (Figure 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 (Figure 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 (Figure 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 (Figure 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 and the canals are quite small, and it is possible that subtle differences such as movement into 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 (Figure 7A). Hence, it is not likely that any suspected redistribution of NHX-9 protein in the CHP family *ok2941* mutant would result in measureable 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, biosynthetic 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 (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 9 and 3 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.

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 (Figure 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’ve 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 a ~50s 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 CHP1 can regulate the exchanger set point for pHi (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 are quite restricted compared to chpf-1. This is also true for the limited tissue
expression profile of mammalian CHP2 and CHP3 compared to CHP1 (18).

Our results demonstrating that chpf-2(lf) mutants were not as effective at surviving hypotonic
exposure suggested a problem with water balance and a defect in excretory cell function (Figure 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 CHPL 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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QW, and RLW designed, performed and analyzed experiments. MA performed and analyzed parts of the
experiments shown in Figures 6 and 7. MAP contributed intellectually to the project and to drafting and
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The abbreviations used are: CHP, calcineurin B homologous protein; CHPF, Calcineurin-like EF-hand protein family; RNAi, RNA interference; NGM, normal growth media; pBoc, posterior body wall muscle contraction
REFERENCES


**FIGURE LEGENDS**

**Figure 1.** PBO-1 promotes plasma-membrane localization of the Na⁺/H⁺ exchanger NHX-7 in live worms. The expression of a recombinant, low-copy NHX-7::mCherry fusion protein was assessed by confocal microscopy in live, immobilized transgenic animals. White arrow heads denote the basolateral membrane of posterior intestinal cells int7, 8 and 9 where NHX-7 is expressed, and dotted lines approximate the intestinal lumen. (A) An extracellular (EC) pH sensor marks the basolateral membrane, where (B) wild-type NHX-7::mCherry clearly resides. (C) Mutation of the PBO-1 binding domain in NHX-7(Δpbo-1) results in redistribution of the exchanger from the basolateral membrane to intracellular organelles and/or aggregates. (D) The wild-type NHX-7 displays a similar distribution in a *pbo-1(tm3716)* mutant background as NHX-7(Δpbo-1) does in a wildtype genetic background. (E) Plasma membrane targeting of the recombinant EC pH sensor is unaffected by the loss of *pbo-1*. (F) Wild-type NHX-7::mCherry distribution is altered following (G) RNAi-mediated knockdown of *pbo-1*. The images in panels A and B are from the same worm, imaged in the green and red spectrum, with the overlay shown as indicated. All other images are individual worms. Please note that the acquisition parameters were identical for images B, C, D, F and G, and that the *pbo-1(tm3716)* mutants are quite small and sickly. Scale bars are 20 µM. (H) Protein sequence alignment of worm NHX proteins with the rat NHE1 amino acids 509-537. The CHP binding region is bounded by a red box. Blue shading indicates sequence identity. Arrows denote key residues positioned on the hydrophobic face in the mammalian exchanger, several of which have been colored blue to indicate specific amino acids that were mutated to disrupt PBO-1 binding. These mutations were based upon the approach used to disrupt CHP1 binding to mammalian NHE1, 2, and 3 in Pang et al 2001 (1). (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 hydrophobic moment and its size corresponds to its overall hydrophobicity. The hydrophobic face of the predicted amphipathic alpha helix in NHX-7 is represented by a curved black arrow and the amino acids that were mutated are indicated by blue arrows, as in panel H.
Figure 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 C-terminus of NHX-2 protein. Antibody target recognition was visualized with an Alexa 555 conjugated secondary antibody via fluorescent micrography. White arrow heads indicate the intestinal apical membrane. (A) control worms, at 400x and 1000x magnification, as well as negative control nhx-2(RNAi) worms, shown in the right inset. 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 auto-fluorescence and the lumen/apical membrane is clearly not detected. (B) pbo-1(sa7) missense mutant worms, and (C) pbo-1(tm3716) null worms. (D) Confocal fluorescent micrograph of transgenic P\textit{vha-6}::VHA-6::mCherry protein expression in a live anesthetized pbo-1(RNAi) worm. The inset is P\textit{vha-6}::VHA-6::mCherry in the pbo-1(sa7) mutant background (E, F).

Confocal fluorescent micrographs of transgenic P\textit{nhx-4}::NHX-4::mCherry protein expression in live anesthetized control and pbo-1(RNAi) worms, as labeled. White arrow heads denote labeling the intestinal baso- and lateral membranes, with the cell junctions being most readily visible. The scale bars are: panels A-C, 5 µM; panel D, 20 µM; and panels E and F, 20 µM. The inset in panel A was acquired using a higher magnification objective, as shown.

Figure 3. Loss of PBO-1 binding causes NHX-7 to accumulate in late endosomes/lysosomes.

Confocal micrographs of fluorescent protein expression. (A) NHX-7(Δpbo-1)::mCherry expression in a strain in which intestinal late endosome/lysosomes are labeled with GFP via an integrated translational fusion to RAB-7 protein. The arrow in the DIC image orients the worm and is pointing toward the head. The white arrowheads in the NHX-7(Δpbo-1)::mCherry image denote the basolateral membrane of int7, 8, and 9. (B) A cross section magnified from the overlap as shown confirms mCherry protein in GFP positive vesicles, quantified by horizontal and vertical histograms. The white arrowheads indicate vesicles of interest through which the histograms were obtained, as shown. (C) NHX-7(Δpbo-1)::mCherry (+),
RAB-7(+) vesicles coincide with anthranilic acid glucosyl ester(+) gut granules, or terminal lysosomes. (D) Wild type NHX-7 fusion protein expressed in the pbo-1(sa7) background co-labels RAB-7::GFP(+) late endosome/lysosomes. Scale bars are 10 µM. (E) Confocal micrographs of V5::PBO-1 expression detected with an anti-V5 antibody and visualized with an Alexa 555 conjugated secondary. Scale bar is 5 µM.

Figure 4. PBO-1 protein stabilizes NHX-2 at the apical membrane. (A) DIC and (B) fluorescent confocal micrograph of recycling endosomes labeled via a GFP::RAB-7 fusion in control RT311 and in (C) pbo-1(RNAi) RT311 worms. The worms were imaged live under anesthetic restraint. White arrow heads are adjacent to the lumen and highlight GFP::RAB-11 positive apical recycling endosomes. (D) DIC image of the intestinal preparation used for immunolabeling, with part of the intestine extruding as a loop after gently slicing the worm’s cuticle. (E-J) Fluorescent detection of endogenous NHX-2 protein and a transgenic GFP::RAB-11 marker of apical recycling endosomes in fixed pbo-1(RNAi) worms, using anti-NHX-2 and anti-GFP antibodies as labeled. The lumen is denoted by a dotted white line. Scale bars are 5 µM.

Figure 5. Sequence alignment of C. elegans and human CHP family proteins. Analysis was performed using ClustalW 2 and formatted using Jalview. Shading indicates sequence identity from low (light blue) to high (dark blue). Structural domains are labeled accordingly, based on mammalian studies, and designated by colored lines. Worm protein names are in red. EF (EF-hand helix-loop-helix); NES (Nuclear Export Signal).

Figure 6. Chpf-1 and chpf-2 expression profile. (A) 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 panels B and C are depicted by solid red arrows. (B, C) Fluorescent micrographs of
transgenic worms expressing wCherry from either (B) the upstream operon promoter, which is widely-
expressed throughout the body of hermaphrodites, or (C) the small intercistronic promoter, which is only
expressed in males, in unidentified cells. Scale bars are 50 µM. (D) A schematic of the chpf-2 gene,
whose promoter and genomic coding sequences are depicted as described in panel 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 panel 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 panel F exhibits specific expression in cells of the male ray, which is involved in
mating behavior. Scale bars are 50 µM and 10 µM, respectively.

Figure 7. Chpf-2 impacts excretory cell function. (A) Worms were allowed to acclimate to
hyperosmotic conditions for 24 hours prior to moving them back to normosmotic media. Survival was
assayed following 12 hours of recovery. Values are averages of 3 paired trials with a minimum of 25
worms per 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.
Basolateral EC sensor
WT

NHX-7:: mCherry WT

Overlay

NHX-7(Δpbo-1):: mCherry WT

pbo-1(tm3716) control

AB

NHX-7(Δpbo-1):: mCherry WT

C

int9

int8

int7

D

E F

G

H

CHP Binding Domain

NHX-2 EKKNVHSDKNMEHIEYSELIDTMAGMEDI
NHX-7 AKKEDHFRLFIE-FNNGMVQHLSEQEIDL
NHX-9 KKKQETKRSINEHTQFLDHLTIEEDI
hNHE1 KKKQETKRSINEHTQFLDHLTIEEDI

510 520 530

I

hNHE1

NHX-7

NHX-7(Δpbo-1)

Hydrophobic

Overlay
Cross Section

RAB-7::GFP pbo-1(sa7)

NHX-7::mCherry pbo-1(sa7)

Overlay

RAB-7::GFP pbo-1(sa7)

NHX-7::mCherry pbo-1(sa7)

OverlayDIC

C

Anti-V5::PBO-1

pbo-1(sa7)
GFP::RAB-11

pbo-1(RNAi)

GFP::RAB-11

control

DIC

NHX-2

overlay

GFP::RAB-11

pbo-1(RNAi)

NHX-2

GFP

overlay
ok2941:

Pharyngeal muscle

Enteric muscle

Body wall muscle cells

Head muscle cells
A. 

Percentage of Death

- WT
- chopf-2
- NHX-9::GFP

B. 

Image of a nematode with aggregates showing Pchpf-2::wCherry and chopf-2(ok2941)

C. 

Images of NHX-9::GFP expression levels in nematodes:
- Control
- chopf-2(ok2941)