HID-1, a new component of the peptidergic signaling pathway

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ABSTRACT

hid-1 was originally identified as a Caenorhabditis elegans gene encoding a novel conserved protein that regulates the decision to enter into the enduring dauer larval stage. We isolated a novel allele of hid-1 in a forward genetic screen for mutants mislocalizing RBF-1 rabphilin, a RAB-27 effector. Here we demonstrate that HID-1 functions in the nervous system to regulate neuromuscular signaling and in the intestine to regulate the defecation motor program. We further show that a conserved N-terminal myristoylated motif of both invertebrate and vertebrate HID-1 is essential for its association with intracellular membranes in nematodes and PC12 cells. Caenorhabditis elegans neuronal HID-1 resides on intracellular membranes in neuronal cell somas; however, the kinesin UNC-104 also transports HID-1 to synaptic regions. HID-1 accumulates in the axons of unc-13 and unc-31 mutants, suggesting it is associated with neurosecretory vesicles. Consistent with this, genetic studies place HID-1 in a peptidergic signaling pathway. Finally, a hid-1 null mutation reduces the levels of endogenous neuropeptides and alters the secretion of fluorescent tagged cargos derived from neuronal and intestinal DCVs. Taken together, our findings indicate that HID-1 is a novel component of a DCV-based neurosecretory pathway, and that it regulates one or more aspects of the biogenesis, maturation, or trafficking of DCVs.
INTRODUCTION

The primary mechanism of intercellular communication between neurons and their target cells is calcium-regulated release of secretory vesicles at synapses. The secretion of fast-acting transmitters such as acetylcholine, GABA, and glutamate is mediated by the fusion of small clear synaptic vesicles (SVs) with the presynaptic membrane while the secretion of peptides and biogenic amine transmitters occurs by the exocytosis of dense core vesicles (DCVs) (BURGOYNE and MORGAN 2003; SUDHOF 2004). Although both SVs and DCVs are released in response to depolarization induced calcium entry, there are significant differences in the many facets of their biology including kinetics of release, biogenesis, and recycling.

DCVs typically contain peptidergic cargo. Thus their biogenesis depends on a regulated secretory pathway that begins with the ER and Golgi in the cell soma. After biogenesis and maturation steps in the cell soma, DCVs are transported to the plasma membrane to be exocytosed. The specialized kinesin motor UNC-104 transports neuronal DCVs to axons for secretion (Jacob and Kaplan, 2003). Prior to transport to the plasma membrane or axons, DCVs undergo a maturation process which includes homotypic fusions (URBE et al. 1998), acidification of the lumen required for the processing of peptide precursors (ORCI et al. 1986) and the removal of soluble components and membrane proteins through clathrin-coated vesicle budding (ARVAN and CASTLE 1998; EATON et al. 2000; HINNERS et al. 2003; TOOZE 1998). All these processes are tightly regulated and coordinated by the concerted action of a host of evolutionary conserved proteins. For example: syntaxin 6 and synaptotagmin IV are necessary for the homotypic fusion (AHRAS et al. 2006; WENDLER et al. 2001), while AP-1, ARF-1, VAMP4, GGA, UNC-108/RAB-2 and myosin Va (KAKHLON et al. 2006; KOGEL et al. 2010) have been found participate in later stages of maturation (EDWARDS et al. 2009; SUMAKOVIC et al. 2009). However, many of these components of the secretory apparatus regulate
post Golgi-membrane trafficking more generally and are not unique to the DCV pathway. DCVs also share several components of the core secretory machinery associated with SVs: synaptobrevins, synaptotagmins and Rab GTPases are all present on both types of vesicles (BITTNER and HOLZ 1988; BURGOYNE and MORGAN 2003; LYNCH and MARTIN 2007; SHOJI-KASAI et al. 1992; TAKAMORI et al. 2006; TSUBOI and FUKUDA 2006). However, the substantial differences between SVs and DCVs must be reflected in differences in the underlying molecular components that control these secretory processes. In contrast to the considerable number of proteins that act in both the SV and DCV secretory pathways, very few proteins apart from DCV cargo proteins are specifically involved in the secretion of only one type of vesicle. One such protein is the calcium-activated protein for secretion UNC-31/CAPS, which has been proposed to play a restricted role in DCV exocytosis (BERWIN et al. 1998; FUJITA et al. 2007; GRISHANIN et al. 2004; SPEESE et al. 2007; SPEIDEL et al. 2005). Another example is PKC-1, which has been suggested to selectively regulate DCV release in C. elegans (SIEBURTH et al. 2007). Thus, the identification of novel regulatory components involved in the secretory machinery remains critical to dissecting the molecular mechanisms that control vesicle release pathways.

Several members of the Rab family have been implicated in the control of secretory granule exocytosis in distinct systems. Specifically, Rab3 and/or Rab27 have been demonstrated to coordinate release of DCVs from PC12 cells (TSUBOI and FUKUDA 2006), insulin granules (YAEKURA et al. 2003; YI et al. 2002), and other types of secretory vesicles (MAHONEY et al. 2006a; ZHAO et al. 2002). These Rab proteins regulate these events through interactions with distinct effectors including Slac2 (FUKUDA and KURODA 2002), granulophilin (YI et al. 2002) and rabphilin (FUKUDA et al. 2004; MAHONEY et al. 2006a). For example, in PC12 cells, rabphilin interacts with SNAP-25 providing a physical link between Rab proteins and the DCV fusion machinery (TSUBOI and FUKUDA 2005).
In a genetic screen for mutants mislocalizing a GFP-tagged form of the RAB-27 effector rabphilin (Mahoney et al. 2006a; Staunton et al. 2001), we isolated a novel allele of hid-1. This novel gene originally identified by Ailion and Thomas (2003) encodes a protein highly conserved from worms to humans. In the present work, we combine studies in C. elegans and PC12 cells to demonstrate that HID-1 is a potentially myristoylated protein that associates with secretory compartments. Furthermore, our studies on neuronal and intestinal systems in C. elegans provide evidence that HID-1 is required for proper function of the peptidergic branch of the secretory signaling network.
MATERIALS AND METHODS

Growth and culture of Caenorhabditis elegans

*C. elegans* cultures were grown at 22.5°C on solid medium as previously described (SULSTON and HODGKIN 1988). Aldicarb (2-methyl-2-[methylthio]proprionaldehyde 0-[methylcarbamoyl]-oxime; Chem Services, West Chester, PA) (100 mM in 70% ethanol) or levamisole (100mM in water) was added to the agar growth medium at the appropriate concentration after autoclaving.

The wild type reference strain was N2 Bristol. The mutant strains used were *aex-3(js815), aex-6(sa24), egl-3(n150), egl-3(ok979), egl-21(n476), hid-1(sa691), hid-1(sa722), ida-1(ok409), rab-3(js49), unc-13(s69), unc-31(e928), unc-104(e1265) and unc-108(n415).* Double mutants were constructed using standard crosses and confirmed by sequencing or PCR analysis of the molecular lesions.

hid-1 mapping

*hid-1(js828)* was isolated in an genetic screen for mutants that mislocalize a GFP-tagged version of the RAB27 effector RBF-1 rabphilin (STAUNTON et al. 2001). *js828* was mapped on the X chromosome and positioned to between -16.26 and -16.19 by single nucleotide polymorphism analysis of *js828* mutant animals derived from an outcross to the polymorphic strain Hawaiian CB4856 (WICKS et al. 2001). *js828* failed to complement *hid-1(sa722)* for the GFP-RBF-1 mislocalization and Unc phenotypes suggesting *js828* was a new allele of *hid-1.* Sequencing of the coding regions of *hid-1* (K02E10.2a) identified two lesions in *hid-1(js828)*; a C to T transition in the second base of codon 274 and G to T transversion in the first base of codon 379 which result in Thr279Ile and Gly379Stop changes in the protein coding sequence.

Transgenic animals
Microinjection was performed as described previously (Mello *et al.* 1991). Plasmid DNAs were purified using Qiagen (Valencia, CA) columns following the protocol of the manufacturer. Plasmid DNA was co-injected with pJM23 *lin-15(+) DNA into lin-15(n765) hid-1(sa722).* Transformed animals were identified as non–Lin progeny of injected animals; the progeny were grown at 22.5°C. The following transgenic strains bearing novel extrachromosomal arrays were created:

**NM 3017 hid-1(sa722) lin-15(n765); jsEx896 (hid-1p-HID-1-GFP)**

**NM3053 hid-1(sa722) lin-15(n765); jsEx897 (rab-3p-HID-1-GFP)**

**NM3139 hid-1(sa722) lin-15(n765); jsEx909 (ges-1p-HID-1-GFP)**

**NM3063 hid-1(sa722) lin-15(n765); jsEx898 (hid-1p-HID-1(G2N)-GFP)**

**NM3148 hid-1(sa722) lin-15(n765); jsEx910 (rab-3p-HID-1(G2N)-GFP)**

**NM3159 jsIs423[rbp-1p,RBF-1-GFP]** was identified as a spontaneous integration of *jsEx86* (Staunton *et al.*, 2001).

**EG3680 oxIs206[aex-3pANF-GFP]** was kindly provided by Erik Jorgensen (Speese *et al.* 2007).

**NM3576 jsIs1072 (vha-6p-AEX-5-VENUS)** was previously described (Mahoney *et al.* 2008).

**KG2361 nuls183 [unc-129-NLP-21-Venus]** was previously described (Sieburth *et al.*, 2007).

*Plasmid constructions*

All molecular manipulations were performed using standard methods. Novel plasmid junctions, PCR amplified coding sequences and site-directed mutagenesis lesions (Fisher and Pei 1997) were all sequenced.

**NM1699 hid-1** promoter driving genomic *hid-1* fused to eGFP.
Plasmid TJ1353 contains the rescuing 8.6-Kb XhoI-BamHI genomic fragment from cosmid KO2E10 inserted into pBluescript KS(+) (AILION and THOMAS 2003). TJ1353 was modified by site-directed mutagenesis to create NheI and SalI sites between codons 551 and 552 of the hid-1 genomic sequence using the oligonucleotides 5’
CTACCATCTCGTCACGATTGTGCGACCTCGGTAGCTTAGCAGCTGCAACACATT 3’ and 5’
AATGTGTTGCAGCTGCTAAGCTAGCGAGGTCGACAATCGTGACGAGATCCTAC 3’. The coding sequence of eGFP was excised from pEGFP-C1 (Clontech, Inc.) with an NheI-SalI digest and inserted into NheI-SalI digested TJ1353.

NM1700 rab-3 promoter driving genomic hid-1 fused to eGFP.
A 1.3kb rab-3 promoter fragment was amplified from NM1141 using oligonucleotides
5’ATCGTGGTACCAGATTCATGATGGGAGCAGTG 3’ and 5’
CTTGTGACGCATCTAGTACAAGCTTTGCTAAAATCCACTCT GCT ACC CTG AGC ACC CAT CTG AAA ATA GGG CTA CTG TAG 3’, digested with KpnI and AatII and used to replace the KpnI-AatII hid-1 native promoter fragment of NM1699.

NM1702 ges-1 promoter driving genomic hid-1 fused to eGFP.
A 2.15 kb ges-1 promoter fragment was amplified from NM1404 using oligonucleotides 5’
ATCGTGTTACCCGATTTCAAACTGATACTTAAG 3’ and 5’
CATGTGACGTACATCTAGTACAAGCTTTGCTAAAATCCACTCTGCTACCCTGACGCACCCAT CTGAATTCAAAGATAAGATATGTAATAG 3’, digested with KpnI and AatII and used to replace the KpnI-AatII hid-1 native promoter fragment of NM1699.
NM1703 hid-1 promoter driving genomic hid-1(G2N) fused to eGFP.

In NM1699, a glycine to asparagine mutation at position 2 was generated by site-directed mutagenesis using the oligonucleotide 5’
GAAAAAAAATGAATGCTCAGGGTAGCAGAGTGGATTTTAAGCAAG 3’.

NM1704 rab-3 promoter driving genomic hid-1(G2N) fused to eGFP.

In NM1700, a glycine to asparagine mutation at position 2 was generated by site-directed mutagenesis using the oligonucleotide 5’
CCCTATTTTCAGATGAATGCTCAGGGTAGCAGAGTGGATTTTAAGC 3’

NM1720 Murine HID-1 in a mammalian expression vector.

A mouse HID-1 cDNA was amplified from a brain cDNA preparation using the oligonucleotides 5’
AATGAGCTAGCCGCCACCATGGGATCCGCAGACTCCAAGCTGAACTTC 3’ and 5’
TAGCTTGAGCTGCACACCCTGGATCTCGATCTCAAACA C 3’, digested with NheI and SacI and inserted in pEGFP-C1 (Clontech, Inc.) replacing eGFP with HID-1. The cDNA clone was sequenced and the coding region is identical to a RIKEN cDNA (Genbank Accession AK138882).

NM1721 Murine HID-1-GFP in a mammalian expression vector.

NM1720 was modified by site-directed mutagenesis to create the AgeI and BsrGI sites between codons 585 and 586 of mouse HID-1 using the oligonucleotide 5’ CGGAGGACACCAGACCCGCTGGATCTCAAACA C 3’, and eGFP was excised from pEGFP-C1 (Clontech, Inc.) with an AgeI-BsrGI digest and inserted into HID-1.
NM1725 Murine HID-1(G2N) fused to eGFP in a eukaryotic expression vector.

NM1721 was modified by site-directed mutagenesis using the oligonucleotide 5’
GCTAGCCGCCACCATGAATGCCGCAGACTCAAGCTGAACTTCCG 3’ to generate a glycine
to asparagine mutation at amino acid 2 of mouse HID-1.

Aldicarb and levamisole assays

Animals were assayed for acute exposure to aldicarb and levamisole (MAHONEY et al. 2006b). In
brief, we assayed the time course of paralysis after exposure of a population of animals to these drugs.
In each experiment, 25-30 worms per genotype were placed on drug plates, and paralysis was assessed
by prodding animals with a platinum wire. Worms that did not respond were classified as paralyzed.

Defecation and locomotion behavioral assays

The posterior body wall muscle contraction (pBoc), anterior body wall muscle contraction
(aBoc) and expulsion (Exp) steps of the defecation cycle were monitored using the timer program Etho
written by J. H. Thomas (University of Washington). The percent of enteric muscle contractions
(EMC) was calculated as the fraction of defecation cycles that contained a pBoc without an Exp,
divided by the total number of pBoc minus 1. Locomotion assays were performed as previously
described (REYNOLDS et al. 2005; SCHADE et al. 2005)

RNAi experiments

RNAi experiments were performed based on feeding protocol (FIRE et al. 1998) modified by Min-Ho
Lee (Washington Univ. School of Medicine). Three L4 worms were transferred onto NGM plates
containing 50 µg/ml ampicillin and 1mM IPTG seeded with E. coli (HT115/DE3) that express double-
stranded RNA targeting hid-1 gene. After three days of incubation at 22°C, 10 F1 young adults
progeny were scored for their Exp defects. Animals fed with bacteria harboring an empty feeding vector were used as control.

Egg retention quantification

L4 stage hermaphrodites were picked onto a plate, left for 40-42 hours and then individually dissected through the body midline, using a tuberculin needle to allow for the release of eggs stored in the uterus, as described (HERMAN et al. 1999). The embryos were then counted and classified according to their developmental stage.

Immunohistochemistry

Whole-mount immunohistochemistry was performed using Bouin’s fixative (NONET et al. 1997). Animals were stained with anti-RAB-3 (NONET et al. 1997), anti-UNC-10 (KOUSHIKA et al. 2001), anti-RAB-27 (MAHONEY et al. 2006a), anti-SNT-1 (NONET et al. 1993), anti-SNB-1 (NONET et al. 1998), and anti-UNC-64 (SAIFEE et al. 1998) polyclonal antibodies and the anti-UNC-17 monoclonal mAb1403 (DUERR et al. 2001). To visualize native neuropeptides we used anti-FMRFamide antiserum kindly provided by C. Li (SCHINKMAN and LI 1992) at 1/2000 and the immunostaining was performed as previously described (EDWARDS et al. 2009). Primary antibodies were visualized with goat anti-mouse IgG Alexa 488 and goat anti-rabbit IgG Alexa 568 (Molecular Probes, Eugene, OR) used at a 1:1000 dilution.

Anti-UNC-29 (KM27A-3.1) is a goat polyclonal antibody raised against GST-UNC-29 (amino acids 348-431). The antibody was affinity purified using purified GST-UNC-29 fusion protein coupled to Aminolink Plus (Pierce) after presorbing 10 mls of serum to 10 mg of pure GST to remove GST-specific antibodies. GST and GST-UNC-29 fusions were produced using methods similar to those previously described (CHARLIE et al. 2006b). To confirm the specificity of the UNC-29
antibody, we tested it on *unc-29(x29)* null mutants. Triple immunostaining of whole animals, imaging, and deconvolution were performed as described (Charlie et al. 2006a; Charlie et al. 2006b).

**Western Analysis**

Western analysis was performed as previously described (Weimer et al. 2003). Briefly, L1-rich samples of each strain were collected (100µl of packed worms) in 500 µl of SHP buffer followed by four 5 second sonication bursts. SHP buffer (360 mM sucrose, 12 mM HEPES solution was supplemented with protease inhibitors (500 X PI cocktail: 2.93 mM leupeptin, 2.07 mM antipain, 1.65 mM chymostatin, 1.82 mM pestatin A, 6.25 mM PMSF). The lysates were spun down at 600X g for 15 minutes to discard the cuticle, nuclei and large cellular debris. The supernatants were centrifuged at 100,000X g for 2 hours at 4°C. Pellets were resuspended in a volume of SHP equal to the supernatant. Comparable amounts of the pellet and supernatant were loaded onto SDS-PAGE gels, and transferred to nitrocellulose membranes (Osmonics, Minnetonka, MN). Western blots were performed using standard methods using an ECL detection kit (Amersham, Piscataway, NJ). Primary rabbit anti-GFP antibody (a gift of Maureen Linder and Phyllis Hanson, Washington University School of Medicine) and rabbit anti-SNB-1 antibody (Nonet et al. 1998) were used at a 1:500 and at a 1:10,000 final dilutions, respectively.

**Cell culture, Transfection and Imaging of PC12 cells.**

PC12 cells were cultured (60-80% confluence) on 35-mm glass-bottom dishes coated with poly-L-lysine (Sigma) in Dulbecco’s modified Eagle’s medium (DMEM) containing 5% horse serum, 5% supplemented calf serum (HyClone, iron supplemented) and 3% fetal bovine serum at 37°C under
5% CO₂. Cells were washed once with serum-free DMEM and transfected with 1 µg of each purified plasmid DNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Before confocal microscopy analysis, cells were fixed in 3% paraformaldehyde in PBS containing 1 mM MgCl₂, 0.2 mM CaCl₂ (PBS-CM) for 20 min, and incubated for 15 min at room temperature in the same buffer containing 50 mM NH₄Cl. PC12 cells were imaged on a Olympus 500 confocal microscope using a 100X oil objective. Confocal images were processed using Metamorph software (Universal imaging).

*Image analysis and quantification*

Image analysis and quantification of puncta fluorescence in the dorsal cord was performed largely as previously described (SIEBURTH *et al.* 2005). *rab3p*-HID-1-GFP and *aex-3p*-ANF-GFP imaging experiments were carried out using an Olympus BX60 compound microscope equipped with an X-CITE 120 (EXFO) metal halide light source, a standard long pass GFP filter, and a Retiga EXi cooled CCD camera (Qimaging). Line scans of dorsal cord fluorescence were analyzed using Metamorph 4.5 software (Universal imaging). *aex-3p*-ANF-GFP imaging experiments of the dorsal nerve cord and coelomocytes were carried out using an Olympus BX51, equipped with a digital camera Micropublisher 3.3 RTV (JH Technologies, San Francisco, CA, USA). Images of coelomocytes from animals expressing ANF-GFP were taken and analyzed as previously described (MAHONEY *et al.* 2008). Images of *rab3p*-HID-1-GFP and *aex-3p*-ANF-GFP in the dorsal nerve cord were captured using a 60X oil objective. Images of *aex-3p*-ANF-GFP in coelomocytes were captured using a 40X objective. Quantification of AEX-5-VENUS was performed as described previously (MAHONEY *et al.* 2008).
Images of the dorsal nerve cord and neurons from immunostained adults for FMRFamide peptides were taken and analyzed as above described for live animals expressing ANF-GFP. Images from DA6 and DB6 motorneuron axons in the region immediately posterior to the vulva were collected at 22°C using a Nikon Eclipse TE2000-E microscope, a Nikon CFI Apo TIRF 100X/1.49 N.A. objective, an X-Cite illuminator (EXFO, Montreal, Canada) and an ORCA-AG camera (Hamamatsu, Bridgewater, NJ) controlled by Metamorph Premier software. Images were processed as previously described (EDWARDS et al. 2009)
RESULTS

hid-1 mutants disrupt neurosecretion

hid-1 mutants were originally isolated in a screen for animals that form dauer larvae constitutively at high temperature (AILION and THOMAS 2003). We isolated a novel hid-1 allele, js828, in a genetic screen for mutants that mislocalize a GFP-tagged version of the RAB-27 effector RBF-1 rabphilin (MAHONEY et al. 2006a; STAUNTON et al. 2001). Ailion and Thomas (2003) previously described hid-1 mutants as lethargic, moderately constipated, touch insensitive and uncoordinated during locomotion. In addition, we detected defects in egg laying behavior (Table 1).

To assess neurotransmission in hid-1 mutant animals we quantified their sensitivity to the acetylcholinesterase inhibitor aldicarb (MAHONEY et al. 2006b; NGUYEN et al. 1995). All of the hid-1 alleles examined (js828, sa722, sa691) were resistant to moderate levels of aldicarb (Figure 1A). They exhibited a sensitivity to aldicarb intermediate between wild type and aex-3(js815), a Rab exchange factor mutation that produces severe aldicarb resistance (IWASAKI et al. 1997; MAHONEY et al. 2006b). This indicates that cholinergic transmission is reduced in these hid-1 mutant animals.

To determine whether the hid-1 defect was presynaptic or postsynaptic we measured the sensitivity of hid-1 mutants to levamisole, an agonist of nicotinic acetylcholine receptors (AChR) in the nematode (FLEMING et al. 1997; LEWIS et al. 1980). hid-1 mutants showed increased sensitivity to levamisole compared to wild type and rab-3 mutant control strains (Figure 1B), the latter of which have a presynaptic defect in neurotransmitter release (NONET et al. 1997). However, this change is modest compared to the complete resistance shown by unc-29 AChR mutants (FLEMING et al. 1997; LEWIS et al. 1980). This change in sensitivity does not appear to be a consequence of altering postsynaptic levamisole receptors in muscle since we observed no detectable changes in the
distribution or the level of this receptor using UNC-29 antibodies (Figure 1C). Thus, loss of hid-1 disrupts normal neuromuscular signaling consistent with HID-1 regulating neurosecretion.

**HID-1 functions in both neurons and the intestine**

Promoter fusion data indicated that HID-1 is expressed primarily in neurons (AILION and THOMAS 2003). To define the cellular site of HID-1 function, we introduced GFP into a full-length hid-1 genomic clone. Since both the N- and C- termini of HID-1 are highly conserved from worm to human, we opted to introduce GFP in frame in a non-conserved domain in the middle of the protein coding sequence (see Materials and Methods for details). Expression of this fusion protein was observed in both wild type (not shown) and hid-1 mutants predominantly in the nervous system, the intestine, the pharynx (Figure 2A) and in uv1 secretory cells (data not shown). In the nervous system HID-1-GFP was primarily localized in the synapse-rich nerve ring, ventral cord and dorsal cord process bundles (Figure 2A). In the nerve cords HID-1-GFP was punctate in distribution (Figure 2A). GFP fluorescence was detected in neuronal cell bodies as well (Figure 2A). HID-1-GFP under the hid-1 promoter was capable of rescuing the uncoordinated movement (data not shown), aldicarb resistance (Figure 2B), the egg laying (Table 1) and the defecation defects (Figure 2D) of hid-1(sa722) null mutants.

To determine the site of HID-1 function in regulating these behaviors, we constructed tissue-specific hid-1 expression vectors. We included the intestine as a possible site of action for hid-1 because an intestinal-derived peptidergic signal has been shown to regulate defecation (MAHONEY et al. 2008). The rab-3 promoter was used for neuron-specific expression (NONET et al. 1997) and the ges-1 promoter for intestine-specific expression (AAMODT et al. 1991). Neuronal expression of HID-1-GFP in null mutants rescued the uncoordinated phenotype, the sensitivity to aldicarb (Figure 2B)
and levamisole (Figure 2C) and the egg laying defects (Table 1), while intestinal expression rescued the defecation defects (Figure 2D). In addition, bacterial feeding RNAi of hid-1 phenocopied the defecation defects (Figure 2D), but not the locomotory phenotypes of hid-1 mutants (data not shown), consistent with HID-1 acting in the RNAi-sensitive intestine to regulate the defecation motor program and the RNAi-resistant nervous system (Simmel et al. 2002; Tavernarakis et al. 2000) to regulate locomotion. These data indicate HID-1 functions presynaptically to regulate neurosecretion since the increase in aldicarb resistance and response to levamisole are the result of loss of neuronal HID-1 function. In summary, these data indicate that HID-1 operates both in the intestine and neurons to regulate distinct functions.

**HID-1 acts in a parallel pathway to RAB-27 and RAB-3 in neurosecretion**

We found that RBF-1-GFP was partially mislocalized from synaptic-rich regions to cell somas in hid-1(sa722) mutant animals (Figure 3A). Recently, RBF-1 rabphilin has been demonstrated to be localized to presynaptic sites through an interaction with aex-6, which encodes RAB-27 in *C. elegans* (Mahoney et al. 2006a). Interestingly, hid-1 and aex-6 RAB-27 mutants share many pleiotropic phenotypes; they have similar defecation and locomotion defects, form dauer larvae at high temperature and both genes appear to act in the insulin branch of the dauer pathway (Aillon and Thomas 2003; Thomas 1990). In addition, we found that hid-1 and aex-6 RAB-27 mutants are similarly resistant to aldicarb (Figure 3B). These data suggested that these genes might act in a common pathway. To test this hypothesis we constructed the double mutant between null alleles of hid-1 and aex-6 RAB-27. Unexpectedly, we observed strong synergistic phenotypes in the double mutant combination. The animals showed a drastic reduction in movement (not shown) and an increase in resistance to aldicarb compared to either single mutant (Figure 3B). Thus, these results
support the idea that hid-1 acts in a parallel pathway to aex-6 RAB-27 and further that both genes regulate neurosecretion.

In vertebrate systems rabphilin acts as an effector not only for rab27, but also for rab3 (FUKUDA 2003). In C. elegans, RAB-3 and RAB-27 GTPases are coordinately regulated by the guanine nucleotide exchange factor AEX-3 and are necessary for the proper synaptic transmission (MAHONEY et al. 2006a). In addition, RAB-3 has been found to have more influence than RAB-27 in the regulation of secretion/transport of INS-22 insulin/IGF in C. elegans (Ch'ng et al. 2008), a signaling ligand of a pathway in which HID-1 has been implicated (Ailon and Thomas 2003). Thus, HID-1 could function in the same pathway as RAB-3. To explore this possibility we assayed the aldicarb resistance of rab-3 (js49); hid-1(sa722) double mutants. While each single mutant showed an intermediate sensitivity to aldicarb, the rab-3(js49); hid-1(sa722) double mutant exhibited a severe resistance to aldicarb (Figure 3C), suggesting that HID-1 acts in parallel to the pathway regulated by RAB-3. In conclusion, these results are consistent with HID-1 operating in a pathway that is parallel to RAB-3 and RAB-27 in the regulation of neurotransmission.

C. elegans and murine HID-1 associates with membranes using a conserved N-terminal myristoylation motif

HID-1-GFP expressed under a neuronal promoter was observed in a punctate pattern in neuronal processes (Figure 2A and 5A). The HID-1-GFP expression was most concentrated in the nerve ring and could also be detected in neuronal cell bodies (Figure 2A). As the nerve ring rich signal was suggestive of synaptic localization, we tested whether HID-1-GFP localization shared other properties with synaptic vesicle associated proteins. In C. elegans, the anterograde transport of synaptic vesicle- and DCV-associated proteins is dependent on the kinesin UNC-104 (Hall and Hedgecock 1991; Jacob and Kaplan 2003; Zahn et al. 2004). Likewise, HID-1-GFP fluorescence
intensity accumulated exclusively in neuronal cell bodies in the *unc-104(e1265)* mutant (Figure 4A). Furthermore, western blotting of fractionated extracts of *neuronal* -HID-1-GFP expressing worms demonstrated that HID-1-GFP was associated exclusively with membranes (Figure 4C). These results are consistent with HID-1-GFP residing on a secretory vesicle or secretory vesicle precursor membrane. HID-1 was previously proposed to be an integral membrane protein based on its hydrophobicity profile (AILION and THOMAS 2003). However, computational analysis using SMART (LETUNIC et al. 2006) and Phobius (KALL et al. 2005) failed to identify transmembrane domain segments in *C. elegans* HID-1 and identified a variable number (none and 3, respectively) in mouse HID-1 though both proteins are relatively hydrophobic. Upon examination of the amino acid sequence of HID-1, we detected an N-terminal consensus motif Met-Gly-XXX-Ser/Thr for protein N-myristoylation (JOHNSON et al. 1994). To define the function of this motif, a glycine to asparagine mutation was generated to disrupt its potential functionality (UTSUMI et al. 2004). When the mutated protein was expressed in the *hid-1* null mutant, most of the *neuronal* -HID-1(G2N)-GFP was missorted. The GFP fusion showed a diffuse localization pattern and its expression was increased in cell bodies (Figure 4B). Consistent with these results, western blots of fractionated *neuronal* -HID-1(G2N)-GFP worm extracts revealed a portion of HID-1(G2N)-GFP in the soluble cytoplasm fraction (Figure 4C). In addition, the locomotion and the sensitivity to aldicarb of *neuronal* -HID-1(G2N)-GFP expressing *hid-1* mutants remained impaired suggesting that HID-1’s function was compromised (data not shown). Similar results were obtained when the HID-1(G2N)-GFP was expressed under the *hid-1* promoter (data not shown).

To further characterize the HID-1 protein, we expressed a full-length murine HID-1 cDNA (amplified from brain cDNA) in PC12 cells. The cDNA contained GFP inserted at the same internal sight shown to preserve function in worm HID-1. Murine HID-1, which is 55% identical to *C. elegans*
HID-1, associated with internal membrane structures. Tubules extending from these mHID-1-GFP labeled vesicular structures were frequently observed (Figure 4D) and detected in time-lapse experiments (data not shown). By contrast, no mHID-1-GFP was detected at the plasma membrane. When murine HID-1(G2N)-GFP, which is equivalent to the mutant in worms, was expressed in PC12 cells, a diffuse cytosolic pattern was observed (Figure 4D). These data provide further support that HID-1 associates with membrane structures through a N-myristoylation motif, although our data do not exclude the possibility that a minor portion of HID-1 is cytosolic. In summary, mammalian and worm HID-1 both associate with an internal secretory membrane compartment using a conserved N-myristoylation motif.

HID-1-GFP accumulates in secretion defective unc-13 and unc-31 mutants

In *C. elegans*, some exocytic defects are associated with abnormal accumulations of secretory vesicle markers at synaptic release sites (SIEBURTH et al. 2005). To better understand the function of HID-1 in neurosecretion, we examined the distribution of neuronal-HID-1-GFP in strains carrying loss-of-function mutations in genes encoding molecular components of the exocytosis machinery. These included *aex-1, aex-3, aex-5, aex-6, cab-1, rab-3, unc-13, and unc-31* (DOI and IWASAKI 2002; IWASAKI et al. 1997; IWASAKI and TOYONAGA 2000; MAHONEY et al. 2006a; NONET et al. 1997; RICHMOND et al. 1999; SIEBURTH et al. 2007). We found that the intensity of HID-1-GFP puncta in the dorsal cord was significantly increased in mutants lacking UNC-31/CAPS and UNC-13 (approximately 80% and 160% respectively; Figure 5). By contrast, no change in the distribution of HID-1-GFP was detected in other mutants used in this study (data not shown). Since UNC-31/CAPS is proposed to promote selectively the secretion of DCVs (ANN et al. 1997; BERWIN et al. 1998; SIEBURTH et al. 2007; SPEESE et al. 2007) and UNC-13 is thought to promote priming of synaptic
vesicles (RICHMOND et al. 1999; RICHMOND et al. 2001), this increase of puncta fluorescence of HID-1-GFP in *unc-31* and *unc-13* mutants suggests that HID-1 associates with some class(es) of exocytic vesicles, or precursor vesicles.

**HID-1 acts in a neuropeptide signaling pathway**

The dauer phenotype associated with HID-1 is consistent with a role in insulin secretion, a DCV-mediated event (KIMURA et al. 1997; LI et al. 2003). In addition *hid-1* mutants display phenotypes associated with alterations in peptide profile such as impaired locomotion, defecation defects and egg laying (HUSSON et al. 2007). We have seen HID-1-GFP accumulates in *unc-13* and *unc-31* CAPS mutants in the dorsal cord. This effect is similar to the accumulation of YFP-tagged neuropeptides in animals lacking PKC-1, which is a putative regulator of DCV secretion (SIEBURTH et al. 2007).

Therefore, following the hypothesis that HID-1 is involved in peptidergic signaling, we explored the relationship of *hid-1* and two genes encoding peptide- processing enzymes (*egl-3* proprotein convertase (PC2) and *egl-21*-carboxypeptidase (CPE)). A major action of neuropeptides is to modulate the efficiency of neurotransmitter release (GREENGARD 2001). In fact, mutations in either of the genes *egl-3* or *egl-21* cause aldicarb resistance (JACOB and KAPLAN 2003; KASS et al. 2001). Therefore, we analyzed the aldicarb responsiveness of *hid-1* double mutants containing mutations in *egl-3* or *egl-21*. Double mutants *hid-1(sa722); egl-3(n150)* showed similar phenotype to each single mutant in aldicarb sensitivity (Figure 6A). Similarly, the aldicarb resistance observed in the *hid-1(sa722); egl-21(n476)* double mutant was no greater than that of an *egl-21* mutant alone (Figure 6B). These results suggest that peptidergic signaling is likely already disrupted in *hid-1*, since the defects observed in *hid-1* are not additive with those of either *egl-3* or *egl-21*. These results are consistent
with the notion that HID-1 acts in the same pathway as EGL-3 and EGL-21-processed peptides to control neural activity.

**HID-1 mutants exhibit reduced levels of ANF-GFP fluorescence in the dorsal nerve cord and coelomocytes.**

To further investigate whether HID-1 is involved in neuropeptide signaling, we examined the expression of the rat prepro atrial natriuretic factor fused to GFP under the control of the neuronal aex-3 promoter (neuronalp-ANF-GFP) in hid-1 mutants. It has been demonstrated that neuronal ANF-GFP is widely expressed in the nervous system and is sorted, packaged, transported and processed into DCVs for release (SPEESE et al. 2007). In the dorsal nerve cord of hid-1 mutants, although a punctate pattern was conserved as in wild type, the GFP fluorescence intensity was significantly decreased (approximately 45%) (Figure 7A and B). This observation could reflect biogenesis defective or increased secretion of DCVs. To distinguish between these possibilities, we analyzed neuropeptide release. The level of secreted GFP-tagged ANF can be estimated by the fluorescence intensity in coelomocytes, which take up soluble GFP released from axons into the pseudocoelomic space (SPEESE et al. 2007). The fluorescence of coelomocytes in hid-1 mutants was significantly reduced compared to wild type (approximately 50%) (Figure 7 C and D) favoring the idea that HID-1 affects the production or content of DCVs. If this hypothesis is correct, blocking DCVs cargo secretion should not result in accumulation of synaptic ANF-GFP in hid-1 mutants. To test this idea we built the unc-31; hid-1 double mutant. It is known that in mutants lacking UNC-31, fluorescent tags derived from different neuropeptides accumulate in the DNC due to the participation of UNC-31 in the docking and release of DCVs (Figure 7 A and B) (SPEESE et al. 2007). Consistent with our prediction, impairing DCVs secretion did not restore the low levels of ANF-GFP fluorescence found in the axons of hid-1
single mutants (Figure 7 A and B). In addition, no significant changes were observed in the fluorescence of coelomocytes in *unc-31; hid-1* double mutants (Figure 7 C and D). These results suggest that the mutation of *hid-1* might affect the biogenesis, maturation, processing or trafficking of DCVs.

Our genetics experiments place HID-1 in the same pathway as EGL-3. This prohormone convertase processes peptide precursors in including neuropeptide fusion proteins such as NLP-21-YFP. In the wild type, a portion of the fluorescent tag of the NLP-21-YFP fusion is removed from DCVs during processing. In *egl-3* null mutants processing is blocked, the FP remains linked to NLP-21, remains in DCVs, and is transported to release sites in the axon (EDWARDS *et al.* 2009). Similar to the behavior of NLP-21-YFP, we observed ANF-GFP signal was also greatly increased in the dorsal nerve cord of *egl-3* mutants (Figure 7E and F). To further investigate the site of action of HID-1 with respect to the pro-protein convertase EGL-3, we analyzed the intensity of GFP signal derived from ANF-GFP in the dorsal nerve cord of *hid-1* mutant animals in absence of EGL-3. Although the intensity of the fluorescent signal of *egl-3(ok979); hid-1(sa722)* double mutants was slightly increased as compared with the *hid-1* single mutant, ANF-GFP levels remained much lower than in the wild type (Figure 7E and F). These results demonstrate that the peptidergic defects observed in *hid-1* are independent of the state of peptide processing.

To provide additional evidence that HID-1 regulates an aspect of the biogenesis, maturation or trafficking of a class of peptidergic DCVs, we turned to the intestine, which contains multiple different secretory pathways that release both peptidergic signals as well as yolk proteins (BEG *et al.* 2008; KIMBLE and SHARROCK 1983; MAHONEY *et al.* 2008). Previously we identified a peptidergic signal pathway using RAB-27 and the SNAP-25 homolog AEX-4, which selectively blocks a peptidergic
signal released from the intestine (MAHONEY et al. 2008). Using a AEX-5-VENUS prohormone convertase fusion coelomocyte uptake assay, we previously demonstrated that aex-4 SNAP-25 mutants reduce coelomocyte uptake and increase internal levels of AEX-5-VENUS (MAHONEY et al. 2008). Similarly, aex-6 RAB-27 mutants also cause a reduction using this indirect secretion assay (Figure 8A and B) indicating that RAB-27 is required for efficient exocytosis of the AEX-5-VENUS containing vesicle population. Surprisingly, we found that HID-1 mutants did not block AEX-5-VENUS secretion suggesting that hid-1 mutants do not directly block this pathway at the secretion point despite the fact that hid-1 mutants share many defecation defects with aex-4 and aex-6. Strikingly, the AEX-5-VENUS secretion defects of aex-6 were partially suppressed in an aex-6; hid-1 double mutant despite the fact that the behavioral defects are actually more severe in the double mutant (see Figure 3B). Taken together, the data indicate that in hid-1 mutants AEX-5-VENUS resides primarily in a class of secretory vesicles that are not in the RAB-27 regulated secretion pathway.

FMRFamide-like neuropeptides levels are reduced in hid-1 mutants

Our evidence suggests that HID-1 functions in DCV biogenesis or in early stages of DCVs maturation. Therefore, we reasoned that the reduction in the expression of ANF-GFP seen in the axons of hid-1 mutants should reflect the state of native neuropeptides. To explore the action of HID-1 on native processed neuropeptides we used an antibody that recognizes the processed form of FMRFamide-like neuropeptides in C. elegans (SCHINKMAN and LI 1992) and analyzed their expression in the dorsal nerve cord of hid-1 mutants. In agreement with our expectations, the levels of anti-FMRFamide immunoreactivity was significantly decreased in the dorsal nerve cord as compared with the wild type (Figure 9A and B). In contrast, in neuronal cell bodies we did not observe a reduction of FMRF-amide staining and interestingly we often observed increased accumulation of the neuropeptide.
(Figure 9C). Thus, our data is consistent with a role for HID-1 in the biosynthesis, sorting, processing or transport of endogenous neuropeptide.

**Relationship between HID-1 and other identified regulators of DCVs signaling**

Recent work has implicated several components in the regulated secretory pathway including the DCVs membrane protein IDA-1 and UNC-108 RAB-2. It has been postulated that IDA-1, detected in a restricted set of neurons, modulates acetylcholine release based on the aldicarb resistance of *ida-1* mutants (CAI *et al.* 2004). To investigate a possible genetic interaction between HID-1 and IDA-1 we assayed the aldicarb response of the *ida-1(ok409);hid-1(sa722)* double mutant. The single mutant *ida-1(ok409)* showed aldicarb resistance that was intermediate between wild type and the moderate aldicarb resistance of *hid-1(sa722)* single mutants (Figure 10A). However *ida-1(ok409); hid-1(sa722)* double mutants showed relatively strong aldicarb resistance that was additive when compared to each single mutant (Figure 10A). This suggests that HID-1 functions in a pathway distinct from the IDA-1 pathway.

Recent studies on *C. elegans* have reported UNC-108/RAB2 as a molecular component regulating the maturation of DCVs (EDWARDS *et al.* 2009; SUMAKOVIC *et al.* 2009). *hid-1* and *unc-108* mutants share common phenotypes; both are uncoordinated and show decreased fluorescence derived from YFP-tagged neuropeptides in both the dorsal nerve cord and coelomocytes. To further investigate the relation between HID-1 and UNC-108, we built the *unc-108 (nu415); hid-1(sa722)* double mutant and analyzed the locomotion phenotype and the fluorescence intensity of NLP-21-Venus in the dorsal nerve cord. The locomotion defect was more severe in the *unc-108 (nu415); hid-1(sa722) double mutant* than in either single mutant suggesting that HID-1 has one or more function that do not overlap with UNC-108 in the control of locomotion (Figure 10B). In both *unc-108* and
*hid-1* single mutants NLP-21-Venus levels were greatly reduced in the dorsal nerve cord, but the levels were not further reduced in *unc-108 (nu415); hid-1(sa722)* double mutants (Figure 10C, D). This suggests that UNC-108 and HID-1 have at least one shared function or that one mutant phenotype dominates over the other. In summary, based upon the synergistic defects observed with both *ida-1* and *unc-108* mutants we conclude *hid-1* regulates a process within the neuropeptidergic signaling pathway distinct from those regulated by either IDA-1 or UNC-108.
DISCUSSION

Regulated secretion of signaling molecules contained in DCVs plays an important role in both cell-cell and tissue-tissue communication in multicellular organisms. The overall knowledge about the DCV secretory pathway results largely from 50 years of investigation mostly performed in mammalian systems (MORVAN and TOOZE 2008). However, more recently new tools used in C. elegans have provided new insights in the dissection of this particular secretion pathway. For example in nervous system UNC-31/CAPS and PKC-1 have been shown to act selectively on DCV exocytosis, while IDA-1 and UNC-108/RAB-2 have been implicated in DCVs biogenesis and maturation, respectively (CAI et al. 2009; EDWARDS et al. 2009; SUMAKOVIC et al. 2009). Here we demonstrate that the novel membrane-associated protein HID-1 regulates early stages of the peptidergic signaling pathway.

**HID-1 is a membrane-associated myristoylated protein**

Several lines of evidence suggest that HID-1 associates with membranes. First, HID-1-GFP behaves as a membrane-associated protein during simple fractionation of worm extracts. Second, the normal localization of HID-1-GFP was dependent on the kinesin motor protein UNC-104 (HALL and HEDGECOCK 1991) suggesting that HID-1 associates with secretory vesicles (DCVs or SVs) and/or secretory vesicle precursor membranes. Third, mammalian HID-1-GFP associated with dynamic vesicular and tubular structures in PC12 cells.

How does HID-1 associate with membranes if it does not contain transmembrane domains? The presence of an N-terminal myristoylation consensus sequence in both C. elegans and vertebrate HID-1 strongly suggests that HID-1 is myristoylated in vivo. An absolute requirement for a glycine at position 2 has been demonstrated for protein N-myristoylation (ROCQUE et al. 1993; TOWLER et al. 1988). Consistent with myristoylation playing a functional role in HID-1, glycine 2 is required for C.
elegans HID-1 function in vivo. Furthermore, the HID-1(G2N) subcellular localization pattern in vivo in both C. elegans and PC12 cells is a diffuse localization pattern consistent with a cytosolic distribution. Lastly, HID-1(G2N) protein fractionates partially into the supernatant strongly indicating HID-1 is unlikely to be an integral membrane protein. We conclude that myristoylation localizes HID-1 to a specific endomembrane compartment.

Neuronal HID-1 is associated with the peptidergic arm of the secretion pathway

What membrane compartments does HID-1 localize to in vivo? Using markers for specific compartments in PC12 cells, we have not been able to identify a marker that co-localizes strongly with HID-1 (Table S1). Nevertheless, our data suggests that HID-1 associates with a compartment(s) in the neuropeptidergic branch of the secretory pathway. This conclusion is supported by several findings. First, hid-1 mutants display defects in phenotypes associated with inappropriate peptidergic functions such as altered dauer formation, uncoordinated locomotion, constipation and egg laying defects. Second, neuronal HID-1-GFP is expressed in both neuronal cell bodies and synaptic rich regions, appears in a punctate distribution in the synaptic rich ventral and dorsal nerve cords, and its localization is dependent on UNC-104. This pattern is similar to that seen for other components of DCVs such as UNC-31, FMRFamide (Phe-Met-Arg-Phe-NH2)-related peptides, EGL-3, EGL-21 and IDA-1 (GRACHEVA et al. 2007; HAMMARLUND et al. 2008; JACOB and KAPLAN 2003; KASS et al. 2001; ZAHN et al. 2004; ZAHN et al. 2001). Third, neuronal HID-1-GFP accumulates in the dorsal nerve cord when secretion from nerve terminals is disrupted in unc-13 and unc-31 mutants. This effect is similar to the accumulation of YFP-tagged neuropeptides in PKC-1 mutants, which is proposed to selectively regulate DCVs secretion (SIEBURTH et al. 2007). Fourth, our genetic interaction experiments between hid-1 and neuronal peptide processing genes egl-3 and
*egl-21* suggest that HID-1 regulates synaptic transmission through a mechanism involving the release of neuropeptides. Together these findings lead to the postulate that the HID-1-GFP positive, UNC-104-kinesin dependent vesicle population is part of the DCV rather than SV pathway.

Analysis of the behavior of dense core cargo in *hid-1* mutants suggests also that HID-1 acts before the secretion step of DCVs. First, the native FMRFamide neuropeptides and the intensity of the fluorescence derived from neuronally expressed NLP-21 and ANF-GFP are decreased at release sites in the DNC in *hid-1* mutants compared with wild type worms. This last observation is accompanied by a decrease in the fluorescence in coelomocytes suggesting that the release of ANF-GFP is reduced in *hid-1* mutants. Furthermore, blocking of peptidergic release using *unc-31* does not restore the level of fluorescently labeled peptide at release sites in the DNC in the *hid-1 unc-31* double mutant. Since *unc-31* mutants accumulate DCVs at synapses (Gracheva et al. 2007; Hammarlund et al. 2008), the data suggest that HID-1 acts prior to DCVs docking. Furthermore, the fact that in *hid-1* mutants the absence of EGL-3 does not restore axonal ANF-GFP to wild type levels, suggests that HID-1 either acts before the peptide processing that takes place during DCVs maturation or disrupts an aspect of cargo recognition, sorting or trafficking that reduces the flux peptidergic granules down the axon. However, the lack of defects in cuticle formation and yolk secretion (data not shown) argue against a broad function of HID-1 in regulating general ER or Golgi trafficking. Our findings do not exclude the possibility that HID-1 also functions at synapses, given the strong expression of neuronal HID-1-GFP in synaptic rich regions, its UNC-104 dependent localization, its punctate distribution in nerve cords and its accumulation at release sites in *unc-31* and *unc-13* mutants. In summary, our analyses of cargo behavior are most consistent with HID-1 regulating an early stage of DCVs maturation, such as biogenesis, acidification, maturation, or trafficking of DCVs.
HID-1 function in the intestine

The defecation motor program is a complex behavior involving multiple tissue-tissue signaling events (THOMAS 1990). We have shown that HID-1 functions in the intestine to regulate defecation suggesting HID-1 participates in an intestinal to muscle and/or an intestinal to neuron signaling event. The defecation cycle is a process strongly regulated by peptidergic signaling as evidenced by the role of the protein convertase aex-5, RAB-27 aex-6 and the aex-2 G-coupled receptor in the pathway (MAHONEY et al. 2008). Thus, HID-1 fits well as a regulator of a pathway that controls secretion of peptide-containing vesicles from posterior intestine that stimulates the aboc and aex steps of the defecation motor program. By contrast with neuronal cargo, intestinal AEX-5-GFP cargo release is not blocked in hid-1 mutants. However, this cargo does appear to be partially diverted from the RAB-27 dependent pathway in hid-1 mutants, as the block observed in rab-27 mutants is partially suppressed in rab-27 hid-1 double mutants. This would be consistent with HID-1 acting in an early step of sorting or maturation of DCVs. The robust constitutive yolk secretion pathway provides a likely alternate secretion pathway for cargo in this double mutant.

Relation of HID-1 and the RAB-3 and RAB-27 GTPases

We isolated hid-1 alleles in a screen for mutants that mislocalized neuronal RBF-1-GFP, an aex-6 RAB-27 effector in C. elegans. This mislocalization, in combination with the similarities in the phenotypes of aex-6 RAB-27 and hid-1, suggested the possibility that these genes function in the same pathway. However, several lines of evidence indicate that the rabphilin mis-localization may be ancillary to hid-1 function in regulating secretion. First, RAB-27 itself is not mislocalized in hid-1. Second, rbf-1 is not expressed in the intestine where aex-6 RAB-27 and hid-1 both function to
regulate defecation. Furthermore, hid-1 aex-6 double mutants exhibit substantially more severe phenotypes than either single mutant suggesting RAB-27 and HID-1 act at largely in parallel. Similarly, we found that in hid-1 rab-3 double mutants the aldicarb resistance and the locomotory defect (not shown) were increased compared to each single mutant. One attractive hypothesis is that HID-1, RAB-27 and RAB-3 regulate distinct classes of DCVs (or precursor compartments) and that RBF-1 redistribution in hid-1 mutants results from the alteration of a HID-1-associated internal membrane population marked by a Rab(s) distinct from RAB-27. In addition to rab27 and rab3, rabphilin has been documented to interact with rab8 and rab37 in vertebrate systems (FUKUDA 2003); homologs of each of these rabs are present in C. elegans (PEREIRA-LEAL and SEABRA 2001). Hence, multiple distinct rabs are implicated in the regulation of peptidergic signaling. Further characterization of the vesicle population(s) associated with HID-1 will be necessary to understand the mechanisms and consequences of rbf-1 mislocalization.

**Where does hid-1 function in the DCV pathway?**

HID-1 shares many phenotypes in common with several mutants lacking components regulating aspects of dense core maturation. Of particular note are the similarities between, unc-108 and hid-1, both of which are uncoordinated, aldicarb resistant and show decreased fluorescence derived from neuropeptides in both dorsal nerve cord and coelomocytes. However, evidence indicates that HID-1 acts in a different pathway than the process of DCV maturation controlled by UNC-108/RAB2. First, HID-1-GFP is detected in cell somas and synaptic rich regions. In contrast, endogenous UNC-108 and/or UNC-108 fusion proteins are concentrated mostly in neuronal cell bodies (EDWARDS et al. 2009; SUMAKOVIC et al. 2009). Second, genetic experiments place UNC-108/RAB2 in a different pathway from EGL-3 (EDWARDS et al. 2009; SUMAKOVIC et al. 2009), while our experiments indicate HID-1 and EGL-3 are acting in the same peptidergic signaling
pathway. Third, the levels of native processed neuropeptides are reduced in hid-1 mutants, while they are unaltered in unc-108. Fourth, the decrease of the neuropeptide-FP fusion in the dorsal nerve cord observed in unc-108 mutants is attributed to the loss of the fluorescent tags derived from neuropeptides after the peptide processing during DCVs maturation (Edwards et al. 2009; Sumakovic et al. 2009). In contrast, we have shown that the decrease in ANF-GFP in dorsal nerve cord in hid-1 mutants is minimally affected by the absence of the PC2 convertase EGL-3 activity. Thus, while hid-1 and unc-108 share many similar phenotypes, they likely act in different steps of neurosecretion.

Although we were unable to place HID-1 in a specific subcellular compartment in PC12 cells, interestingly we observed that the level of murine HID-1-GFP was minimal in compartments positive for the cis/medial Golgi protein Giantin. In contrast, we saw a partial colocalization with CI-M6PR, a marker of the trans-Golgi network (TGN) as well as several other post-Golgi compartments (Table S1 and Figure S2). Moreover, HID-1 shows some modest structural similarities with dymeclin (see Figure S1; El Ghouzzi et al. 2003), a Golgi protein which has been found to be involved in bidirectional trafficking within the Golgi apparatus (Dimitrov et al. 2009; Osipovich et al. 2008). Thus our data correlates well with the idea that the localization of HID-1-GFP seen in neuronal cell bodies in C. elegans corresponds to the trans Golgi apparatus.

Aspects of cargo trafficking are clearly disrupted in hid-1 mutants. The decrease of FMRFamide neuropeptides, NLP-21-Venus and ANF-GFP at release sites in the dorsal nerve cord and the failure of ANF-GFP to accumulate at release sites when DCV secretion is blocked in UNC-31 mutants both provide strong evidence for this. Mechanistically, the most likely possibilities are that one or more of DCVs formation/maturation, trafficking, or pro-protein processing are disrupted in hid-1. This idea is supported by the fact that interruption of the processing of peptide precursors does not
greatly affect the axonal ANF-GFP phenotype seen in *hid-1* mutants, suggesting that HID-1 may function before this crucial step in the formation of mature DCVs.

How new DCVs form and emerge from the TGN remains poorly understood. Granin proteins have been found to be important factors regulating DCVs biogenesis. The granin proteins aggregate at low pH and high calcium concentrations (Chanat and Huttner 1991) and are capable of interacting with TGN- membrane components and drive the sorting of other proteins destined for DCVs (Mahapatra et al. 2008; Montero-Hadjadje et al. 2009). It has been proposed that these protein aggregates, physically induce the TGN-membrane budding to form dense-core granules (Courel et al. 2010; Montero-Hadjadje et al. 2009). In this process HID-1 at the TGN might participate in the appropriate sorting of peptides into DCVs and/or in the induction of the DCVs budding. If these steps do not occur properly DCVs proteins may be overexposed and hydrolyzed by local enzymes at the TGN (Kim and Loh 2006; Koshimizu et al. 2010) or they may be mis-sorted to other compartments to be degraded. Our genetic experiments that place HID-1 in the same pathway interrupted by mutations of crucial proteins for generating functional neuropeptides in *C. elegans* are consistent with a role of *hid-1* in DCV formation or sorting, as is the behavior of AEX-5-VENUS cargo in the intestine of *hid-1* mutants. Furthermore, considering these data and the presence of neuronal HID-1-GFP both in axons and at synaptic release sites, it is also possible that HID-1 plays a role in the trafficking of DCVs from the TGN to post-Golgi processing compartments or to plasma membrane release sites.
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FIGURE LEGENDS

Figure 1: Synaptic function defects in hid-1 mutants

(A) hid-1 mutants are moderately resistant to aldicarb. Wild-type (open circle), hid-1(sa722) (closed circle), hid-1(sa691) (open box), hid-1(js828) (open triangle) and aex-3(js815) (closed box) animals were exposed to 1mM aldicarb and scored periodically for the percentage of animals still responding to gentle touch. Errors bars represent SEM.

(B) hid-1 mutants are hypersensitive to levamisole. Wild-type (open circle), hid-1(sa722) (closed circle) and rab-3(js49) (open box) animals were exposed to 100 µM levamisole and scored for the percentage of animals still responding to gentle touch. Errors bars represent SEM.

(C) UNC-29 distribution is not altered in hid-1 mutants: Wild-type (top) and hid-1(sa722) (bottom) immunostaining in the anterior regions of the ventral nerve cord. UNC-17 (red) and UNC-10 (blue) mark cholinergic synapses and active zones, respectively. UNC-29 (green) is usually concentrated at or near the UNC-10 puncta in both strains. No significant differences were observed in the UNC-29 pattern between wild-type and hid-1 mutant animals. Scale bar= 10 µm.

Figure 2: Sites of HID-1 function

(A) HID-1 is broadly expressed in secretory tissues. Top left panel: a young adult hid-1(sa722) animal expressing HID-1-GFP driven by the hid-1 promoter. Expression is seen in neuronal and intestinal cells. In the inset a solid arrowhead indicates expression in the nerve ring, an open arrowhead indicates expression in the anterior intestinal cells and an open arrow indicates expression in the pharynx. Scale bar=50 µm. Bottom left panel: expression of HID-1-GFP driven by the rab3 promoter in the nerve ring, the dorsal nerve cord and the ventral nerve cord of a young adult hid-1(sa722) animal. Scale bar=50 µm. Top right panel: punctate pattern of neuronal HID-1-GFP in the...
dorsal (DC) and ventral nerve cords (VC) of a young adult *hid-1(sa722)* animal. Scale bar= 40 µm.

Middle right panel: neuronal HID-1-GFP is shown concentrated in small spots (arrowheads) in cell bodies of motor neurons. Scale bar= 5 µm. Bottom right panel: neuronal HID-1-GFP expressed in nerve ring and cell bodies of the head neurons. The pharynx is outlined as an anatomical guide. Scale bar= 10 µm.

(B) The aldicarb resistance of *hid-1(sa722)* is rescued by expression of GFP-tagged HID-1 in neurons. Wild-type (open circle), *hid-1(sa722)* mutants (closed circle) and *hid-1(sa722)* mutants carrying the transgenes driving the expression of wild type HID-1-GFP under the *hid-1* promoter (open box), the pan-neuronal *rab-3* promoter (closed box), and intestinal *ges-1* promoter (star) were exposed to 0.5mM aldicarb and scored periodically for the percent of animals still responding to gentle touch. Errors bars represent SEM.

(C) The increased sensitivity to levamisole of *hid-1(sa722)* is rescued by expression of GFP-tagged HID-1 in neurons. Wild-type (open circle), *hid-1(sa722)* mutants (closed circle) and *hid-1(sa722)* mutants carrying a transgene driving the expression of HID-1-GFP in neurons under the *rab-3* promoter (closed box) were exposed to 100µm levamisole and scored periodically for the percent of animals still responding to gentle touch. Errors bars represent SEM.

(D) Rescue of the defecation defects of *hid-1(sa722)* mutants by tissue specific expression plasmids and replication of the defecation phenotype by *hid-1* RNAi. The percentage of enteric muscle contractions (EMC) in ten defecation cycles is shown on the y axis. *hid-1;HID-1, hid-1;neuronal*, *HID-1, or hid-1;intestinal*, *HID-1* indicates *hid-1(sa722)* animals carrying HID-1-GFP under a native, neuronal, or intestinal promoter, respectively. WT (RNAi vector) and WT (RNAi *hid-1*) indicate wild-type animals exposed to bacteria transformed with an empty RNAi vector as a control and an
RNAi vector targeting *hid-1*, respectively. Ten animals were observed for each strain. The error bars represent SEM.

Figure 3: HID-1 functions in parallel to *aex-6 RAB-27* and *RAB-3* in neurosecretion
(A) *hid-1* mutant animals partially mislocalize a GFP-tagged version of the RAB-27 effector RBF-1 rabphilin. Top panel: Image of an integrated GFP-tagged RBF-1 transgenic animal (*jsIs423*) showing primarily nerve ring staining. Bottom panel: RBF-1-GFP is partially mislocalized to neuronal cell bodies in *hid-1(js828)* mutant animals. The same defect was observed in *hid-1(sa722)* and *hid-1(sa691)* mutants. Scale bar=20 µm.

(B) The combination of *hid-1* and *aex-6 RAB-27* mutations causes a severe resistance to aldicarb. Wild-type (open circle), *hid-1(sa722)* (closed circle), *aex-6(sa24)* RAB-27 (open triangle) and *aex-6(sa24) RAB-27; hid-1(sa722)* double mutants (closed box) were exposed to 1mM aldicarb and scored periodically for the percent of animals still responding to gentle touch. Errors bars represent SEM.

(C) The combination of *hid-1* and *rab-3* mutations causes a severe resistance to aldicarb. Wild-type (open circle), *hid-1(sa722)* (closed circle), *rab-3(js49)* (open box) and *rab-3(js49); hid-1(sa722)* double mutants (closed box) were exposed to 1mM aldicarb and scored periodically for the percent of animals still responding to gentle touch. Errors bars represent SEM.

Figure 4: HID-1 is a potentially myristoylated protein associated with membranes.
(A) HID-1-GFP localization is UNC-104 dependent. Left panel: Image of GFP-tagged HID-1 localized in the synaptic-rich nerve ring of a *hid-1(sa722)* mutant animal. Right panel: HID-1-GFP is mislocalized to the ganglia of neuronal cell bodies surrounding the nerve in *unc-104(e1265)* mutant animals. An arrow points to a representative cell body. Scale Bar= 10 µm.
(B) HID-1(G2N)-GFP mutant protein is missorted in the nervous system. Left panel: image of the ventral nerve cord of a *hid-1(sa722)* animal showing the punctate pattern of wild type HID-1-GFP expressed in neurons. Right panel: The expression of neuronal HID-1(G2N)-GFP (mutated in its potential myristoylated site) in the ventral nerve cord of *hid-1(sa722)* animals shows a diffuse localization pattern and a redistribution to neuronal cell bodies. An arrow points to a representative cell body. Scale bar=10 µm.

(C) Neuronal HID-1-GFP is associated with membrane fractions. *C. elegans* extracts were sonicated and separated by ultracentrifugation. Comparable amounts of the supernatant (S) and pellet (P) were analyzed by immunoblotting with anti-GFP and anti-SNB-1. Neuronal HID-1-GFP was exclusively membrane-associated, while a portion of neuronal HID-1(G2N)-GFP was recovered in the soluble fraction. The vesicular marker SNB-1 synaptobrevin was used as a control for proteins retained in the pellet. The data shown is representative of four independent experiments with similar results.

(D) Murine HID-1 is a potentially myristoylated protein associated with membranes

PC12 cells were transfected with a mHID-1-GFP construct and 24 hrs later the distribution of GFP fluorescence was analyzed. Left panel: wild type murine HID-1 protein associates with internal membrane structures. Tubules extending from these murine HID-1-labelled vesicular structures were frequently observed. Inset show tubular projections attached to a vesicle. Right panel: the distribution of the murine HID-1(G2N)-GFP mutant is diffused. Scale bar=5µm.

Figure 5: Neuronal HID-1-GFP accumulates in *unc-13* and *unc-31* mutants

(A) neuronal HID-1-GFP distribution in the dorsal cord of *lin-15(n765),unc-31* CAPS; *lin-15(n765) and unc-13(s69); lin-15(n765)* mutant animals. Scale bar=10 µm.
(B) Quantification of neuronal HID-1-GFP puncta fluorescence. The number of worms analyzed are as follows: lin-15(n765)=19, unc-31 CAPS; lin-15(n765) =13, unc-13(s69); lin-15(n765) =8. Asterisks indicate a significant difference from the lin-15 control for p<0.001 (Student’s t-test). AU, arbitrary units. Errors bars represent SEM.

Figure 6: HID-1 acts in a neuropeptide processing pathway

(A) Double mutants containing mutations in hid-1 and egl-3 PC2 show similar aldicarb resistance to each single mutant. Wild-type (open circle), hid-1(sa722) (closed circle), egl-3(n150) (open box) and egl-3(n150); hid-1(sa722) double mutants (closed box) were exposed to 1mM aldicarb and scored periodically for the percent of animals still responding to gentle touch. Errors bars represent SEM.

(B) Double mutants containing mutations in hid-1 and egl-21 CPE show similar aldicarb resistance to each single mutant. Wild-type (open circle), hid-1(sa722) (closed circle), egl-21(n476) (open box) and egl-21(n476); hid-1(sa722) double mutants (closed box) were exposed to 1mM aldicarb and scored periodically for the percent of animals still responding to gentle touch. Errors bars represent SEM.

Figure 7: hid-1 mutants exhibit reduced levels of ANF-GFP fluorescence in the dorsal nerve cord and coelomocytes.

(A) Representative images of the ANF-GFP derived fluorescence in the dorsal nerve cord of wild-type, unc-31 (e928), hid-1(sa722) and unc-31(e928); hid-1 mutant animals. Scale bar = 5µm.

(B) Quantification of fluorescence normalized to wild type in the dorsal nerve cord. The number of worms analyzed are as follows: wt=20, hid-1(sa722)=17, unc-31(e928); hid-1=15, unc-31(e928)=10.
Asterisks indicate significant difference from the wild type control for \( p<0.001 \) using a Student’s t-test. Errors bars represent SEM (Standard Error of the Mean).

(C) Representative images of the ANF-GFP derived fluorescence in posterior coelomocytes of wild-type, \( unc-31(e928) \), \( hid-1(sa722) \) and \( unc-31(e928); hid-1 \) mutant animals. Scale bar = 5µm.

(D) Quantification of fluorescence, normalized to the wild type, measured from posterior coelomocytes of young adults worms expressing neuronal ANF-GFP. The number of worms analyzed are as follows: wt =50, \( hid-1(sa722) = 48 \), \( unc-31(e928); hid-1 = 15 \), \( unc-31 (e928) = 10 \). Asterisks indicate significant difference from the wild type control for \( p<0.001 \). Student’s t-test. Errors bars represent SEM (Standard Error of the Mean).

(E) Representative images of the ANF-GFP derived fluorescence in the dorsal nerve cord of wild-type, \( hid-1(sa722) \), \( egl-3(ok979) \) and \( egl-3(ok979);hid-1(sa722) \) mutant animals. Scale bar = 5µm.

(F) Quantification of fluorescence in the dorsal nerve cord normalized to the wild type. The number of worms analyzed are as follows: wt=25, \( hid-1(sa722)=23 \), \( egl-3(ok979) = 21 \), \( egl-3(ok979);hid-1=22 \). Asterisks indicate significant difference from the wild type control for \( p<0.001 \) using a Student’s t-test. Errors bars represent SEM.

Figure 8  \textit{hid-1} mutants alter intestinal secretion patterns

(A) Representative DIC (top) and fluorescent (bottom) images of the AEX-5-VENUS derived fluorescence in coelomocytes of wild-type, \( aex-6, hid-1(sa722) \) and \( aex-6; hid-1 \) mutant animals. Coelomocytes are outlined in the DIC image with a dashed white line. Scale bar = 5µm.

(B) Quantification of fluorescence measured from coelomocytes of young adults worms expressing intestinal expressed AEX-5-VENUS. The number of worms analyzed are as follows: wt =13, \( aex-
6=16, hid-1=15, aex-6; hid-1=15. Asterisks indicate significant difference among the pairs at $p < 0.001$. Student’s t-test.

Figure 9: hid-1 mutants exhibit reduced levels of FMRFamide neuropeptides in the dorsal nerve cord. A) Representative images of anti-FMRFamide immunostaining in the dorsal nerve cord of wild-type and hid-1(sa722) mutant animals. Scale bar=5\(\mu\)m

B) Quantification of fluorescence in dorsal nerve cord normalized to wild type. The number of worms analyzed are as follows: wt=30, hid-1(sa722)=15. Asterisks indicate significant difference from the wild type control for $p<0.001$ using a Student’s t-test. Errors bars represent SEM.

C) Images of anti-FMRFamide immunostaining in the cell bodies of neurons located in the left lateral posterior ganglia of wild-type and hid-1(sa722) mutant animals. Arrows indicate the increased accumulation of anti-FMRFamide immunostaining observed in hid-1 mutants compared with wild type. Scale bar=5\(\mu\)m

Figure 10: Relationship between HID-1 and other regulators of DCVs signaling

(A) The combination of hid-1 and ida-1 mutations increases the sensitivity to aldicarb compared to each single mutation. Wild-type (open circle), hid-1(sa722) (closed circle), ida-1(ok409)(open box) and ida-1(409); hid-1(sa722) double mutants (closed box) were exposed to 0.5 mM aldicarb and scored periodically for the percent of animals still responding to gentle touch. Errors bars represent SEM.

(B) The combination of hid-1 and unc-108 mutations causes an increase in the locomotion defect compared with each single mutation. Spontaneous (unstimulated) locomotion rates of wild-type
compared with *hid-1(sa722), unc-108(nu415) and unc-108(nu415); hid-1(sa722)* mutant animals. Errors bars are SEMs of 10 animals per genotype. Triple asterisks indicate that the difference relative to the wild type is significant with a P value of <0.002. P values are from the unpaired t tests with Welch corrections.

(C) Representative images of Venus-tagged NLP-21 neuropeptides in DA/DB motor neuron axons in wild-type, *unc-108(nu415), hid-1(sa722) and unc-108(nu415); hid-1(sa722)* mutant animals. Images are scaled identically for brightness. Scale bar=1µm.

(D) Quantification of NLP-21-Venus fluorescence in the dorsal axons of the indicated genotypes. Graph shows the total integrated fluorescence per micron of dorsal axon length. Data are means and SEMs from images acquired from 11-13 animals per genotype. Triple asterisks indicate that the difference relative to the wild type is significant with a P value of <= 0.001. P values are from the unpaired t test with Welch correction.
Table 1: *hid-1(sa722)* mutants show defective egg laying phenotype

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Embryos in utero (mean)$^1$</th>
<th>Percent worms with late stage embryos in utero$^2$</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2</td>
<td>15.23 ± 0.87</td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td><em>hid-1</em>(sa722)</td>
<td>22.43 ± 1.0</td>
<td>63.2</td>
<td>36</td>
</tr>
<tr>
<td><em>hid-1</em>;HID-1-GFP</td>
<td>14.48 ±0.77</td>
<td>1</td>
<td>27</td>
</tr>
<tr>
<td><em>rab-3</em>;HID-1-GFP</td>
<td>14.0 ± 0.73</td>
<td>0</td>
<td>24</td>
</tr>
</tbody>
</table>

$^1$L4 stage hermaphrodites of each genotype were aged for 40-42 hours and individually dissected to release embryos from the uterus. The average number of embryos is shown (mean ±S.E.M.).

$^2$The developmental stage of the embryos was assessed under a dissecting scope and those at the comma stage and older were classified as late-stage embryos.