Alteration in cellular acetylcholine influences dauer formation in Caenorhabditis elegans

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Altered acetylcholine (Ach) homeostasis is associated with loss of viability in flies, developmental defects in mice, and cognitive deficits in human. Here, we assessed the importance of Ach in Caenorhabditis elegans development, focusing on the role of Ach during dauer formation. We found that dauer formation was disturbed in choline acetyltransferase (cha-1) and acetylcholinesterase (ace) mutants defective in Ach biosynthesis and degradation, respectively. When examined the potential role of G-proteins in dauer formation, goa-1 and egl-30 mutant worms, expressing mutated versions of mammalian Gα and Gβ homolog, respectively, showed some abnormalities in dauer formation. Using quantitative mass spectrometry, we also found that dauer larvae had lower Ach content than did reproductively grown larvae. In addition, a proteomic analysis of acetylcholinesterase mutant worms, which have excessive levels of Ach, showed differential expression of metabolic genes. Collectively, these results indicate that alterations in Ach release may influence dauer formation in C. elegans. [BMB Reports 2014; 47(2): 80-85]

INTRODUCTION

Facing adverse conditions such as deprivation of foods, increased population density and heat stress, C. elegans enters a non-aging state termed dauer larvae (1). Previous reports have shown that sensory neuronal function is also important for dauer formation (2). During dauer formation (3), communication among neuronal pathways, including sensory neurons, interneurons, as well as neurosecretory cells, is likely to utilize neurotransmitters, such as acetylcholine (Ach). Indeed, Ach is reported to be important for the dauer recovery pathway (4).

Ach was the first neurotransmitter identified and has since been shown to play an important role in both the peripheral nervous system (PNS) and central nervous system (CNS) in many organisms (5). Ach is synthesized from choline and acetyl-CoA in cholinergic neurons by the enzyme choline acetyltransferase (ChAT), and is loaded into synaptic vesicles by various transporters. Upon presynaptic activation, vesicles fuse with the plasma membrane and release Ach into the synapse. The released Ach acts through Ach receptors (AChRs) on post-synaptic neurons to generate action potentials. Synaptic Ach is ultimately cleared by the action of acetylcholinesterase (AChE), a synaptically abundant enzyme that degrades Ach into the inactive metabolites choline and acetate. Each step in this process is essential for proper synaptic function (6). Ach directly activates muscles in the PNS, but is involved in learning and short-term memory in the CNS. A defective ChAT leads to episodic apnea, a form of congenital myasthenic syndrome characterized by use-dependent fatigue (7). A decline in ChAT has been identified previously in the brains of patients with Rett syndrome and Alzheimer’s disease (8). In mice, neuromuscular junctions lacking ChAT generate abnormal structures (9). In Drosophila melanogaster, the ChAT gene is necessary for viability (10).

In C. elegans, defects in ChAT, typified by cha-1 mutants, are not lethal; these mutants survive with minor defects and mild growth retardation, a phenotype similar to that of Unc and Egl mutants (http://www.wormbase.org/). Ach is synthesized in about one third of the 302 neurons that compose the nervous system of an adult C. elegans hermaphrodite (11). Four AChEs are responsible for degrading and inactivating Ach. Most cholinergic neurons also express unc-17, which is responsible for loading Ach into synaptic vesicles (12), and unc-104, which mediates synaptic vesicle anterograde transport (13). G-proteins are also known to be involved in Ach release (14, 15). In the egg-laying system, the G-proteins egl-30 and goa-1 are important mediators of synaptic vesicle release through a pathway involving regulators of G-protein signaling (RGS), phospholipase-Cβ (PLC-β) and diacylglycerol binding protein (Unc-13 (15)).

The formation of dauer larvae, an important life cycle response of C. elegans to an unfavorable environment (16), is thought to reflect the function of various sensory and interneurons, which determine the balance of environmental fac-

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tors and execute the dauer formation process. Indeed, a previous report has shown that sensory neuronal function is very important for dauer formation (17). During dauer formation, communication among neuronal pathways, including sensory neurons and interneurons, as well as neurosecretory cells, is likely to involve neurotransmitters such as Ach. Consistent with this, Ach is reported to be important for the dauer recovery pathway (4).

In this paper, we addressed a question as to how quantitative change in Ach release influence dauer formation (non-aging state) in C. elegans.

RESULTS

Ach level modulates dauer formation

During dauer formation (3), the communication between neurons, such as neurotransmitter secretion, could be important. Consistent with this, Ach is reported to be important for the dauer recovery pathway (4). Therefore, to investigate the importance of Ach during dauer formation, we performed sub-optimal daumone assays using various Ach metabolic mutants; cha-1 mutants defective in synthesis of Ach (11), ace-1;ace-2 mutants defective in degrading and inactivating Ach, unc-17 mutant defective in loading Ach into synaptic vesicles (12), and unc-104 mutant defective in synaptic vesicle anterograde transport (13). The dauer formation rate of wild-type N2 worms in sub-optimal daumone assays (18) was about 30% (Fig. 1A). In these same assays, the dauer formation rate was enhanced ∼3.3-fold (97.2%) in the cha-1 mutant and 2.3-fold (66.8%) in the unc-104 mutant. The dauer formation rate also trended higher (53.3%) in the unc-17 mutant, although the observed 1.8-fold increase did not reach statistical significance. Moreover, dauer formation was partially defective in ace-1;ace-2 mutants, which have elevated levels of Ach due to defects in AchEs; in these worms the dauer formation rate was only 10.8%, approximately a 2.8-fold decrease compared to wild-type N2 worms. None of these mutants showed dauer formation abnormalities under normal conditions, confirming the value of the suboptimal daumone assay in detecting subtle changes in dauer formation that might be overlooked (18).

These data show that a reduction in Ach biosynthesis and release enhances dauer formation, and an increase in Ach levels results in partial defects in dauer formation under conditions in which daumone concentrations are suboptimal. Taken together, these results demonstrate that the level of Ach release modulates dauer formation in C. elegans.

Neurotransmitter release mutants show defects in dauer formation

Ach plays an important role in the egg-laying system, and its release is regulated by two G-proteins, goa-1 and egl-30 (6). Both goa-1 and egl-30 are known to antagonize each other’s function: mutations in goa-1 cause the Egl-c phenotype, while mutation in egl-30 causes an Egl-d phenotype (19). Since goa-1 and egl-30 are also highly expressed in the nerve ring, it seems unlikely that the roles of goa-1 and egl-30 are restricted to HSN neurons (http://www.wormbase.org/). We hypothesized that the G-proteins and acetylcholine release might be utilized in the process of dauer formation as well. To determine whether G-protein-dependent regulation of Ach release is involved in dauer formation, we performed sub-optimal daumone assays using goa-1 and egl-30 mutants. goa-1 mutants, which exhibit enhanced Ach release, showed a partial dauer-defective phenotype (2.9% dauers) compared to wild-type N2 (29.1% dauers) (Fig. 1B). The egl-30 mutant, in which Ach release is suppressed, showed a 2.5-fold increase in the dauer formation rate (74.1%). A gain-of-function mutation of egl-30, which is constitutively active regardless of upstream signaling, showed a dauer-defective phenotype similar to that of the goa-1 mutant (1.2% dauers). These data are consistent with those of previous experiments (see Fig. 1A), which showed that Ach, particularly the level of Ach release, could modulate dauer formation. Each of these mutants behaved as predicted in conjunction with the egg-laying system, strengthening the notion that Ach release could be important during the dauer formation process.

Dauer larvae contain less Ach than developing worms

Since Ach affects dauer formation, we hypothesized that the regulation for the amount of Ach might be a critical step during dauer formation. To measure the amount of Ach in vivo during dauer formation, we used quantitative mass spectrometric analysis to compare the amount of Ach between reproductively growing and dauer stage worms (Fig. 2A). In order to collect the well-defined stage of worms, the indicated samples were synchronized and harvested at the L3 stage. For dauer stage worms, the samples were cultured with a limited

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Acetylcholine could regulate *C. elegans* dauer formation
Jee Yong Lee, et al.

Fig. 2. Dauer larvae contain less Ach than reproductively grown worms. The indicated samples were analyzed by LC-MS/MS, and the measured values of Ach levels (upper traces) were normalized to trimethyl-D9 choline (ISTD; lower traces). (A) Typical peaks generated by LC-MS/MS for reproductively grown wild-type N2 worms (top left), N2 dauers (top right), cha-1 mutants (bottom left) and AchE (ace-1;ace-2) mutants (bottom right) are shown. A black arrow indicates the predicted Ach peak; numbers indicate peak intensities for Ach and ISTD. (B) The graph shows the relative Ach levels (mean ± SD) in arbitrary units (y axis) for the indicated genotype normalized to that for reproductively grown N2 worms.

2DE analysis of the AchE mutants
To determine whether the expression of any proteins was influenced by an increase in Ach levels in *C. elegans*, we performed 2DE analysis (in two independent sets) using N2 wild type and the AchE double mutant (ace-1;ace-2), which contained excessive Ach (Fig. 3A). Among the differentially expressed proteins (Fig. 3A, right panel), we selected spots for those whose expression was changed by more than 4-fold, and further analyzed them by LC-MS/MS (Fig. 3B). Lipid binding protein (lbp-9), which is involved in fat transfer, and alcohol dehydrogenase (H24K24.3), which is involved in amino acid catabolism (20), were down-regulated 4.2-fold and 4.9-fold, respectively (Table 1). RAP homolog (rap-1), which is a small amount of food source and harvested when they form dauers. For normalization of measurements, the same dried weight was used for each measurement and internal standard, trimethyl-D9 choline, was added into each sample prior to extraction. The results clearly show that dauer larvae contain only residual levels of Ach compared to reproductively growing worms (Fig. 2B). cha-1 and AchE mutants were also measured. Ach levels were almost undetectable in cha-1 mutants, whereas Ach levels in the AchE mutant (ace-1;ace-2) were about 2-fold higher as in N2 (Fig. 2B). These data indicated that the amount of Ach was down-regulated in dauer larvae, and suggested that the down-regulation of Ach may promote dauer formation.

Fig. 3. 2DE analysis of AchE (ace-1;ace-2) mutant *C. elegans*. Lysates from the indicated genotypes were resolved by 2DE, and gels were stained with Coomassie Brilliant Blue G-250. (A) A representative image from two independent experiments is shown. (B) Black arrows shown in panel (A) indicate spots for proteins differentially expressed by more than 4-fold that were selected for further analysis. Molecular weight (MW) and pl values are also indicated. The identities of spots are shown in Table 1. (C) A proposed working model. Various daunone analogues capable of inducing dauer formation are likely detected by a receptor(s) in sensory neurons. The cholinergic neurons, which regulate Ach production and release, could detect daunone directly, or alternatively are notified by sensory neurons. Then, the cholinergic neurons reduce acetylcholine release via goa-1/egl-30 circuit. The reduced Ach release could affect the sensory neurons to secrete insulin-like peptides as suggested by Tissenbaum et al. (4) or could affect the target cells to respond to the insulin-like peptide. Finally, the reduced Ach level induces catabolic enzymes, such as alcohol dehydrogenase and lipid binding protein. Collectively, these changes are predicted to enhance dauer formation in *C. elegans.*

Fig. 2. Dauer larvae contain less Ach than reproductively grown worms. The indicated samples were analyzed by LC-MS/MS, and the measured values of Ach levels (upper traces) were normalized to trimethyl-D9 choline (ISTD; lower traces). (A) Typical peaks generated by LC-MS/MS for reproductively grown wild-type N2 worms (top left), N2 dauers (top right), cha-1 mutants (bottom left) and AchE (ace-1;ace-2) mutants (bottom right) are shown. A black arrow indicates the predicted Ach peak; numbers indicate peak intensities for Ach and ISTD. (B) The graph shows the relative Ach levels (mean ± SD) in arbitrary units (y axis) for the indicated genotype normalized to that for reproductively grown N2 worms.
GTP binding protein and regulates cell-cell contact and epithelial integrity of hypodermal cells (21,22), was highly up-regulated (7.6-fold) (Table 1). This may coordinate hypodermal cell movement during embryonic development (21,22). Taken together, these changes in protein abundance may cooperatively inhibit dauer formation by interfering with the proper catabolism and cell movement.

**DISCUSSION**

Earlier works indicate that Ach is involved in dauer recovery in which Ach treatment wakes up both *C. elegans* and the parasitic nematode *Ancylostoma caninum* from a dormant state (4). Signaling within sensory neurons involves various Ach receptor genes, which regulate behavior, neuronal degeneration, and embryonic development (5). Thus, Ach may be a key regulator of the nematode life cycle, and this association is strongly supported by a number of our findings. First, the level of Ach affected dauer formation (Fig. 1A). Second, the Ach release also affected dauer formation process (Fig. 1B). Third, dauer larvae contained lower levels of Ach than worms reproducibly grown (Fig. 2). Finally, a number of proteins were differentially expressed in the context of excessive Ach (Table 1). The excessive Ach could down-regulate alcohol dehydrogenase (H24K24.3) and lipid binding protein (lbp-9), and up-regulate RAP homolog (rap-1). H24K24.3 has not been studied yet, although yeast homolog is involved in amino acid catabolism (18). In case of *lbp-9*, *Ha et al* investigated the involvement of cellular senescence (23). They reported that the knock-down of *lbp-9* led to shorter lifespan and earlier accumulation of lipofuscin when compared with the control. Therefore, the excessive Ach could induce down-regulation of *lbp-9* and subsequently promote the reproductive aging process. In addition, rap-1 is known to coordinate hypodermal cell movement and elongation during embryonic development (24). During dauer formation, massive hyperdermal rearrangement is involved. Although we are unable to pinpoint the effect of up-regulation of rap-1, it seems that the up-regulation might interfere with hypodermal cell movement and elongation during dauer formation. Overall, our study provides novel insights into the role of Ach during dauer formation in *C. elegans*.

We have shown that Ach release affected the process of dauer formation. Ach release has been studied intensively in the egg-laying system (6). We demonstrated that many participants in the egg-laying machinery also involved in dauer formation. goa-1 and egl-30 antagonize each other in the egg-laying circuit (including in HSN neurons). Similarly, egl-30 and goa-1 mutants show the opposite phenotype in sub-optimal daurome assays (Fig. 1B). In addition to goa-1 and egl-30, other genes implicated in the egg-laying system (6, 15) are involved in dauer formation. Among these are *eat-16*, which is a regulator of G-protein signaling (RGS) for egl-30; egl-10, which is an RGS for goa-1; *unc-13*, which is a neurotransmitter release regulator; and *egl-8*, phospholipase C-β which is a target of activated egl-30. Mutant strains for the genes were tested for defects in dauer formation with suboptimal dauer formation assays (Fig. S1A). Each of these mutant behaviors as predicted in conjunction with the egg-laying system, strengthening our notion that Ach release could be important during the dauer formation process as well (Fig. S1B). However, it should be emphasized that dauer formation is independent of the egg-laying system, because the HSN is not fully differentiated during the dauer induction period (25); thus, only the core proteins in the egg-laying machinery are shared with the dauer pathway.

It has been reported that ASJ, ASK and ASI amphid neurons are important for dauer formation (26). Interestingly, none of the sensory neurons are known to be cholinergic; therefore, the effect of goa-1 and egl-30 is likely to be mediated by nearby interneurons which are cholinergic neurons. Many sensory neurons are connected with interneurons via gap junction and synapses (27). Therefore, activation of sensory neurons could activate the specific interneurons, and *C. elegans* neuronal circuit could be important to mediate the daurome signaling between the individual neurons in this picture. These interneurons must be responsible for either the activation of Cha-1 or/and inactivation of AchE in order to control the level of Ach during development. Especially, it was reported that the acetylcholine signaling pathway activates an insulin-like signal during dauer recovery (4), it would be reasonable to predict that the similar pathway works during the dauer induction pathway. One possibility is that reduced Ach release could potentiate the dauer-inducing ability of sensory neurons, which are responsible for down-regulation of insulin-like peptides during dauer formation. Alternative possibility could be that

Table 1. Identification of 2DE spots that are differentially expressed between wild-type (N2) and AchE mutant (ace-1;ace-2) *C. elegans*

| Spot ID | Protein name | Gene name | NCBI accession number | Fold changed$^a$ | Number of matched peptides | Sequence coverage | MASCOT score | MW (KDa) | pl |
|---------|--------------|-----------|-----------------------|-----------------|--------------------------|-----------------|-------------|----------|----|
| 1       | Alcohol dehydrogenase | H24K24.3 | gi|25146526 | 4.9 ↓ | 84 | 41% | 1,549 | 41.3 | 5.96 |
| 2       | Lipid binding protein | lbp-9 | gi|86564433 | 4.2 ↓ | 93 | 73% | 459 | 15.3 | 5.88 |
| 3       | RAP homolog | rap-1 | gi|17542026 | 7.6 ↑ | 6 | 26% | 418 | 21.2 | 5.15 |

*Average fold changes from two independent experiments.*

protein identifications were determined based on MASCOT scores, predicted molecular weights and predicted pl values.

http://bmbreports.org
Acetylcholine could regulate C. elegans dauer formation
Jeeyong Lee, et al.

the low Ach secretion affects the insulin-like signaling transduction in various insulin target cells. It would be interesting to see whether the mutation in goa-1/egl-30 circuit affects insulin-like peptide secretion.

In summary, our current model of dauer formation is presented schematically in Fig. 3C. Various daumone analogues (>160 ascarosides) capable of inducing dauer formation have been identified in C. elegans (28-32). The cholinergic neurons, which regulate Ach production and release, could detect daumone directly, or are alternatively notified by sensory neurons, which detect daumone. The notified cholinergic neurons reduce acetylcholine release via the goa-1/egl-30 circuit. The reduced Ach level could affect the sensory neurons to secrete insulin-like peptides as suggested (4) and/or the target cells to respond the insulin-like peptide. Furthermore, the reduced Ach level up-regulates H24K24.3 and lbp-9, and down-regulates rap-1. Our data suggest that these negative changes in Ach level collectively enhance dauer formation in C. elegans; however future studies will be required to confirm the details of this model.

MATERIALS AND METHODS

General methods and strains
Nematodes of the C. elegans Bristol variety, strain N2, were used as wild types. C. elegans were grown at 20°C on nematode growth media (NGM) agar plates containing Escherichia coli (OP50). C. elegans mutant strains were obtained from the Caenorhabditis Genetics Center (CGC). The strains used for this work are egd-30(n686) I, egl-30(js126) I, goa-1(n1134) I, unc-104(e1265) II, cha-1(p1152) IV, unc-17(e245) IV, ace-1(p1000) X and ace-2(g272) I. Chemicals, suboptimal daumone assays, 2DE analysis and protein Identification by mass spectrometry are described in ‘Supplemental Information’.

LC-MS/MS conditions for Ach measurement
The indicated strains were synchronized as described previously (28). Eggs were collected and cultured for 1.5 days. The samples were harvested at the L3 stage and stored in −70°C. For dauer stage worms, eggs were cultured with a limited amount of food source and harvested when they form dau-
ers (33). The harvested samples were dried in a speed vacuum. The samples were carefully weighed and solubilized with 0.1% formic acid in acetonitrile containing 100 ng of the trimethyl-D9 choline (internal standard). The upper phases were dried and re-solubilized with 20 μl of 0.1% formic acid in water, and a 1-μl aliquot was injected into a triple-quadrupole mass spectrometer (API 4000, AB/MD SCIEX) for LC-MS/MS quantitation of Ach using a multiple reaction monitoring (MRM) method. The column used for LC-MS/MS was a PC-HILIC (150 × 2.0 mm I.D., 5 μm, Shiseido, Japan). The mobile phase for LC separation was a mixture of 0.1% formic acid in 10 mM ammonium acetate and 0.1% formic acid in acetonitrile (30:70, v/v). The flow rate was maintained at 0.35 ml/min. Electrospray ionization was performed, and the temperature of the nebulizer was set at 300°C. Analyses were carried out in the positive ion mode using the MRM method with mass transitions of m/z 146.1→87.1 for Ach and m/z 113.2→66.2 for trimethyl-D9 choline (internal standard). Two independent measurements were performed for each sample, and the data were processed using Microsoft Excel 2003.

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Acetylcholine could regulate C. elegans dauer formation
Jeeyoung Lee, et al.

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