**Communication**

**A Caenorhabditis elegans Insulin-Like Peptide, INS-17: Its Physiological Function and Expression Pattern**

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The insulin/insulin-like growth factor-1 signaling pathway of *Caenorhabditis elegans* regulates larval diapause and adult lifespan through the sole insulin receptor-like protein, DAF-2. In the present study, the physiological function and expression pattern of INS-17, one of the *C. elegans* insulin-like peptides, were examined by disruption and overexpression of the gene, and by the use of a reporter gene. INS-17 might function as a DAF-2 antagonist in the regulation of larval diapause, but not of the adult lifespan. The reporter protein was intensively expressed during larval diapause. It showed a drastic decrease in amount after larval diapause, which matches well the physiological function of INS-17.

**Key words:** antagonist; *Caenorhabditis elegans*; insulin-like peptide; larval diapause; spatio-temporal expression

In the nematode *Caenorhabditis elegans* (*C. elegans*), environmental conditions determine whether the worm develops directly into an adult or is arrested at the alternative L3 larval stage to form a dauer larva. Dauer larvae are induced by harsh environments such as starvation, a high concentration of dauer pheromones secreted by crowded worms, or high temperature.1,2 The worms can survive in these adverse conditions because of their distinctive adaptive morphological, behavioral, and metabolic features. When environmental conditions improve, the worms resume normal growth. This survival system, known as larval diapause, is controlled by pathways including the transforming growth factor (TGFB)–β related pathway3 and the insulin/insulin-like growth factor (IGF)-1 signaling (IIS) pathway. In these pathways, the IIS pathway plays an important role in controlling not only larval diapause but also adult lifespan.4 It contains the sole *C. elegans* insulin/IGF-1 receptor-like protein, DAF-2,4 and the forkhead box O (FOXO) transcription factor homolog, DAF-16.5,6 DAF-2 inactivation results in DAF-16 nuclear translocation,7–9 which not only induces larval diapause but also leads to increased longevity.10–12 Thus IIS is an important pathway controlling larval diapause and adult lifespan in *C. elegans*. DAF-2-ligands, insulin-like peptides (ILPs), control the activation and inactivation of this signaling pathway. To date, 40 insulin-like genes have been identified in the *C. elegans* genome, and the predicted peptides encoded by the genes are classified into type-α, -β, and -γ according to their predicted disulfide bond patterns.13,14 Type-γ ILPs have three canonical disulfide bonds that are conserved among vertebrate insulin family peptides. We have identified *ins-18* (*Ceinsulin-1*) and *ins-17* (*Ceinsulin-2*), which encode type-γ ILPs, INS-18 and INS-17 respectively.15,16 These ILPs are structurally similar to each other, and include a characteristic insertion of three amino acid residues (-Pro-Pro-Gly-) in the B domain. In addition, their predicted tertiary structures match well. Our recent study indicated that INS-18 functions as a DAF-2 antagonist and is required not only for larval diapause but also for extension of the adult lifespan.17 In addition, INS-18 is intensively expressed in neurons at the diapause stage, and shows a drastic decrease in amount after recovery from diapause, but further examination of INS-17 has not yet been done.

In this study, first we examined the physiological function of INS-17 by gene disruption and overexpression. Subsequently we examined the spatio-temporal expression of INS-17 and compared it with that of INS-18. In addition, we investigated the regulation of *ins-17* transcription.

To elucidate the physiological functions of INS-17, first we disrupted the *ins-17* gene by the TMP/UV method.18 To screen a deletion mutant by *sib*-screening, we used the primer sets shown in Table S1 (see *Biosci. Biotechnol. Biochem.* Web site) for PCR amplification of the genomic region. A 585-bp *ins-17* deletion removed the entire second exon, indicating that the
mutant did not synthesize the INS-17 peptide. This deletion mutant was named tm790 (Fig. 1A). The mutant was backcrossed with wild-type animals 5 times to remove an unexpected mutation. Then this backcrossed worm (the ins-17 KO animal) was used in subsequent experiments. We also produced nematode strains to rescue the defect caused by ins-17-deletion and to overexpress ins-17. For producing these strains, the ins-17 transgene (ins-17p::ins-17) was constructed as follows: a genomic fragment containing the ins-17 coding region and a 5.4-kb upstream sequence was amplified by PCR and inserted into pGEM T-easy vector (Promega, WI). This ins-17 transgene was verified by sequencing, and then microinjected (15 ng/µL) into the germ line of ins-17 KO and wild-type animals along with the efi-3p::venus marker (20 ng/µL). The wild-type animals injected with this marker did not show any influence on larval diapause or adult lifespan.

Next we examined to determine whether INS-17 modulates larval diapause. When ins-17 KO animals were reared on standard nematode growth medium (NGM) plates seeded with Escherichia coli OP50 bacteria at 20 °C, the animals showed no larval diapause. Hence we measured the appearance of dauer larvae in the presence of a crude extract of dauer pheromones prepared as described previously to produce a diapause-inducing condition. NGM plates containing the extract at a final concentration of 1% (v/v) were seeded with Escherichia coli OP50 bacteria. Five to 10 adult hermaphrodites were placed on the plates and incubated at 20 °C. After a short period of egg laying, adult worms were removed from the plates and the progeny were allowed to develop to the young-adult stage at 20 °C. We identified dauers (worms in diapause) and non-dauers using a microscope and counted them. The dauer stage was confirmed by the presence of dark pigment granules, constriction of the body and pharynx, and a loss of pharyngeal pumping. As shown in Fig. 1B, ins-17 deletion resulted in a 32.8% reduction in the larval diapause as compared to the wild-type animals. This reduction was reversed by injection of the ins-17 transgene. There was no statistically significant difference between the ins-17 rescue animals and wild-type animals (p = 0.247), resulting in a rescue of the phenotype. In addition, ins-17-overexpressing (ins-17 O/E) animals generated by ins-17 transgene injection into wild-type animals showed a 35.4% increase in larval diapause as compared to the wild-type animals. Deletion and overexpression of ins-17 showed opposite phenotypes in larval diapause, as expected. These results suggest that INS-17 is involved in the regulation of the larval diapause.

Recently, it was reported that INS-6 and DAF-28 modulate larval diapause function as agonists in the same neurons. In other words, several insulin-like peptides might modulate larval diapause cooperatively through the IIS pathway. Our previous study reported that INS-18 is required for larval diapause as well as longevity. Hence, we attempted to determine whether INS-17 and INS-18 contribute cooperatively to the regulation of larval diapause. For this, first we examined the effect of the ins-17;ins-18 double mutation on larval diapause. A double-mutant animal was produced by crossing the single mutants (the ins-17 KO and tm339 animals). As shown in Fig. 1B, ins-18 deletion (tm339) resulted in a 58.6% reduction in larval diapause as compared to the wild-type animals. This reduction matches well the results of our previous study.

These results suggest that INS-17 is lesser relevance to larval diapause than INS-18. In addition, the ins-17;ins-18 double mutant animals showed a 71.6% reduction in larval diapause as compared to the wild-type animals. Although more reduction of larval diapause was observed in the double mutant animals, no statistically significant difference was found between the double mutant animals and the ins-18 mutant animals (p = 0.264). Then we investigated the effects of ins-17 deletion and overexpression on adult lifespan. Neither deletion nor overexpression showed any effect on it (Table S2 and Fig. S1). In sum, INS-17 modulates larval diapause but not adult lifespan, while INS-18 modulates both.

Many insulin-like molecules are thought to modulate the insulin/IGF-1 signaling (IIS) pathway of C. elegans by functioning as agonists or antagonists of sole insulin/IGF-1 receptor DAF-2. When agonists bind predominantly to the receptor (the normal state), IIS increases, leading to normal development. In contrast, when antagonists bind predominantly to DAF-2, IIS decreases, leading to larval diapause. In our study, ins-17 deletion

Fig. 1. Effects of ins-17 Disruption and Overexpression on Larval Diapause.

A. Schematic structure of ins-17. Black boxes represent exons in ins-17. The broken line with end caps indicates the ins-17(tm790) deletion site. B. Dauer formation in the wild-type, the ins-17 KO, and the ins-18 mutant animals was measured in the presence of a crude pheromone extract. Relative percentages of dauer formation in the mutants compared with the wild-type animals are shown. ins-17 rescue animals represent ins-17 KO animals into which an ins-17 transgene was introduced. ins-17-overexpressing (ins-17 O/E) animals represent wild-type animals into which an ins-17 transgene was introduced. Each mean ± SEM includes at least two independent experiments with more than 100 worms per experiment. Multiple comparisons between groups were made by Dunn’s test (*p < 0.05; NS, not significant). The number under each sample name represents the total number of animals scored.
reduced larval diapause in the presence of dauer pheromone, probably due to the relative dominance of agonist binding to DAF-2. Overexpression of ins-17 induced larval diapause, probably due to high levels of INS-17 binding to DAF-2. Based on these data, we assumed that INS-17 can function as a DAF-2 antagonist at the larval 1 and 2 stages.

The information as to when and where the hormonal peptides are expressed is important for further functional analysis. Hence we investigated the spatio-temporal expression of INS-17 by generating transgenic animals expressing VENUS driven by the intact ins-17 promoter. For this, we constructed a reporter gene as follows: For an ins-17 reporter gene (ins-17p::venus), a genomic fragment containing a 5.3-kb upstream sequence of the initial ATG in ins-17 was amplified by PCR from genomic DNA and inserted into pPDvenus vector, which contains a multiple cloning site followed by the venus coding sequence and the unc-54 3’-untranslated region (Fig. 2A). The primers used for PCR amplification are listed in supplemental manuscript (Table S1).

To produce animals expressing ins-17p::VENUS, the ins-17 reporter gene and co-injection marker pRF4 [rol-6(su1006)] (15 ng/µL, each) were microinjected into the germ line of wild-type animals. Fluorescent images were obtained with an IX71 differential interference fluorescence microscope (Olympus, Tokyo). On analysis of the expression pattern of the VENUS in this study, we observed that it was expressed in head neurons at every developmental stage. At the adult stage, VENUS expression was also seen in the uterus (Fig. 2B), suggesting the hypothesis that INS-17 is involved in reproduction. However, when we measured the numbers of laid eggs of the ins-17 KO animals, we did not find any significant difference with the wild-type animals (data not shown). At the dauer stage, VENUS was more intensively expressed in the head and tail neurons than at normal developmental stages. VENUS expression became weak during recovery from the dauer stage (Fig. 2C). This expression pattern matches well the proposed physiological function of INS-17. In a further observation, VENUS was expressed in at least amphid neurons (not chemosensory neurons), the ventral nerve cord, and AVG interneurons at the dauer stage (Fig. S2). We identified cells according to DiI staining and the anatomy database for C. elegans. As far as we know, INS-17 is the sole ILP that shows such expression patterns among 40 ILPs. Environmental cues, including dauer pheromones, might induce INS-17 expression in interneurons through chemosensory neurons releasing a second messenger, leading to the dauer stage.

As we described above, INS-17 might function as a DAF-2 antagonist, like INS-18. Hence we compared the expression pattern of INS-17 with that of INS-18. For this, we constructed a reporter gene as follows: For an ins-18 the reporter gene (ins-18p::mrfp), a genomic
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fragment containing a 5.2-kb upstream sequence of the initial ATG in ins-18 was amplified by PCR from genomic DNA and inserted into the phK_mrfp vector, which contains a multiple cloning site followed by the mRFP coding sequence and the unc-54 3′-untranslated region (Fig. 2A). The primers used for, PCR amplifications are listed in the supplemental manuscript (Table S1). To produce animals expressing both ins-17p::VENUS and ins-18p::mRFP, a mixture of both reporter genes and co-injection marker pRF4 [rol-6(su1006)] (15 ng/μL, each) was microinjected into the germ line of the wild-type animals. Fluorescent images were obtained with an FV1000 confocal laser scanning microscope (Olympus). We focused on the dauer stage, since INS-17 as well as INS-18 is required for larval diapause. In the merged image, a few cells appeared to express both (Fig. 2D). However, the 3D images indicated that VENUS was expressed in cells different from those in which mRFP was seen. Hence we confirmed that INS-17 and INS-18 were produced in the respective cells and separately induced larval diapause, unlike INS-6 and DAF-28.

Finally, we investigated the regulation of ins-17 transcription. At the dauer stage, DAF-16, a transcription factor downstream of the DAF-2 receptor, is translocated into the nucleus, suggesting that nuclear DAF-16 promotes transcription of the genes required for larval diapause and suppresses transcription of the genes that prevent diapause. Hence we hypothesized that ins-17 transcription is upregulated by DAF-16 due to the proposed physiological function of INS-17 as a DAF-2 antagonist. In fact, the DAF-16 consensus sequences (GTAAAt/cA and CTTATCA) have been identified in the ins-17 upstream sequence. To test this hypothesis, we measured the relative amounts of the ins-17 transcript in the absence and the over-preservation of nuclear DAF-16 by qRT-PCR. For this, we utilized the daf-16(mu80) mutant, in which the gene is deleted and hence DAF-16 is never produced. We also used the daf-2(e1370) mutant, containing a temperature-sensitive point mutation (P146SS) located in the intercellular kinase domain. In this mutant, IIS signaling is reduced, leading to nuclear localization of DAF-16 at non-permissive temperatures due to incomplete DAF-2 function. Synchronized L1 larvae (10,000–15,000) were grown to the L2 stage over 12 h at 20 °C (a non-permissive temperature for the daf-2 mutant). Subsequently, the grown animals were stored in 100 μL of RNAlater solution (Takara, Shiga, Japan) at 4 °C. mRNA samples were prepared using an Isogen Poly(A)⁺ Isolation Pack (Nippon Gene, Tokyo), and cDNA was synthesized using a cDNA synthesis kit (Roche, Mannheim, Germany). The PCR mixture consisted of 0.3 μM primers, 0.5 μL of dT-30-primed cDNAs, and 5 μL of THUNDERBIRD™ SYBR® qPCR Mix (Toyobo, Osaka). qPCR was monitored using a LineGene real-time thermal cycler (BioFlux, Tokyo) under the following reaction conditions: denaturation, 95 °C, 15 s; annealing, 55 °C, 15 s; extension, 72 °C, 30 s (40 cycles). act-1 was used as internal control. The primers used for qPCR amplification are listed in the supplemental manuscript (Table S1). Amplification was performed in triplicate. Initial data analysis was carried out using the Fluorescent Quantitative Detection System (BioFlux, Tokyo), which calculated Ct values and extrapolated the relative levels of the PCR products from standard curves. To discount the possibility of contamination and of primer dimers, we analyzed the melt curves. As shown in Fig. 3, the amount of ins-17 transcript decreased to approximately 0.34-fold as compared to that of the wild-type animals, indicating that a lack of the DAF-16 transcription factor suppresses ins-17 expression. This confirms our hypothesis. In contrast, the ins-17 transcript levels in the daf-2(e1370) mutants increased to 2.1-fold as compared to that for the wild-type animals. We also confirmed this phenotype by VENUS expression. ins-17p::VENUS was downregulated in the daf-16(mu80) mutant background, but upregulated in the daf-2(e1370) mutant background (Fig. S3). These results indicate that ins-17 transcription is positively regulated by DAF-16 nuclear translocation, like ins-18 transcription.

In this study, we elucidated the involvement of INS-17 in the regulation of larval diapause, but not of adult lifespan, and then we assumed that INS-17 can function as a DAF-2 antagonist. INS-17 is of lesser relevance to larval diapause than INS-18, another DAF-2 antagonist. These ILPs, which are intensively expressed in different cells during the dauer stage, probably modulate diapause separately. INS-17 modulates only larval diapause, while INS-18 modulates adult lifespan in addition to larval diapause through the DAF-2 receptor. On the other hand, the expression of both genes is regulated by DAF-16. Recently, it was reported that DAF-16 isoforms selectively function to modulate larval diapause and/or adult lifespan. Although the underlying mechanism that causes a physiological difference between INS-17 and INS-18 remains unknown, these ILPs might regulate larval diapause and/or adult lifespan through different DAF-16 isoforms. This study perhaps provides insight into the hormonal regulation of larval diapause and adult lifespan in C. elegans.

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