Biochemical and Biophysical Research Communications 423 (2012) 478–483

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Physiological function, expression pattern, and transcriptional regulation of a *Caenorhabditis elegans* insulin-like peptide, INS-18

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ARTICLE INFO

Article history:
Received 20 May 2012
Available online 5 June 2012

Keywords:
Caenorhabditis elegans
Insulin-like peptide
Diapause
Lifespan
Gene-expression

ABSTRACT

In *Caenorhabditis elegans*, insulin/insulin-like growth factor (IGF)-1 signaling (IIS) is an important pathway that controls larval diapause and adult lifespan. The IIS pathway is modulated by many insulin-like peptides (ILPs) through the DAF-2 receptor, the sole insulin/IGF-1 receptor-like protein in *C. elegans*. We previously identified the ILP, INS-18, and predicted its tertiary structure to be similar to the crystal structures of human insulin and IGF-1. In this study, the physiological function of INS-18 was first examined by gene disruption and overexpression, and we identified INS-18 as a DAF-2 antagonist required for larval diapause and longevity. Analysis of the INS-18 expression pattern using a reporter gene showed it to be expressed in nerve cells, including hermaphrodite-specific neurons (HSNs) at the adult stage. Other ILP expressions have not been previously observed in HSNs, and we believe that INS-18 expression in these cells may contribute to longevity by regulating reproduction. Loss of the DAF-16 transcription factor located downstream of the IIS pathway completely blocked *ins-18* expression. We propose a positive feedback model for the regulation of *ins-18* expression in which an antagonist binding to the DAF-2 receptor increases *ins-18* gene expression, thus leading to increased INS-18 protein levels and increased DAF-2 receptor binding. Thus, this study provides a new insight into the hormonal regulation of insulin, an important and widespread process in the animal kingdom.

1. Introduction

Environmental conditions affect the development of most species. In *Caenorhabditis elegans* (*C. elegans*), environmental conditions determine whether the worm develops directly into an adult or is arrested at an 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 temperatures [1,2]. Worms can survive these adverse conditions because of their distinctive adaptive morphological, behavioral, and metabolic features. When environmental conditions improve, worms resume normal growth. This survival system, known as larval diapause, is controlled by many signaling cascades including the insulin/insulin-like growth factor (IGF)-1 signaling (IIS) pathway. This pathway contains the sole *C. elegans* insulin/IGF-1 receptor-like protein, DAF-2 [3], and the forkhead box O (FOXO) transcription factor homolog, DAF-16 [4,5]. DAF-2 inactivation results in DAF-16 nuclear translocation [6–8], which induces larval diapause and leads to increased longevity [9–11]. Thus, IIS is an important pathway controlling larval diapause and adult lifespan in worms.

The activation or inactivation of this signaling pathway is controlled by DAF-2-ligands, the insulin-like peptides (ILPs). Agonistic ILPs are thought to promote signaling and antagonistic ILPs to suppress signaling through the DAF-2 receptor. To date, 40 insulin-like genes have been identified in the *C. elegans* genome and predicted peptides encoded by the genes are classified into types α, β, and γ according to their disulfide bond pattern [12,13]. Type-γ ILPs have three canonical disulfide bonds that are conserved among vertebrate insulin family peptides. We previously identified INS-18 as a type-γ ILP and predicted its tertiary structure to be similar to the crystal structures of human insulin and IGF-1. In addition, we speculated that INS-18 may function as a DAF-2 agonist because *ins-18* knockdown in wild-type animals slightly increases longevity [14]. In contrast, Pierce and coworkers showed that a high *ins-18* gene dosage enhances the *daf-2* mutant phenotype, indicating that INS-18 may function as a DAF-2 antagonist [13]. Thus, the function of INS-18 remains unclear.

Abbreviations: C. elegans, *Caenorhabditis elegans*; DAF, dauer formation; FOXO, forkhead box O; HSN, hermaphrodite-specific neuron; IGF-1, insulin-like growth factor-1; IIS, insulin/IGF-1 signaling; ILP, insulin-like peptide; INS, Insulin; qPCR, quantitative PCR; RNAi, RNA interference.

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http://dx.doi.org/10.1016/j.bbrc.2012.05.145

In this study, we first determined whether INS-18 functions as a DAF-2 agonist or antagonist. We subsequently examined the spatio-temporal expression of INS-18 and investigated the transcriptional regulation of *ins-18*. Our data suggests that INS-18 is a DAF-2 antagonist that functions mainly in nerve cells. In addition, our data also allows us to propose a positive feedback model for the regulation of INS-18 expression.

2. Materials and methods

2.1. Strains

C. elegans were grown on standard nematode growth medium (NGM) [15]. Unless otherwise stated, NGM plates were seeded with *E. coli* OP50 bacteria and maintained at 20 °C [15]. Worm strains and alleles used in this study were described in the Supplemental Manuscript.

2.2. Construction of plasmids and transgenic worms

Amplified genomic fragments were subcloned into pBluescript SK(+) (Stratagene, Santa Clara, CA) or pPD_venus (provided by Dr. Ishihara of Kyushu University) vectors. A plasmid overexpressing *ins-18* was constructed to rescue the *ins-18* phenotype. Briefly, a genomic fragment containing the *ins-18* coding region and a 5.0 kb upstream sequence was amplified by PCR and inserted into the pBluescript SK(+) vector. The *ins-18* reporter gene (*ins-18p*::*ins-18*::*venus*) was constructed as follows: a genomic fragment containing the *ins-18* coding region up to a site just before the stop codon and a 5.0 kb upstream sequence was amplified by PCR from genomic DNA and inserted into the pPD_venus vector, which contains a multiple cloning site followed by the venus [16] coding sequence and the *unc-54* 3′-untranslated region. Each plasmid was verified by sequencing.

To rescue the *ins-18* phenotype, the *ins-18* transgene (*ins-18p*::*ins-18*) was microinjected into germ lines (15 ng/μl) [17] of *ins-18*(*tm339*) and *ins-7*(*tm1907*);*ins-18*(*tm339*) double mutant animals along with co-injection of the *eft-3p*::*venus* (10 ng/μl) marker. To produce *ins-18*-overexpressing animals, the transgene was microinjected into the germ line (15 ng/μl) of wild-type animals along with the *eft-3p*::*venus* (10 ng/μl) marker. Wild-type animals injected with this marker (10 ng/μl) did not have any influence on larval diapause or adult lifespan. To produce mutants expressing INS-18::VENUS, the *ins-18* reporter gene (*ins-18p*::*ins-18*::*venus*) was microinjected into the germ lines (25 ng/μl) of wild-type, *daf-2*(*e1370*), and *daf-16*(*mu86*) animals along with the *pRF4* [*rol-6*(*su1006*)] marker (20 ng/μl) [18]. Fluorescent images were obtained with an IX71 differential interference fluorescence microscope (OLYMPUS, Tokyo, Japan). The primers used for PCR amplification are described in Table S2.

2.3. Dauer formation assay

Dauer-forming ability was determined by two methods. Dauer formation was first tested in the presence of a crude pheromone extract that induces its formation [19]. The crude pheromone extract prepared by our method [20] was added to a final concentration of 1% (V/V) liquid NGM that had been autoclaved and cooled to 60 °C. The medium was immediately dispensed into petri plates. Second, the dauer-forming ability was tested under the *daf-2* (-) condition at 20 °C, 22.5 °C, and 25 °C. For each temperature tested, 5–10 adult hermaphrodites were placed on standard NGM plates seeded with *E. coli* OP50 bacteria at 20 °C. After a short period of egg laying, adult worms were removed from the plates and the progeny were allowed to develop at 20 °C, 22.5 °C, and 25 °C. When worms developed to the young-adult stage, dauers and non-dauers were identified using a microscope and counted. The dauer stage was confirmed by the presence of dark pigment granules, constriction of the body and pharynx, and the loss of pharyngeal pumping. Differences were considered statistically significant if *P*-values were < 0.05.

2.4. Lifespan assay

In the lifespan assay, synchronous L4-stage worms estimated as 0-day adults were incubated on NGM containing 40 μM 5-fluorodeoxyuridine (FUdR) to prevent self-fertilization. The number of surviving worms was monitored until death, judged by nonresponse to a mechanical stimulus. The results of the survival assays were analyzed using the Kaplan-Meier method and significance was measured by the log-rank test using the statistical analysis add-in soft for Excel, Excel Statistics 2010, (SSRI, Tokyo, Japan). Differences were considered statistically significant if *P*-values were < 0.05. Details were described in the Supplemental material.

2.5. qPCR

Synchronized L1 larvae (10,000–15,000) were grown to the L4 stage at 15 °C. The animals were cultured for two days at 20 °C and subsequently 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, Japan) 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, Japan). qPCR was monitored using a Line-Gene real-time thermal cycler (BioFlux, Tokyo, Japan) with 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 an internal control. The amplification was performed in triplicate. Initial data analysis was carried out using the Fluorescent Quantitative Detection System (BioFlux, Tokyo, Japan), which created Ct values and extrapolated the relative levels of PCR products from standard curves. Melt curves were routinely done to discount the possibility of both contamination and primer dimers. Differences were considered statistically significant if *P*-values were < 0.05.

3. Results and discussion

3.1. INS-18 functions as a DAF-2 antagonist to modulate larval diapause and adult lifespan

To elucidate the physiological functions of INS-18, we first mutated the *ins-18* gene using the TMP/UV method [21] followed by *sib*-screening. A 1.4 kb *ins-18* deletion removed half of the second and the entire third exon, indicating that the mutant could not synthesize the INS-18 peptide. This deletion mutant was named *tm339* (after the reference list [22]) and used in subsequent experiments.

We first examined whether INS-18 modulates larval diapause. At 20 °C and 25 °C, *ins-18*(*tm339*) animals showed no larval diapause. Hence, we measured appearance of dauer larvae in the presence of a crude extract of dauer pheromones [20]. Although 42.0% of wild-type animals entered the dauer stage, *ins-18* deletion resulted in a 30.6% reduction in the larval diapause compared with the wild-type animals. This reduction was reversed by injection of the *ins-18* transgene (*ins-18p*::*ins-18*), resulting in a complete rescue of the phenotype. In contrast, *ins-18*-overexpressing animals generated by transgene injection into wild-type animals showed a 30.2% increase in larval diapauses compared with the

wild-type animals (Fig. 1A). These results indicate that INS-18 is required for larval diapause under dauer-inducing conditions.

We next investigated the effect of *ins-18* deletion on the adult lifespan. At 25 °C, both *ins-18* and *ins-18*-rescue animals had almost the same lifespan as wild-type animals. In contrast, *ins-18* overexpression extended the mean lifespan by 3.1 days ($P < 0.013$) (Fig. 1B). However, we could not detect the effect of the *ins-18* deletion on the adult lifespan. Therefore, to investigate the effect, we measured the adult lifespan of *ins-18*(*tm339*) animals under an *ins-7*(*tm1907*) background, which provides a lifespan-extending condition. *ins-7* is one of the insulin-like genes and its

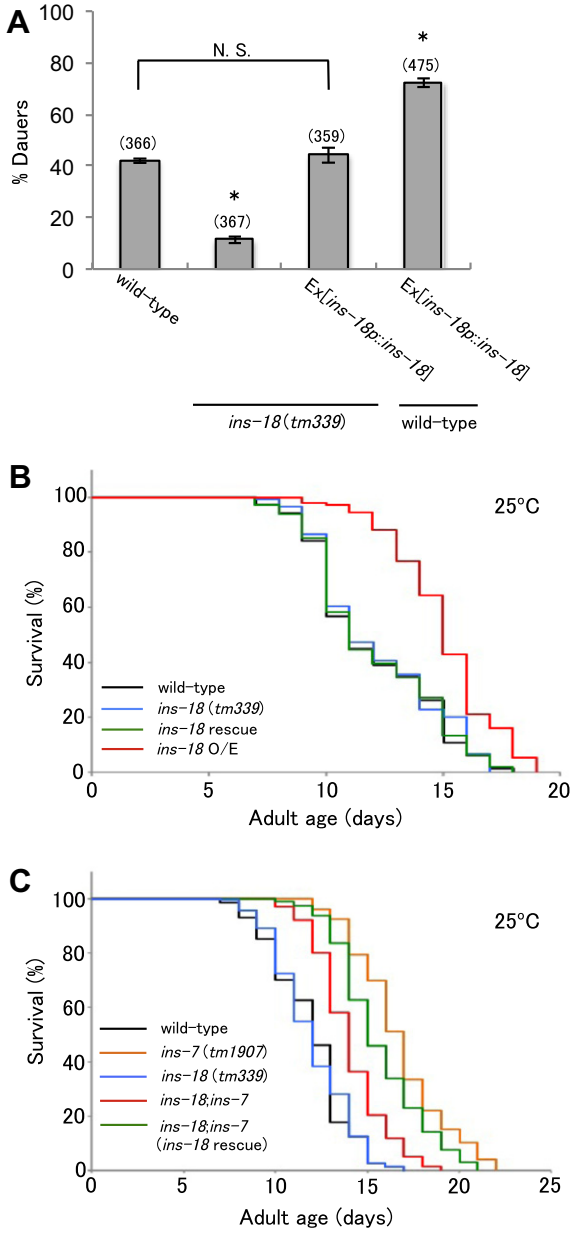

Fig. 1. INS-18 modulates larval diapause and adult lifespan to function as an antagonist. (A) The percentage of larval diapause was measured in wild-type and mutant animals in the presence of a crude pheromone extract. Each mean ± SEM includes at least three independent experiments using approximately 100 worms per experiment. Multiple comparisons between groups were made using Dunnett's test (*, $P < 0.01$; N.S., not significant). The number above each column represents the total number of animals scored. (B, C) Survival curves of wild-type and *ins-18* mutant animals are shown. Worms in lifespan assay were cultivated at 25 °C from the L4 stage until death. Day 0 corresponds to the L4 molt. The results of the survival assays were analyzed using the Kaplan-Meier method. Detailed parameters are shown in Table S1.

RNAi knockdown has been reported to induce an extended adult-lifespan [23]. As expected, the mean lifespan of *ins-7*(*tm1907*) animals was extended by 4.8 days ($P < 0.001$). In contrast, the mean lifespan of *ins-18*;*ins-7* double mutant animals was 2.7 days ($P < 0.004$) shorter than that for *ins-7* animals. However, double mutants expressing the *ins-18* transgene had almost the same lifespan as *ins-7* animals ($P < 0.28$), indicating a significant rescue of the phenotype (Fig. 1C). These results indicate that the INS-18 is required for lifespan extension at the adult stage.

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 the sole insulin/IGF-1 receptor DAF-2. When agonists predominantly bind to the receptor (the normal state), IIS signaling increases leading to normal development and normal adult lifespan. In contrast, when antagonists predominantly bind to DAF-2, IIS signaling decreases, larval diapause occurs, and the adult lifespan is extended. Under dauer-inducing conditions, the availability of agonists presumably decreases, leading to an increase in antagonist binding to DAF-2. In our study, *ins-18* deletion reduced larval diapause in the presence of dauer pheromone probably due to the relative dominance of agonist binding to DAF-2. The overexpression of *ins-18* induced larval diapause and longevity probably due to high levels of INS-18 binding to DAF-2 and inhibiting agonist binding. Under *ins-7*(-) conditions, a lack of the dominant agonist INS-7 reduces IIS signaling leading to adult lifespan extension. In this case, INS-18 predominantly binds DAF-2 and thus prevents the binding of other DAF-2 agonists. Therefore, a lack of INS-18 enables other agonists to bind DAF-2 leading to recovery of the adult lifespan extension. Based on these data, we concluded that INS-18 functions as a DAF-2 antagonist.

3.2. INS-18 modulates the larval diapause and adult lifespan through DAF-2 receptor

It was recently suggested that several insulin-like molecules, including DAF-28 can bind to other receptor tyrosine kinases (RTKs) and may function through signaling cascades acting in parallel to the DAF-2/DAF-16 pathway [12]. Therefore, we examined whether INS-18 binds only to DAF-2 using epistatic analysis. For this, we used the *daf-2*(*e1370*) mutant, containing a temperature-sensitive point mutation (P1465S) located in the intercellular kinase domain [3]. At a permissive temperature (15 °C), DAF-2 functions normally; however, it loses its tyrosine kinase activity at a restrictive temperature (25 °C). We produced *ins-18*;*daf-2* double mutants and measured larval diapause and adult lifespan in *ins-18*(*tm339*), *daf-2*(*e1370*), and double mutant animals.

We first measured larval diapause at 20 °C, 22.5 °C, and 25 °C, and found that *ins-18* animals do not undergo larval diapause at these temperatures. In contrast, *daf-2* animals showed an increase in larval diapause proportionate to the increase in temperature (Fig. 2A). Although double mutant animals also underwent larval diapause in proportion to the temperature, the extent of larval diapause was lesser than that of *daf-2* animals at 20 °C and 22.5 °C. These results indicate that INS-18 is required for larval diapause in *daf-2* animals. Conversely, *daf-2* and double mutant animals showed complete diapause at 25 °C, indicating that INS-18 does not antagonize larval diapause in the absence of DAF-2 activity.

We next measured the adult lifespan of each mutant at 20 °C and 25 °C (Fig. 2B, C). As expected, *daf-2* animals showed an increased longevity at both temperatures. In contrast, the mean lifespan of *ins-18*;*daf-2* double mutants was shortened by 7.0 days ($P < 0.0014$) at 20 °C compared with *daf-2* animals. These data indicate that INS-18 is required for longevity of *daf-2* animals. However, *daf-2* and double mutant animals had the same lifespan at 25 °C, indicating that INS-18 does not affect longevity in the absence of DAF-2 activity.

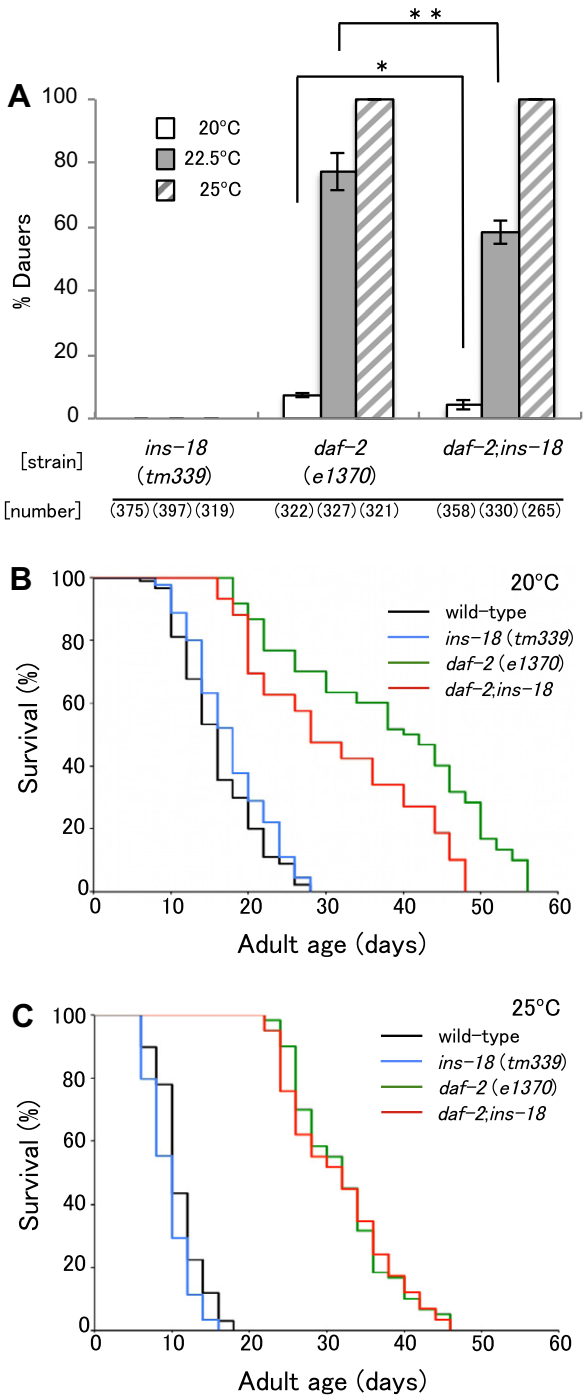

Fig. 2. *ins-18* mutation reduces larval diapause and adult lifespan in *daf-2* mutant animals. (A) The percentage larval diapause in wild-type and mutant animals. Data represents the mean ± SEM of at least three independent experiments using approximately 100 worms per experiment. Multiple comparisons between groups were made using Dunnett's test (∗, $P < 0.05$; ∗∗, $P < 0.01$). (B, C) Survival curves of wild-type and mutant animals are shown. Worms in lifespan assay were administered at 20 °C (B) and at 25 °C (C). Experiments were performed as described in the legend to Fig. 1. Detailed parameters are shown in Table S1.

Taken together, we found that complete inactivation of the DAF-2 tyrosine kinase prevents the decrease of larval diapause and adult lifespan extension caused by loss of INS-18. This indicates that *daf-2* is epistatic to *ins-18*. If INS-18 bound to other receptors to regulate larval diapause and adult lifespan, the inactivation of DAF-2 would incompletely suppress the effect of INS-18 loss. Therefore, we speculated that DAF-2 is the sole receptor of INS-18 in the context of dauer formation and lifespan.

3.3. The expression pattern of INS-18

The information of when and where the hormonal peptides are expressed is important for a complete functional analysis. Therefore, we investigated the spatio-temporal expression of INS-18 by generating transgenic animals expressing INS-18::VENUS driven by the intact *ins-18* promoter. We observed INS-18::VENUS expression in the head and tail neurons of adult-stage worms (Fig. 3A, B). This expression pattern matched that of whole body staining in wild-type animals obtained by using an anti-INS-18 polyclonal antibody (Fig. S1). Thus, the expression of INS-18::VENUS was expected to reflect the original expression of INS-18. On analysis of the expression pattern of INS-18::VENUS in our study, we observed that INS-18 was expressed in head and tail neurons at every developmental stage. Because INS-18 is required for larval diapause and longevity, we focused on the dauer and adult stages.

At the dauer stage, INS-18::VENUS was more intensively expressed in head and tail neurons than at normal developmental stages. It was also expressed in ventral cells. This expression was never seen at any developmental stage. During recovery from the dauer stage, INS-18::VENUS expression gradually weakened (Fig. 3C, D). In 5 days after worms reached the young adult stage, we observed INS-18::VENUS expression in pairs of ventral cells near the vulva (Fig. 3E). Based on their shape and anatomical location, we speculate these cells to be HSNs (after the reference list [24]). To support this hypothesis, we analyzed INS-18 expression in male transgenic worms. These worms expressed INS-18::VENUS

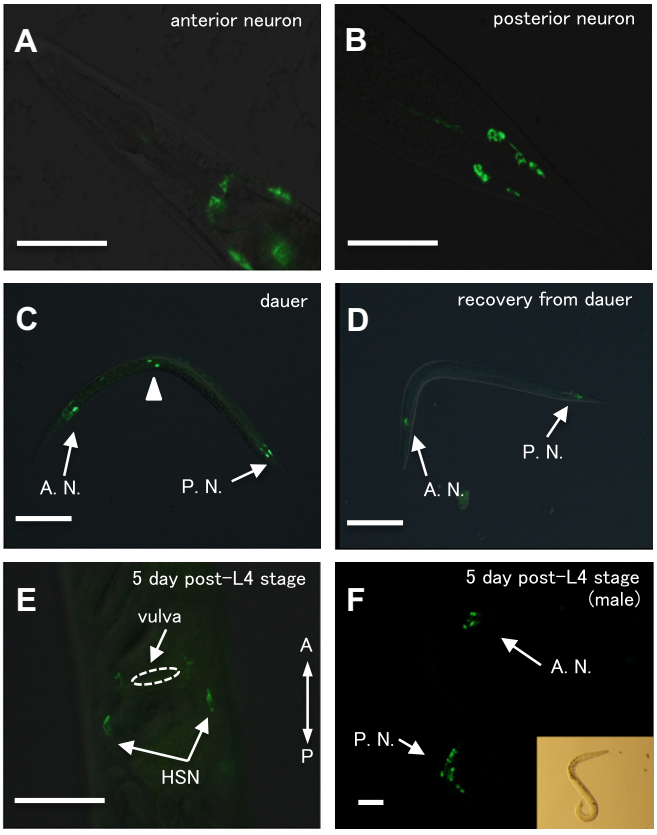

Fig. 3. The expression pattern of INS-18 at dauer and adult stages. (A, B) INS-18::VENUS expression in head (A) and tail (B) neurons of adult-stage worms. (C, D) The INS-18::VENUS expression pattern from dauer to the recovery stage. The arrowhead indicates a pair of cells specifically expressing INS-18::VENUS at the dauer stage. (E, F) INS-18::VENUS expression 5 days post-L4 stage in HSNs near the vulva (E). The expression in ventral cells was not observed in male worms (F). A. N., anterior neuron; P. N., posterior neuron. The scale bar represents 50 μm.

only in head and tail neurons and not in ventral cells (Fig. 3F). Therefore, we concluded that INS-18::VENUS is expressed in HSNs at the adult stage.

It has been reported that many ILPs are mainly expressed in head and/or tail neurons [13]. As far as we know, INS-18 is the sole ILP expressed in HSNs, and the relevance of INS-18 expression in HSNs to the regulation of adult-lifespan is unclear. However, since these neurons contribute to the regulation of egg laying, INS-18 may also modulate reproduction. In fact, *ins-18* overexpression reduced the numbers of eggs laid (Fig. S2A) and animals overexpressing INS-18 displayed a delayed egg-laying phenotype (Fig. S2B). These phenotypes are similar to those of *daf-2*(*e1370*) [9,25]. In addition, phenotypes resulting from INS-18 overexpression were suppressed by *daf-16* RNAi treatment (Fig. S2) as was observed for *daf-2* mutants [25], and in the *daf-16*(*mu86*) background. Interestingly, INS-18::VENUS expression in HSNs was not observed in *daf-16* RNAi-treated worms (Fig. S3). Thus, HSN INS-18 expression may contribute to adult lifespan regulation through IIS pathway-dependent control of reproduction.

3.4. *ins-18* transcription is controlled by DAF-16

In *daf-16* RNAi-treated animals, INS-18::VENUS expression was suppressed not only in HSNs but also in head and tail neurons (Fig. S3). In addition, the DAF-16 consensus sequences (GTAAAt/cA and CTTATCA) have been identified in the *ins-18* upstream sequence [23]. Based upon this information, we hypothesized that *ins-18* transcription is regulated by DAF-16. To test this hypothesis, we measured the change in *ins-18* transcript levels and INS-18::VENUS fluorescence in adult-stage *daf-16*(*mu86*) animals. qRT-PCR showed that the amount of *ins-18* transcript significantly decreased compared with that of wild-type animals (Fig. 4A). In addition, INS-18::VENUS fluorescence was scarcely visible in mutant animals, but easily observed in wild-type animals (Fig. 4B upper and middle panels). These results indicate that a lack of the DAF-16 transcription factor suppresses *ins-18* expression, thereby supporting our hypothesis. In other words, nuclear DAF-16 promotes *ins-18* expression. To confirm this, we utilized the *daf-2*(*e1370*) mutant in which IIS signaling is reduced leading to nuclear localization of DAF-16. Using this system, we examined whether DAF-16 nuclear translocation promotes *ins-18* expression by measuring the change in *ins-18* transcription and INS-18::VENUS fluorescence in *daf-2*(*e1370*) animals. We observed that *ins-18* transcript levels increased 3.2-fold compared with that in the wild-type animals at 20 °C where DAF-2 function is incomplete (Fig. 4A). In addition, a more intense INS-18::VENUS fluorescence could be seen in mutant animals compared with wild-type animals (Fig. 4B upper and bottom panels). These results indicate that *ins-18* transcription is positively regulated by DAF-16 nuclear translocation.

In this study, we propose a positive feedback model for the regulation of *ins-18* expression (Fig. 4C). Binding of the antagonist INS-18 to the DAF-2 receptor leads to inactivation. Subsequently, the DAF-16 transcription factor is incompletely phosphorylated and is translocated into the nucleus where it activates *ins-18* transcription leading to INS-18 protein synthesis. Increased levels of INS-18 further inactivate the DAF-2 receptor leading to a further increase in INS-18 production. Thus, the DAF-2 receptor can be inactivated in a cell-autonomous and/or non-cell-autonomous manner. On the other hand, a positive feedback model for the regulation of *ins-7* expression, which encodes a DAF-2 agonist, has been proposed by Murphy and coworkers [23]. In this model, the DAF-2 agonist phosphorylates the DAF-16 transcription factor through activated DAF-2 by its binding. Subsequently, translocation of the DAF-16 into the nucleus where it negatively regulates *ins-7* transcription is prevented. As a result, the transcription continues leading to an increase in INS-7 production, which contributes to normal development and prevention of larval diapause. Thus, based upon our and Murphy's models, binary switch of predominant agonist

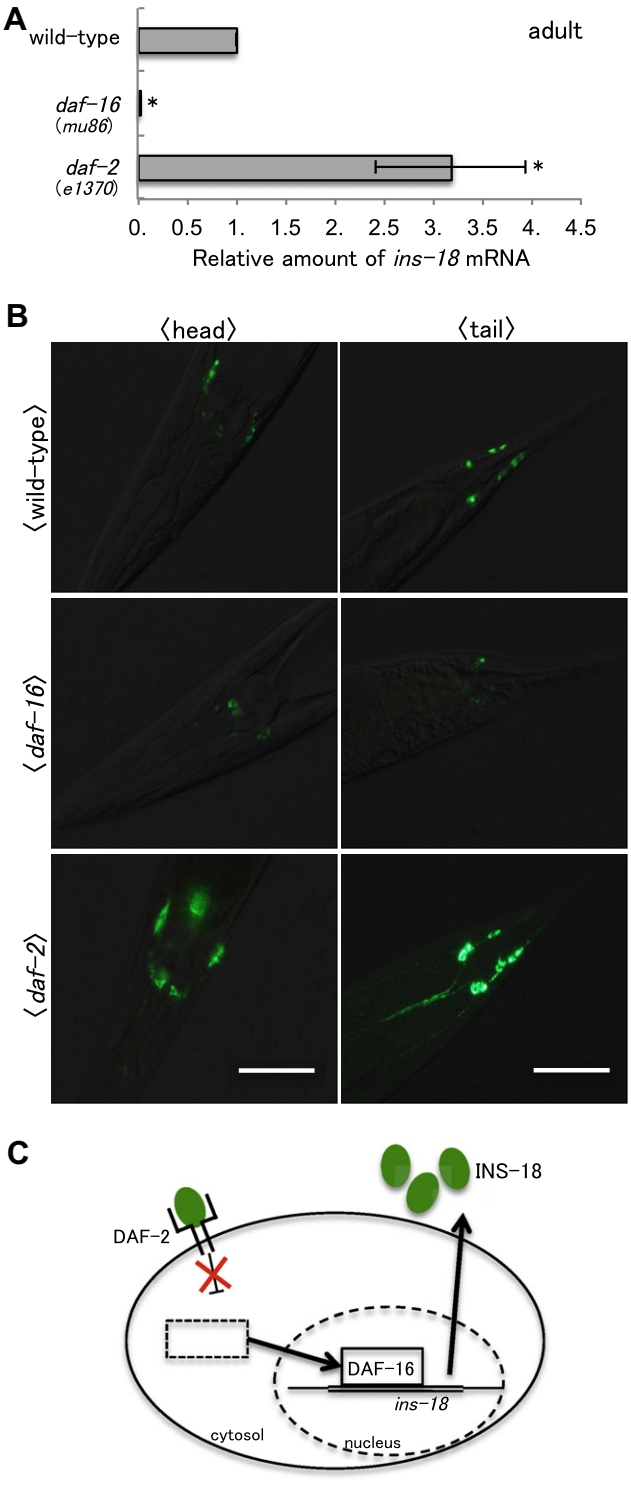

Fig. 4. DAF-16 regulates *ins-18* expression. (A) Relative quantitation of *ins-18* transcript in adult-stage worms. Each column indicates the relative value, which is standardized by the amount of transcript of wild-type animals. Each mean ± SEM includes at least three independent experiments. Multiple comparisons between groups were made using Dunnett's test (∗, $P < 0.01$). (B) INS-18::VENUS expression in wild-type, *daf-16*(*mu86*), and *daf-2*(*e1370*) animals at the adult stage. The scale bar represents 50 μm. (C) A proposed positive feedback model of regulation of *ins-18* expression.

(INS-7) and antagonist (INS-18) may provide a shrewd regulation of development and survival by regulating the mutual expression in *C. elegans*.

Although insulin-like molecules exist in diverse species of animals, insulin-like antagonists have not yet been identified in other animals. Therefore, this study provides new insights into hormonal regulation by insulin-like molecules.

Acknowledgments

We are grateful to Dr. Andrew Fire of Stanford University for providing us with the RNAi vector L4440. We thank Dr. Takeshi Ishihara of Kyusyu University for providing us with the reporter plasmid pPD_venus. We also thank Dr. James M. Kramer of Northwestern University for providing us with the maker plasmid *pRF4*. We thank Caenorhabditis Genetic Center for providing us with the *E. coli* strain HT115 and, the *C. elegans* strains *daf-2*(*e1370*) and *daf-16*(*mu86*). We thank Mr. Kenji Gouda and Ms. Yukari Ohmichi for their contribution to lifespan assays. We also thank Ms. Maki Komatsu and Mr. Kensuke Nakajima for their input and analysis of lifespan data. This work was supported in part by a grant-in-aid (No. 23580150, to T.K.) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.05.145.

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