Identification of a DAF-16 Transcriptional Target Gene, scl-1, that Regulates Longevity and Stress Resistance in Caenorhabditis elegans

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Summary

In Caenorhabditis elegans, an insulin-like signaling pathway, which includes the daf-2 and age-1 genes, controls longevity and stress resistance [1, 2]. Down-regulation of this pathway activates the forkhead transcription factor DAF-16, whose transcriptional targets are suggested to play an essential role in controlling the phenotypes governed by this pathway [3, 4]. We have surveyed the genes that have the DAF-16 consensus binding element (DBE) [5] within putative regulatory regions. Here, we show that one such gene, termed scl-1, is a positive regulator of longevity and stress resistance. Expression of scl-1 is upregulated in long-lived daf-2 and age-1 mutants and is undetectable in a short-lived daf-16 mutant. SCL-1 is a putative secretory protein with an SCP domain and is homologous to the mammalian cysteine-rich secretory protein (CRISP) family. scl-1 is required for the extension of the life span of daf-2 and age-1 mutants, and down-regulation of scl-1 reduces both life span and stress resistance of this animal. SCL-1, whose expression is dependent on DAF-16, is the first example of a putative secretory protein that positively regulates longevity and stress resistance.

Results and Discussion

The ageing process is being analyzed genetically in multicellular organisms, and the best-characterized pathway is an insulin/insulin-like growth factor (IGF-1)-like endocrine system that regulates the life span of C. elegans. Mutations that lower the level of daf-2, which encodes an insulin/IGF receptor homolog, cause the animal to live more than twice as long as normal [6, 7]. DAF-2 has been shown to activate a conserved phosphatidylinositol-3-kinase (PI(3)K)/3-phosphoinositide-dependent kinase-1 (PDK1)/Akt signal transduction pathway [7–10]. Reduction-of-function mutations in components of this pathway, such as mutations in age-1, which encodes a PI(3)K [8], and pdk-1, which encodes a PDK1 homolog [9, 10], are also shown to extend life span. These long-lived mutants in the insulin/IGF-1 pathway are of great importance because they are not only long-lived, but they are also active and youthful much longer than wild-type. Their longevity requires the activity of DAF-16, a forkhead/winged-helix family transcription factor [3, 4]. Remarkably, a pathway comprising PI(3)K, Akt, and the forkhead transcription factor is also evolutionarily conserved. Thus, in C. elegans, the DAF-2/AGE-1 pathway functions to shorten life span by downregulating DAF-16. In other words, DAF-16 appears to extend life span by inducing expression of those genes that are essential for the extension of life span.

To find out which genes are necessary to extend life span, we surveyed the complete genome sequence of C. elegans at Wormbase in search of genes that have a DBE (DAF-16 consensus binding element) sequence within putative promoter regions. We then extracted 159 genes that have a DAF-16 sequence and have putative mammalian homologs. In genetic mosaic analysis, removing daf-2 activity from individual lineages can cause the whole animal to be a long-lived adult [11]. This result, together with other observations [12], suggests that the DAF-2/AGE-1 pathway acts non-cell autonomously. Thus, it is likely that the targets of DAF-16 will be genes that encode, or regulate, a secreted factor. In the 159 genes tested, we found a gene (F49E11.9) that has a signal sequence and termed it scl-1 for the SCP-like extracellular protein. It has one copy of DBE at 110 bp upstream of its first exon (Figure 1A). We then examined the level of scl-1 mRNA in various mutants by RT-PCR analysis. At 20°C (a permissive temperature), scl-1 mRNA was highly expressed in daf-2(e1370) and age-1(hx546) mutants, but it was very low in N2 (wild-type) and was undetectable in a daf-16(mgDf50) mutant (Figure 2A). At exposure to 27°C (a nonpermissive temperature) for 12 hr, the level of scl-1 mRNA was elevated in N2, daf-2(e1370), and age-1(hx546) (Figure 2A). In daf-16(mgDf50), however, the expression of scl-1 mRNA was fully repressed even at 27°C (Figure 2A). These results strongly suggest that expression of scl-1 is regulated by the DAF-2/AGE-1 pathway and that scl-1 could be a target of the transcription factor DAF-16, although indirect regulation of scl-1 expression by DAF-16 could also be possible.

SCL-1 comprises 207 amino acids with a hydrophobic signal sequence at the N termini and has an SCP domain in the middle portion (Figure 1B). An SCP domain is an evolutionarily conserved motif and is found in secretory proteins of many species, but its function is unknown. SCL-1 is especially homologous to the mammalian cysteine-rich secretory protein (CRISP) family (Figure 1C). This family includes the sperm-coating glycoprotein TPX-1 [13], the cancer-specific, highly expressed protein CRISP-3 [14], the glioma pathogenesis-related protein GliPR [15], and so on.

In order to understand the function of scl-1 in the growth of C. elegans, we examined the level of expression of scl-1 mRNA in N2 (wild-type) and the daf-2(e1370) mutant at various developmental stages. In N2, scl-1 mRNA was expressed mainly in the egg stage and almost never in larvae or adult stages (Figure 2B). In

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current, the level of scl-1 mRNA was increased in the late adult stages in the daf-2(e1370) mutant (Figure 2B).

We considered the possibility that scl-1 is required for the long-lived phenotype of the daf-2 mutant. To test this possibility, we carried out an RNA interference (RNAi) method for scl-1 in N2 and daf-2(e1370) (Figure 3A). The RNAi for scl-1 had no apparent effect on development in either the N2 or daf-2(e1370) mutant (data not shown), but it had a significant effect on their life span. Under our conditions, the life span of non-RNAi-treated daf-2(e1370) was about 2-fold of that of non-RNAi-treated N2. The life span of scl-1 RNAi-treated daf-2(e1370), namely daf-2; scl-1(RNAi), was reduced by about 80% compared with the non-RNAi-treated daf-2(e1370) and became similar to that of non-RNAi-treated N2 (Figure 3B). Therefore, scl-1 is required for the long life span of daf-2(e1370). In N2, the scl-1 RNAi treatment slightly but reproducibly reduced the life span (not statistically significant) (Figure 3B). Interestingly, the life span of the scl-1 RNAi-treated N2 was nearly identical to that of daf-16(mgDf50), in which scl-1 is not expressed at all (Figure 2A, and data not shown). In addition, scl-1 RNAi treatment reduced the life span of age-1(hx546) by about 80% (data not shown). Thus, scl-1 is required for the extension of life span of daf-2 and age-1 mutants and may generally function to extend life span of C. elegans. It has been shown that daf-2(e1370) adults have dark intestinal pigmentation [16]. The scl-1 RNAi treatment markedly reduced the dark intestinal pigmentation (Figure 3C). However, the physiological significance of this observation is not obvious at present, as previous genetic studies have shown that intestinal pigmentation can be uncoupled from life span [11]. Thus, we observed the accumulation of lipofuscin-like intestinal fluorescence, which is one of the major markers of ageing in C. elegans [17]. At 5 days after L4, no autofluorescence was observed in daf-2(e1370) or daf-2; scl-1(RNAi), and only a slight fluorescence could be observed in N2. At 10 days after L4, marked accumulation of intestinal fluorescence was observed both in daf-2;
scl-1(RNAi) and N2, but not in daf-2(e1370) (Figure 3D). Additionally, in 12-day-old daf-2; scl-1(RNAi), N2-like tissue deterioration occurred in the intestine (Figure 3E). These data indicate that daf-2; scl-1(RNAi) animals age faster than daf-2 animals.

Longevity is often correlated with stress resistance in C. elegans [18]. We tested whether scl-1 has any function in stress resistance of wild-type animals. RT-PCR analysis showed that scl-1 mRNA is upregulated markedly after exposure to three stressers, heat, UV light, and starvation, in N2 (Figure 4A). RNAi for scl-1 decreased resistance of N2 to these stresses. At a high temperature, i.e., at 35°C, the mean surviving time of N2 was 14.6 ± 0.5 hr, while that of N2; scl-1(RNAi) was decreased to 9.8 ± 0.1 hr (Figure 4B). After exposure to UV light (0.2 J), the mean surviving time of non-RNAi-treated N2 was 3.5 ± 0.1 days, while it was decreased to 2.7 ± 0.1 days in N2; scl-1(RNAi). These results indicate that expression of scl-1 positively regulates stress resistance in C. elegans. RNAi treatment for scl-1 did not apparently influence C. elegans development, although scl-1 mRNA is expressed at embryonic stages in both
wild-type and the dat-2 mutant. Therefore, the role of SCL-1 in embryonic development remains unclear. It may be speculated, however, that expression of scl-1 may have a protective function from various stressors during embryogenesis, when cell division is rapid and replication errors may occur at a higher frequency.

Our results here have identified a novel transcriptional target of DAF-16, scl-1, that regulates longevity and stress resistance of C. elegans. scl-1 encodes a protein with an SCP domain and a signal sequence and has similarity to a diverse family of proteins referred to as the CRISP superfamily (see Figure 1C). These proteins are in general secreted proteins and possess several conserved cysteine residues [19]. This family includes the human TPX-1, GliPR, CRISP-3, protease inhibitors [20], several plant pathogenesis-related (PR) proteins [21], and C. elegans lon-1 [22]. TPX-1 (also identified as CRISP-2) is a testis-specific protein involved in differentiation of spermatogonial cells [23]. The human GliPR protein is highly expressed in the tumor glioblastoma multiforma, but not in normal fetal or adult brain tissues [15]. CRISP-3 has a putative role in the pathophysiology of Sjogren’s syndrome [24] and is highly expressed in cancers [5]. In addition, SCL-1 shows similarity to protease inhibitor 15 preproprotein. In mice, a mutation in the gene encoding the protein p66shc extends the life span and adds resistance against apoptosis induced by oxidative damage [25]. If stress-induced apoptosis accelerates ageing, protease inhibitors may retard ageing by inhibiting apoptosis. In this context, it should be noted that plant PR-1 proteins, which have a high degree of identity with GliPR, are shown to be important in mechanisms involved in plant defense systems [21]. Thus, many proteins of the CRISP family seem to be involved in host defense systems of various organisms. lon-1 is negatively regulated by the Sma/Mab signaling pathway and regulates body size in C. elegans [22]. All of these results suggest that the CRISP family proteins play a crucial role in control of diverse fundamental biological processes. It is possible that SCL-1 is secreted and functions as a ligand for another signaling pathway, as a protease inhibitor, or as a matrix scaffolding protein to regulate life span and stress resistance of C. elegans. Further biochemical studies for SCL-1 and the homologs may provide us with novel molecular understanding of evolutionarily conserved systems of ageing, stress resistance, and host defense.

Experimental Procedures

General Worm Procedures
C. elegans strains were maintained on NGM agar plates with fresh E. coli, OP50, as described [26, 27]. The assessment of life span and stress resistance was performed on NGM agar plates spread with OP50 as described [28].

Detection of the scl-1 mRNA
Total RNA was isolated from stage-mixed or synchronized cultures by the guanidinium-acid-phenol-chloroform method. First, cDNA was synthesized by using random primers and Mmlv polymerase (Invitrogen) following the manufacturer’s recommendations. The oligo DNA primers used for scl-1were 5'-CCAGCAACTGTATGAGG-3' and 5'-TTATCCGCAAAAGCCAGAAGC-3', and those used for EF-1α (R03G5.1) were 5' -CTAATGTGCTCAGCAGATCGTACGC-3' and 5'-TGCTCGGTGAGTTCTGATGCAAG-3'. A typical condition for the PCR was 94°C for 2 min, 32 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min, followed by 72°C for 5 min.

RNA Interference
RNAi was performed essentially as described [29]. Briefly, a scl-1 cDNA segment was cloned into the feeding vector pPD129.36 and was transformed to HT115 bacterial cells. NGM plates containing 1 mM IPTG and 50 µg/ml Ampicillin were seeded with the bacterial culture grown for 8–18 hr. Seeded plates were allowed to dry at room temperature, and induction was continued at room temperature overnight. The DNA primers used for scl-1 RNAi were 5'-CCC TCGAGACTTCTATTCTGCGTCCTC-3' and 5'-CCTCGAGTTATCGCAGAAAGCC-3'.

Photography of Autofluorescence
Endogenous gut fluorescence was photographed with the DM505 filter (BP460–490 and BA510IF).
Life Span and Stress Resistance Assays

Life span assays were done at 20°C. L4s or young adults were picked to normal or RNAi plates containing 400 µg/ml 5’-fluoro-2’-deoxyuridine (FUDR) at 20°C. Animals were tapped every 2–4 days and were scored as dead when they did not move after repeated taps with a pick. After scoring, they were transferred to new plates. Before the stress resistance assay, eggs were put on normal or RNAi plates without FUDR and were allowed to grow until they reached young adulthood. In the heat resistance assay, they were transferred to new plates without FUDR (normal, RNAi) and were set at 35°C. Dead animals were scored every hour. In the UV stress assay, the young adults were washed in M9 buffer and were exposed to UV light (0.2 J) on NGM plates without food. After exposure, animals were transferred to new plates with FUDR (normal, RNAi) and were set at 16°C. Dead animals were scored every day. In both the life span and stress resistance assays, a difference in diet bacteria, OP50 or HT115, did not affect the results for non-RNAi-treated animals.

Heat Shock, UV Stress, and Starvation

Mixed-stage cultures were stressed by heat (27°C or 35°C, 2 hr) on an NGM plate with food. For UV stress, mixed-stage cultures were stressed by UV light (0.2 J) as described above. For starvation, mixed-stage cultures were washed three times and were incubated on an NGM plate without food at 20°C.

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