Regulation of *Caenorhabditis elegans* RNA Interference by the *daf-2* Insulin Stress and Longevity Signaling Pathway

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A conserved insulin-like signaling pathway regulates metabolism, development, stress resistance, and life span in *Caenorhabditis elegans* (Lee et al. 2003). This pathway involves an insulin-like receptor DAF-2, a phosphatidylinositol-3-OH kinase (PI3K) AGE-1, and the kinases AKT-1, AKT-2 (Paradis and Ruvkun 1998), and PDK-1 (Paradis et al. 1999), as well as the fork head transcription factor DAF-16 (Ogg et al. 1997; Lee et al. 2003). Loss-of-function mutations in *daf-2* or *age-1* cause the worm to arrest at the dauer diapause stage rather than proceeding to reproductive development (Ogg et al. 1997). In addition, the *daf-2* and *age-1* mutants have increased life span and stress resistance, compared with wild type (Ogg et al. 1997; Honda and Honda 2002; Lee et al. 2003). Both the dauer-constitutive and life span phenotypes of the *daf-2* or *age-1* mutants are suppressed by loss-of-function mutation in the downstream *daf-16* gene (Ogg et al. 1997).

RNA interference, or RNAi, is well conserved across phylogeny and protects the genome from invasive genetic elements such as transposons and viruses (Denli and Hannon 2003). Exogenous double-stranded RNA (dsRNA) is cleaved by the RNase III Dicer into 22-nucleotide short interfering RNA (siRNA) (Bernstein et al. 2001). siRNA is incorporated into a protein complex called RNA-induced silencing complex (RISC), which recognizes and destroys the target mRNA based on the sequence homology between the target mRNA and the trigger dsRNA (Song et al. 2004).

Here we show that *C. elegans* mutants with decreased insulin-like signaling have a more intense RNAi response than wild type. Such regulation of RNAi by this stress and longevity signaling pathway suggests a role in response to pathogens such as viruses.

SECTION THEMES

Decreased *age-1*/PI3-Kinase Signaling Enhances RNAi

Mutants lacking *age-1* activity show enhanced response to RNAi. We used dsRNAs, which cause less severe or penetrant phenotypes than the corresponding loss-of-function mutations, to test the insulin signaling-defective mutants. *age-1(mg305)* is a temperature-sensitive allele that causes dauer arrest at 25°C or higher temperatures. *age-1(mg305)* animals display a tempera-

ture-sensitive enhanced RNAi response at the nonpermissive temperature of 25°C. For instance, loss-of-function mutation in the *lin-1* ETS transcription factor gene causes production of multiple vulvae (Muv). RNAi of *lin-1* causes multiple vulvae in 96% of the *age-1(mg305)* mutants at 25°C, but 0% at 20°C, and 0% of wild-type animals at both temperatures. Similarly, mutations in the *daf-2*/insulin-like receptor gene show enhanced response to RNAi (Table 1). The increase in longevity, stress resistance, and fat storage caused by *daf-2* or *age-1* mutations is strongly suppressed by mutations in *daf-16*, a fork head transcription factor coding gene. The enhanced RNAi phenotype is also mediated by signaling via DAF-16: the *age-1;daf-16* double mutant shows wild-type response to *lin-1* dsRNA (Table 2). These data suggest that insulin-like signaling normally inhibits RNAi via the DAF-16 transcriptional cascade.

age-1 Mediates RNAi Response via mRNA Abundance

The phenotypic difference between *age-1* and wild type in response to dsRNA correlates with the different change of the target mRNA level. RNAi of the histone gene *his-44* causes 100% early larval arrest in *age-1(mg305)*, but does not cause this phenotype in wild type. Similarly, other RNAi of a wide range of histone genes is enhanced in *age-1(mg305)*. Consistent with the enhanced RNAi phenotype, northern analysis shows that after feeding *his-44* dsRNA, *his-44* mRNA level is significantly decreased in *age-1(mg305)*, whereas no change is observed in wild type (Fig. 1). This decrease in *his-44* mRNA level was similar to that induced by the previously known RNAi enhancer mutant *rrf-3(pk1426)* (Simmer et al. 2002), which also enhances the lethality induced by *his-44* RNAi. Therefore, the insulin-like pathway enhances RNAi by facilitating the degradation of target mRNA.

The Dauer-Regulatory Pathways Overlap with the RNAi Pathway

Three parallel signaling cascades regulate dauer arrest in *C. elegans*: the *daf-2*/insulin-like, *daf-7*/TGF- β -like, and *daf-11*/cyclic GMP pathways (Li et al. 2003). Mutants lacking activity of either pathway arrest at the dauer stage; however, only mutations in the *daf-2* insulin-like pathway

Table 1. The Dauer-Constitutive Mutants Show Different Sensitivity to RNAi

Strains	Feeding RNAi (phenotype)			
	<i>his-44</i> (% L1/L2 arrest)	<i>lin-1</i> (% Muv)	<i>hmr-1</i> (% embryonic lethal)	<i>col-183</i> (dumpy)
<i>age-1(mg305ts)</i>	75 ± 2	96 ± 7	81 ± 8	+
<i>daf-2(e1370ts)</i>	91 ± 6	65 ± 6	79 ± 5	+
<i>daf-7(mg1372ts)</i>	7 ± 3	22 ± 5	41 ± 9	—
<i>daf-11(mg295ts)</i>	9 ± 6	0 ± 0	35 ± 7	—
<i>rrf-3(pk1426)</i>	86 ± 11	77 ± 10	96 ± 2	++
Wild type (N2)	12 ± 9	0 ± 0	28 ± 6	—

Animals were fed bacteria expressing dsRNA targeting *his-44*, *lin-1*, or *col-183* from the L1 stage at 25°C. For the *lin-1* and *col-183* experiment, dauers from 25°C were recovered at 20°C and allowed to proceed to adulthood when the multiple vulval (Muv) and dumpy body shape phenotypes were scored. For the *col-183* experiment, —, +, and ++ indicate no (—), dumpy (+), and extremely dumpy (++) body shape. For the *hmr-1* experiment, animals at young adult stage were fed *hmr-1* dsRNA and the percentage of embryonic lethality among their progeny was scored.

increase longevity and stress resistance of reproductively growing animals (Tissenbaum and Ruvkun 1998; Wolkow et al. 2000). To decide whether the RNAi-enhanced phenotype is caused by some general dauer-inducing signal shared by all three pathways, we tested the RNAi response of dauer-constitutive mutants in the other two pathways, *daf-7/TGF-β* and *daf-11/guanylate cyclase*. *daf-11(mg295ts)* is no more sensitive to RNAi than wild type. *daf-7(mg1372)* is slightly more sensitive than wild type to *lin-1* (22% Muv vs. 0% in wild type) and *hmr-1* (41% embryonic lethal vs. 28% in wild type) dsRNAs, but no different than wild type in response to *his-44* dsRNA (7% embryonic lethal vs. 12% in wild type) or *col-183* dsRNA (not dumpy) (Table 1). The weak RNAi enhancement of a *daf-7* mutant may reflect the known cross-talk between the insulin and TGF-β pathways (Lee et al. 2001; Li et al. 2003). We conclude that RNAi, like stress resistance and longevity, is most affected by mutations in the insulin-like pathway.

CONCLUSIONS

Mutations in the insulin-like pathway enhance RNAi response and this enhancement is dependent on the DAF-16 fork head transcription factor. The insulin-like metabolic and longevity signaling is transduced by the fork head transcription factor DAF-16 (Ogg et al. 1997).

Table 2. The *age-1* Mutant Has Enhanced Response to *lin-1* dsRNA

Strains	% of Muv animals	
	20°C	25°C
<i>age-1(mg305ts)</i>	0 ± 0	96 ± 7
<i>daf-16(mgDf47)</i>	1 ± 3	5 ± 5
<i>age-1(mg305ts); daf-16(mgDf47)</i>	1 ± 2	7 ± 3
<i>rrf-3(pk1426)</i>	64 ± 6	77 ± 4
Wild type (N2)	0 ± 0	0 ± 0

Animals of the indicated genotypes were fed bacteria expressing *lin-1* dsRNA at 20°C or 25°C since the L1 larval stage. The percentage of animals with multiple vulval structures (Muv) was scored. For the experiment with *age-1(mg305ts)* at 25°C, animals arrested as dauers and were then recovered at 20°C to proceed to adulthood when the Muv phenotype was scored.

The nuclear localization of DAF-16 is regulated by insulin-like signaling (Lee et al. 2001). One model for how insulin-like signaling affects RNAi is that components that positively regulate RNAi, like *dcr-1* and *rde-1*, are positively regulated by DAF-16; or components that negatively regulate RNAi, like *eri-1* (Kennedy et al. 2004) and *rrf-3* (Simmer et al. 2002), are negatively regulated by DAF-16. The DAF-16 binding site has been determined and 947 *C. elegans* genes were identified to bear at least one DAF-16 binding site within 1 kilobase (kb) upstream of their predicted transcriptional start sites. Among these genes, 17 genes are orthologous between *Drosophila* and *C. elegans*, highlighting the response pathways that may be conserved (Lee et al. 2003). None of those conserved genes with consensus DAF-16 binding sites correspond to known RNAi factors, but many regulatory steps in RNAi have yet to be identified.

Recent study shows that a conserved and pancreatic islet-specific microRNA, miR375, regulates the insulin signaling pathway in mammals (Poy et al. 2004). Overexpression of miR375 suppresses glucose-induced insulin secretion and, conversely, inhibition of endogenous *miR-375* function enhances insulin secretion (Poy et al. 2004). MicroRNA processing shares the same machinery with RNAi, which includes the RNase III Dicer and the *Argonaute* proteins ALG-1 and ALG-2 (Grishok et al. 2001). The fact that mutation in the insulin-like pathway enhances RNAi suggests a possible negative feedback between insulin signaling and miRNA/siRNA process-

Figure 1. Mutation in *age-1* enhances the suppression of the target mRNA of RNAi. *age-1(mg305)*, *rrf-3(pk1426)*, and wild type were fed bacteria expressing *his-44* dsRNA (+) or a control vector L4440 (–) for 12 hr at 25°C. The change of mRNA was determined by northern blot.

ing. Such a feedback loop could control the amount of insulin signaling via regulation of microRNAs that in turn regulate the secretion of insulins.

Alternatively, the interaction between the insulin and RNAi pathways may occur at the chromatin level. In *C. elegans*, dauer larvae express histone H1 at a higher level than nondauer animals (Jones et al. 2001). The *C. elegans* homolog of SIR-2, which mediates chromatin silencing in yeast, functions in the insulin-like signaling pathway. Overexpression of the *sir-2* gene mimics the loss-of-function mutation in *daf-2* or *age-1* to extend the life span and favors the dauer entry, and both phenotypes are suppressed by a mutation in the downstream *daf-16* gene (Tissenbaum and Guarente 2001). A distinct chromatin packaging in dauers and animals deficient in insulin signaling may enhance the action of siRNAs in chromatin remodeling to suppress the transcription of RNAi target genes. On the other hand, components of the RNAi machinery, including Dicer and Argonaute, are required for the assembly and localization of heterochromatin in flies (Pal-Bhadra et al. 2004) and fission yeast (Verdel et al. 2004). There may be a competition between heterochromatin formation and RNAi. It is possible that the change in chromatin structure in the *daf-2* mutants releases RNAi components from heterochromatin to now allow a more robust silencing of mRNAs.

Mutants lacking the insulin-like signaling, such as *daf-2* and *age-1*, are more resistant than wild type to environmental stresses (Honda and Honda 2002) as well as pathogens (Garsin et al. 2003). RNAi components also mediate silencing of transposons in *C. elegans* (Sijen and Plasterk 2003) as well as resistance to virus infection in *Arabidopsis* (Mourrain et al. 2000). Thus the coupling of RNAi responses to the stress resistance pathway of *daf-2*/insulin-like signaling makes biological sense—as part of a stress resistance pathway, RNAi responses may be enhanced. It is intriguing to contemplate that the aging regulation by the *daf-2*/insulin signaling pathway could also depend in part on RNAi pathways.

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