ROP-1, an RNA quality-control pathway component, affects *Caenorhabditis elegans* dauer formation

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Edited by William B. Wood, University of Colorado, Boulder, CO, and approved September 13, 2000 (received for review June 19, 2000)

***Caenorhabditis elegans* dauer formation is an alternative larval developmental pathway that the worm can take when environmental conditions become detrimental. Animals can survive several months in this stress-resistant stage and can resume normal development when growth conditions improve. Although the worms integrate a variety of sensory information to commit to dauer formation, it is currently unknown whether they also monitor internal cellular damage. The Ro ribonucleoprotein complex, which was initially described as a human autoantigen, is composed of one major 60-kDa protein, Ro60, that binds to one of four small RNA molecules, designated Y RNAs. Ro60 has been shown to bind mutant 5S rRNA molecules in *Xenopus* oocytes, suggesting a role for Ro60 in 5S rRNA biogenesis. Analysis of ribosomes from a *C. elegans rop-1*(−) strain, which is null for the expression of Ro60, demonstrated that they contain a high percentage of mutant 5S rRNA molecules, thereby strengthening the notion of a link between the *rop-1* gene product and 5S rRNA quality control. The Ro particle was recently shown to be involved in the resistance of *Deinococcus radiodurans* to UV irradiation, suggesting a role for the Ro complex in stress resistance. We have studied the role of *rop-1* in dauer formation. We present genetic and biochemical evidence that *rop-1* interacts with dauer-formation genes and is involved in the regulation of the worms' entry into the dauer stage. Furthermore, we find that the *rop-1* gene product undergoes a proteolytic processing step that is regulated by the dauer formation pathway via an aspartic proteinase. These results suggest that the Ro particle may function in an RNA quality-control checkpoint for dauer formation.**

Ro ribonucleoprotein complex | aspartic proteinase | ribosome biogenesis | 5S rRNA | *daf-2*

The dauer pathway is a specialized stress-resistant, alternative developmental stage of the nematode *Caenorhabditis elegans* (for review, see ref. 1). Developing worms are prone to form dauer larvae when environmental conditions become unfavorable for growth and reproduction. To make this developmental decision, the worms integrate a variety of sensory information about environmental conditions, including the density of conspecific animals, food availability, and temperature (2, 3). When growth conditions improve, dauer larvae can exit the dauer stage and resume normal development. The subsequent life history parameters of adult animals who passed through the dauer stage, such as brood size and life span, are independent of the time spent as dauer larva. The worm monitors external conditions to determine whether it is favorable to enter the dauer stage rather than to proceed along the normal developmental program, but it is currently not known whether internal conditions, such as previously sustained molecular damage, are also monitored.

A complex cascade of genetic interactions regulates entry into the dauer stage, and at least three pathways have been defined: *daf-2*, *daf-7,* and *daf-11*. The *daf-2* gene encodes an insulin receptor-like tyrosine kinase and regulates growth and development by modulating the activity of *daf-16. daf-16* encodes a member of the Forkhead family of transcription factors (4, 5), a signaling pathway that is conserved in mammals (6). *daf-7* encodes a member of the transforming growth factor β family of signaling proteins, and *daf-11* encodes a guanylyl cyclase molecule (7, 8). The *daf-7* and *daf-11* pathways mediate some of their effects through the gene *daf-12*, which encodes a nuclear hormone receptor (9, 10).

The Ro ribonucleoprotein complex was initially identified as an autoantigen in disorders such as systemic lupus erythematosus and Sjögren's syndrome (11–13). It has been found in several eukaryotes, from nematodes to human, and in one prokaryote, *Deinococcus radiodurans* (14). It is composed of at least one protein of ≈60 kDa (Ro60) that binds to a small RNA polymerase III transcript (Y RNA; for review, see ref. 15). Although the exact function of the complex remains elusive, Ro60 has been shown to bind misfolded copies of 5S rRNA in *Xenopus* and thus appears to play a role in 5S rRNA quality control (16, 17). Ro60 has also been shown to associate with the 5′ untranslated region of the mRNA encoding L4 ribosomal protein in *Xenopus*, again implying a role for Ro60 in ribosome biogenesis (18). No function has been proposed for Y RNA molecules, although the levels of the *C. elegans* Y RNA depend on the presence of Ro60, suggesting that Ro60 is necessary for Y RNA stability and, perhaps, function (19). Recently, characterization of the Ro particle in the radiation-resistant eubacterium *D. radiodurans* revealed a role for this complex in resistance to UV radiation (14).

We studied the role of *rop-1* in the formation of *C. elegans* dauer larvae. We report herein that *rop-1*(−) animals do not readily form dauer larvae under conditions that induce dauer formation in wild-type animals and that *rop-1* genetically interacts with *daf-2* and *daf-7*, two genes involved in the regulation of dauer formation. These results are strengthened by the observation that the *rop-1* gene product is processed by an aspartic proteinase during larval development and that this proteinase is regulated by the activity of genes involved in dauer formation.

Methods

Strains. *C. elegans* strains were grown as described by Brenner (20). All animals were grown at 18°C unless otherwise stated. The wild-type strain used was the Bristol N2 strain. The mutations used in this study were as follows: LGI, *daf-16(m26)*; LGII, *age-1(hx546)*, *daf-5(e1386)*; LGIII, *daf-7(e1372)*, *dpy-1(e1)*, *daf-2(e1370, m41, e1365, m579, m596)*, *unc-32(e189)*; LGV, *unc-42(e270)*, *rop-1(pk93)*; LGX, *daf-12(m20)*.

Construction of *daf-2; rop-1* and *daf-7; rop-1* Double Mutants. *daf-2* or *daf-7* mutant animals were mated with *unc-42* males at 25°C,

This paper was submitted directly (Track II) to the PNAS office.

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Article published online before print: *Proc. Natl. Acad. Sci. USA*, 10.1073/pnas.230284297. Article and publication date are at www.pnas.org/cgi/doi/10.1073/pnas.230284297

and F_1 animals that did not form dauers were isolated singly on plates. These F_1 heterozygotes were allowed to lay eggs at 25°C, and the F_2 dauer animals that displayed an Unc-42 phenotype were isolated and recovered at 15°C. These *daf-2; unc-42* or *daf-7; unc-42* hermaphrodites were mated with *rop-1* males at 25°C, and F_1 animals that did not form dauers were isolated singly on plates. These F_1 heterozygotes were allowed to lay eggs at 25°C, and the F_2 dauer animals that did not display an Unc-42 phenotype were isolated and recovered at 15°C. The F_2 animals that did not give rise to *unc-42* mutants in their progeny had the *daf-2; rop-1* or *daf-7; rop-1* genotype, as confirmed by PCR analysis of the *rop-1* locus (19).

Dauer Formation Assays. Dauer formation of all strains was assayed as described (21). To disrupt the activity of *daf-16*, both sense and antisense strands of *daf-16* coding region were synthesized *in vitro* and annealed. Young adult animals were injected with the double-stranded RNA as described (22) and allowed to recover for 6–12 h, and their progeny were scored for dauer formation. This method has been shown to phenocopy strong loss-of-function alleles of targeted genes (for review, see ref. 23). To rescue the *rop-1* dauer phenotype, we injected *daf-2:rop-1* animals with a mixture of wild-type *C. elegans* genomic DNA, the wild-type *rop-1* gene, and the plasmid pRF4 [containing a dominant mutation in the collagen gene, *rol-6(su1006)*], using the injection procedure and complex array method as described (24, 25). As a control to rule out a possible influence of genomic DNA and pRF4 on dauer formation, *daf-2; rop-1* animals were injected with these two components alone. The percentage of dauer formation in these transgenic strains was the same as nontransgenic animals, which confirms that wild-type *rop-1* alone is necessary and sufficient for the rescue of the dauer formation phenotypes scored.

To score dauer formation of *rop-1(pk93)* animals, plates with identical bacterial lawns were seeded with various numbers of worms were then placed at 25°C. These populations were left to multiply on the plate until food was exhausted. Seeding the plates with various numbers of worms ensured that at least some plates would have many worms capable of becoming dauer larvae upon starvation of the population. Dauer formation was determined by visual inspection of mutant animals and by resistance to SDS treatment.

Preparation of Dauer Extracts. For dauer larvae preparation, wild-type animals were grown in liquid culture at 20°C until food was exhausted. Fresh food was then added to the medium. The worms were then incubated at 20°C until food was again exhausted, and many dauer larvae had formed. Worms were subsequently collected, resuspended in M9 buffer containing 1% SDS, and incubated in this solution for 15–30 min at room temperature. The suspension was then washed with M9 buffer and worms were placed on a fresh unseeded plate at 20°C for 12 h. Because dauer larvae are resistant to SDS (2), the dauer larvae that had crawled away from the layered dead worms could be collected in M9 buffer.

For dauer recovery, dauer larvae were transferred to a fresh plate containing food and incubated for 8 h at 20°C. The recovery from dauer stage was confirmed by visual inspection of the worms and by the resuming of food uptake by the animals.

Proteinase Assays. Staged worm extracts were grown and prepared as described (26). Briefly, worm pellets were ground in liquid nitrogen until nematode cuticles were opened. The ground material was resuspended in NET-2 buffer (40 mM Tris·HCl, pH 7.4/150 mM NaCl/0.05% Nonidet P-40), insoluble molecules were removed by centrifugation, and supernatants were stored at −80°C. To reconstitute the aspartic proteinase activity *in vitro*, 100 μg of embryo extract was mixed with 50 μg of wild-type or *rop-1*(−) L4 extract in 100 mM Tris acetate buffer (pH 6.00). The reaction mixtures were incubated at 25°C for 2 h and then stopped by the addition of 1× SDS sample buffer (12.5 mM Tris·HCl, pH 6.8/4% glycerol/0.4% SDS/1.25% 2-mercaptoethanol/0.005% bromophenol blue) followed by 3 min of boiling. Addition of Mg^{2+}, Ca^{2+}, or Zn^{2+} to the reaction mixture had no effect on the enzymatic activity detected. Proteins were resolved by SDS/PAGE on 8% gels. Western blot analyses were carried out as described with a ROP-1-specific antibody (27), and bound antibodies were detected with the Renaissance system (Dupont), according to the manufacturer's recommendations.

The processing of ROP-1 that we observed occurs *in vivo* and is specific for the following reasons: (*i*) We still observed processing of ROP-1 when adult animals were collected, resuspended in SDS sample buffer, and denatured by boiling (data not shown); this would require that the processing of ROP-1 occurred within 5 min at 100°C in denaturing conditions, which is quite unlikely. (*ii*) We did not detect major degradation of the protein extracts, even if incubated at 25°C for 2 h without adding any proteinase inhibitors (data not shown). (*iii*) We did not observe processing of ROP-1 in embryo extracts, even when incubated at 25°C for 2 h without adding any proteinase inhibitors (Fig. 2, lanes 3 and 7). (*iv*) If we mixed embryo extracts with L4 extracts, we observed some processing, demonstrating that ROP-1 can be processed in embryo extracts (Fig. 2, lanes 5 and 8). (*v*) When we prepared extracts from both embryo and adult animals simultaneously, and ran this mixed extract without an incubation step, we observed both ROP-1 bands. This demonstrates that the proteinage activity present in the adult does not process the ROP-1 protein in the embryos unless they are incubated together at 25°C (data not shown). (*vi*) When we incubated embryo extracts with L4 extracts at pH 7.00 (a pH close to that of NET-2 buffer in which the worm extracts are resuspended) for 2 h at 25°C, we did not observe any processing of ROP-1 (Fig. 2, lane 6).

Results

***rop-1* Interacts with Components of the *daf-2* and *daf-7* Dauer Formation Pathways.** We have shown that the allele *pk93* is a null allele of *rop-1* (19). To test a possible role for *rop-1* in the dauer pathway, we first scored dauer formation in a strain mutant for *rop-1*. Plates with identical bacterial lawns were seeded with various numbers of worms and then placed at 25°C. Under these conditions, most plates containing wild-type animals yielded large number of dauers, but none of the plates containing *rop-1(pk93)* had dauers. Therefore, *rop-1*(−) animals do not form dauer larvae under starved and crowded conditions at 25°C. To understand the basis of this phenomenon, we investigated whether *rop-1* interacts with *daf-2*, one of the genes that is crucial to dauer formation. *daf-2* encodes an insulin receptor-like tyrosine kinase and has been shown to regulate a variety of developmental and metabolic processes, including life span (6, 28). Conditional mutations in *daf-2* result in a dauer-constitutive phenotype where animals enter the dauer stage at the restrictive temperature even under environmental conditions that normally do not induce dauer formation. All phenotypes of *daf-2* alleles are suppressed by mutations in *daf-16*, which encodes a transcription factor of the Forkhead family that appears to be one of the most downstream effectors in the *daf-2* signal transduction pathway (4, 5).

To study in more detail the effect of *rop-1* disruption on dauer formation, we constructed *daf-2; rop-1* double mutant strains with four different alleles of *daf-2*: *e1370*, *m41*, *m579,* and *m596*. As shown in Table 1, the absence of *rop-1* function has very different effects on dauer formation in combination with various *daf-2* alleles. At 20°C and 22.5°C, we observed that the disruption of *rop-1* in combination with the *daf-2* alleles *e1370* and *m579* led to a significant increase in the percentage of dauer formation

Table 1. Percentage of dauer formation in wild-type and mutant strains

Strain	Genotype	% dauer formation 20°C*	22.5°C†
N2	Wild type	0	0
MQ470	*rop-1(pk93)*	0	0
CB1370	*daf-2(e1370)*	4.4 ± 2.1‡ (6443; 8)	40.0 ± 2.6‡ (1350; 3)
MQ844	*daf-2(e1370); rop-1(pk93)*	42.3 ± 6.4 (6010; 8)	89.3 ± 3.4 (1826; 3)
CB1370+	*daf-16(RNAi); daf-2(e1370)*	0.8 ± 0.9 (820; 1)	ND
MQ844+	*daf-16(RNAi); daf-2(e1370); rop-1(pk93)*	0.7 ± 1.5 (350; 1)	ND
MQ845	*daf-2(e1370); rop-1(pk93); qmEx202 [rop-1(+); pRF4]*	6.4 ± 1.2 (2177; 4)	59.7 ± 2.0 (740; 3)
DR1566	*daf-2(m579)*	4.2 ± 1.7‡ (3149; 3)	60.9 ± 4.4‡ (2500; 3)
MQ846	*daf-2(m579); rop-1(pk93)*	74.7 ± 9.3 (2975; 3)	99.1 ± 0.5 (1981; 3)
DR1564	*daf-2(m41)*	9.2 ± 1.2§ (4031; 4)	99.5 ± 2.6§ (2019; 3)
MQ841	*daf-2(m41); rop-1(pk93)*	0.1 ± 0.1 (3836; 4)	5.6 ± 1.9 (2713; 3)
DR1565	*daf-2(m596)*	2.2 ± 0.1§ (3179; 3)	69.4 ± 3.9§ (2078; 3)
MQ847	*daf-2(m596); rop-1(pk93)*	0.2 ± 0.1 (2362; 3)	15.4 ± 4.8 (1725; 3)
CB1372	*daf-7(e1372)*	16.1 ± 3.9 (5601; 7)	89.7 ± 4.5¶ (1242; 2)
MQ804	*daf-7(e1372); rop-1(pk93)*	32.6 ± 3.8 (3982; 7)	65.7 ± 4.3¶ (1001; 2)

Experiments were performed as described by Gems *et al.* (21). The value corresponds to the percentage of dauer larvae formed from broods of 5–10 individual animals, depending on the trial (mean ± SEM across one–eight trials). The total number of animals scored is the first number given in parentheses. The number of trials is the second number given in parentheses.

*Scored 96 h after hatching.

†Scored 72 h after hatching.

‡Many dauer-like animals with intermediate characteristics of L3 or L4 stage larvae.

§Animals take 4 to 5 days to reach adulthood.

¶This particular set of experiments was performed at 25°C.

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when compared with animals containing either mutation alone (Table 1). In addition to dauer larvae, morphologically abnormal L2 and L4 larvae are also formed by the *daf-2(e1370)* and *daf-2(m579)* strains. This partial dauer phenotype is suppressed by *rop-1(pk93)* and all of the dauer animals formed in the double mutant strains appeared morphologically as normal dauer larvae.

Dauer formation in combination with two other alleles of *daf-2, m41* and *m596,* was also scored at 20°C and 22.5°C, and we observed that disruption of *rop-1* in these backgrounds caused a strong suppression of the dauer-constitutive phenotype of both alleles when compared with either mutation alone (Table 1). The suppression of *daf-2(m41)* and *daf-2(m596)* by *rop-1(pk93)* also restored the time needed by the animals to reach adulthood. Indeed, while *daf-2(m41)* and *daf-2(m596)* animals take more time than the wild type to reach adulthood (4–5 days instead of 3 days), the developmental rate of *daf-2(m41); rop-1(pk93)* and *daf-2(m596); rop-1(pk93)* double mutants is like that of wild-type animals (Fig. 1 *a* and *b*).

The dauer formation phenotype of the *daf-2(e1370); rop-1(pk93)* double mutant strain was rescued by an array expressing transgenic *rop-1*(+), thereby indicating that the dauer formation effect of *rop-1(pk93)* on *daf-2* mutations is specifically caused by the disruption of the *rop-1* gene and not to a nonspecific strain effect (Table 1). Similarly, we succeeded in rescuing the slow growth of *m41*, but not the dauer formation phenotype, by expressing *rop-1*(+) in the *daf-2(m41); rop-1(pk93)* double mutant (Fig. 1). The reason for this only partial rescue might be that *daf-2(m41)* is presumably more severely affected by *rop-1(pk93)* than *daf-2(e1370)*, because *pk93* prevents the expression of the normal phenotype of the mutant (Daf-c). We found previously that *rop-1*(+) extrachromosomal arrays did not produce ROP-1 at high level and did not rescue *rop-1*(−) phenotypes very efficiently (19). Similarly, a *rop-1*(+) array might not fully rescue all phenotypes of the most severely affected double mutant. Using RNA interference (22), we found that the phenotype of *daf-2(e1370)* and *daf-2(m41)* alone, or in combination with *rop-1*, was also suppressed by the disruption of *daf-16*, indicating that the effect of *rop-1* disruption on *daf-2* is dependent on the downstream Forkhead transcription factor (Table 1 and data not shown).

To determine whether *rop-1* could also affect dauer formation in presence of the wild-type allele of *daf-2*, we tested possible interactions of *rop-1* with *daf-7*, a gene encoding a transforming growth factor-β-like protein that regulates dauer formation by a pathway that appears to be independent of *daf-2* (7, 29). As shown in Table 1, *rop-1* affects dauer formation in combination with *daf-7(e1372)*. At 20°C, disruption of *rop-1* enhanced dauer formation in a *daf-7* background. However, at 25°C, the converse effect was observed, and *rop-1* suppressed the dauer formation

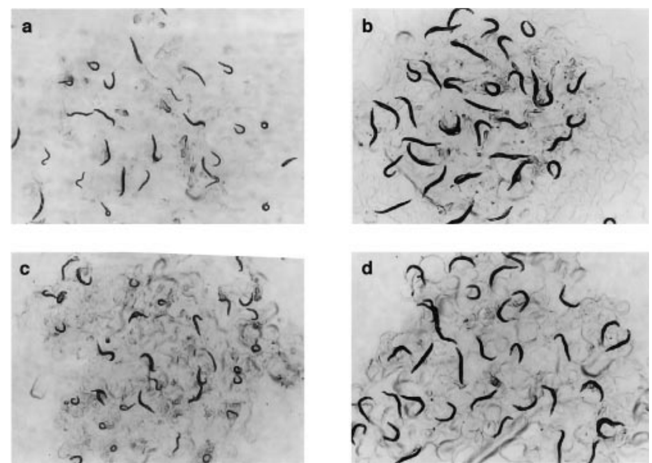

Fig. 1. Growth rate of *daf-2(m41)* strains. After 3 days of growth at 20°C, most of the *daf-2(m41)* animals were at the L3–L4 larval stages (*a*), while all of the *daf-2(m41); rop-1(pk93)* animals had reached adulthood (*b*). This phenotype is caused by the disruption of *rop-1*, because it could be rescued by a transgenic array containing *rop-1*(+) and the dominant marker *rol-6(su1006)* (*c*) but not by the dominant marker alone (*d*). Animals in all panels were synchronized at the start of their development.

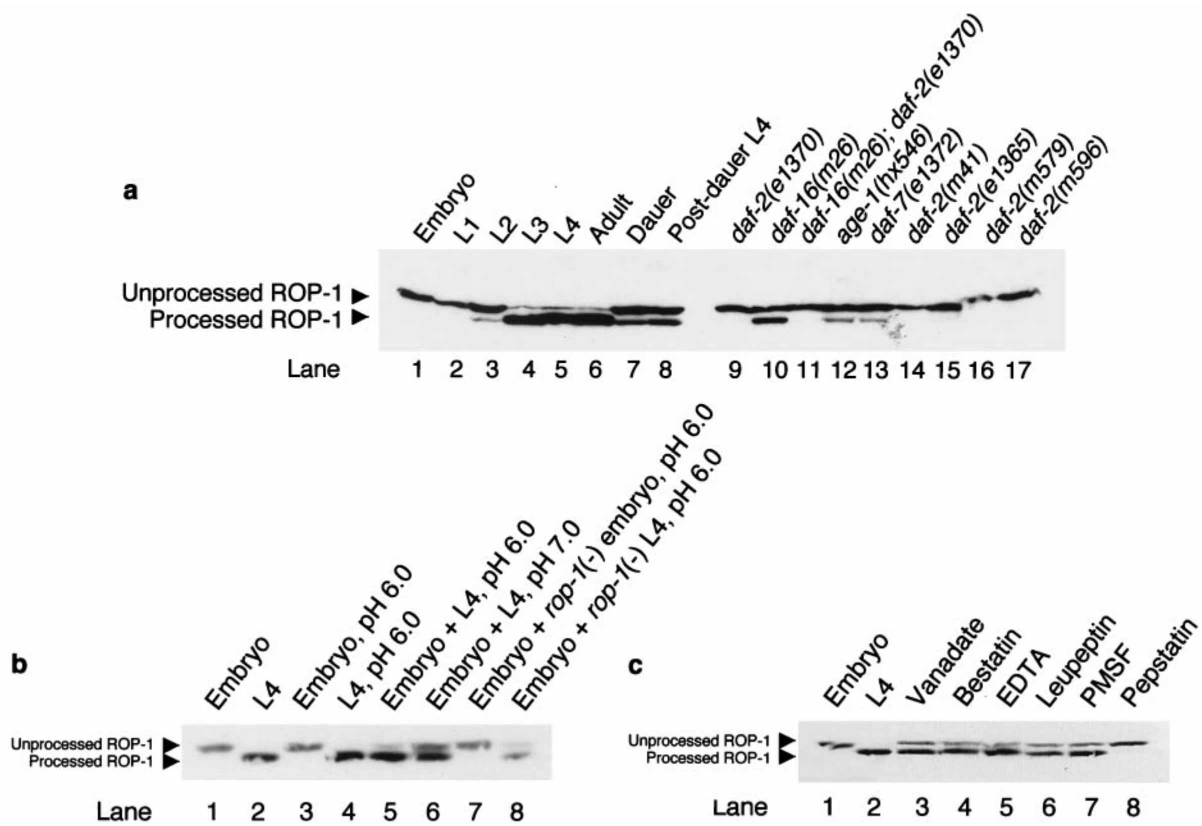

Fig. 2. Dauer genes regulate the processing of ROP-1 by an aspartic proteinase during *C. elegans* larval development. (*a*) Western blot analysis of ROP-1 on 100 μg of total protein extract from staged animals revealed that ROP-1 undergoes a mobility change during *C. elegans* larval development. In the wild type, the mobility shift of ROP-1 occurs at the L2–L3 stage transition (lanes 3 and 4). This mobility shift is influenced by components of the dauer formation pathway (lanes 9–17). All mutant extracts were obtained from L4 animals grown at 20°C in the same conditions as wild-type animals and, thus, should be compared with the wild-type L4 extract (lane 5). (*b*) The *in vitro* reconstitution of the ROP-1 processing activity revealed that although embryo extracts alone did not contain the activity (lane 3 and 7), addition of L4 extracts from wild type or *rop-1*(−) was sufficient to allow the processing of ROP-1 at pH 6.00 (lanes 5 and 8). Increasing the pH to 7.00, completely inhibited the processing of ROP-1 (lane 6). (*c*) The *in vitro* reconstitution of the ROP-1 processing activity in the presence of several inhibitors for various modification enzymes demonstrated that inhibitors of phosphatases, exoproteases, serine proteases, cysteine proteases, and metalloproteases had no effect on the processing of ROP-1 (lanes 3–7, respectively). However, addition of a specific inhibitor of aspartic proteinases (Pepstatin, lane 8) efficiently inhibited the processing reaction, demonstrating that ROP-1 is processed by an aspartic proteinase. Processed and unprocessed forms of ROP-1 are indicated.

phenotype of *daf-7.* A similar temperature-dependent interaction with *daf-7* has been reported for the gene *ttx-3*, a LIM homeobox gene involved in the differentiation of the AIY thermosensory neuron (30). We have investigated the morphology of the AIY neuron in *rop-1* mutants, by visualization with a *ttx*::*gfp* reporter gene and found no anomaly, at least at this level of analysis (data not shown). Our results suggest that the effects of *rop-1* on dauer formation are not specific to particular alleles of *daf-2* but impinge on the whole dauer formation process. Thus, these results indicate that *rop-1* interacts genetically with both *daf-2* and *daf-7* and participates in the regulation of *C. elegans* dauer formation.

The *rop-1* Gene Product Is Processed During *C. elegans* Larval Development. Because *rop-1* and dauer formation genes are interacting genetically, we examined the expression of *rop-1* during development and dauer formation. We first monitored the abundance of the *rop-1* gene product (ROP-1) during normal development and during the alternative dauer development by using anti-ROP-1 antibodies. We observed that during normal development the abundance of ROP-1 is constant but that ROP-1 undergoes a mobility shift (due to proteolytic processing, see below) of about 3.5 kDa between the L2 and L3 larval stages. In embryos and L1 larvae, ROP-1 is detected as a single molecular band (unprocessed; Fig. 2*a*, lanes 1 and 2), but at the L2 stage, although ROP-1 is present mainly as a single form, a minor, faster migrating form can also be detected (processed; Fig. 2*a*, lane 3). This faster migrating form becomes the main form at the L3, L4, and adult stages (Fig. 2*a*, lanes 4–6). This mobility shift is specific, occurs *in vivo,* and is not a consequence of the lysis procedure.

The developmental period between the L2 and L3 larval stages is also the time at which worms either continue normal development or enter the dauer stage (for review, see ref. 1). We therefore looked at ROP-1 mobility in dauer larvae and in L4 larvae whose development had transited through the dauer stage. Although ROP-1 is almost completely processed at the L3 stage, it is mainly unprocessed in the dauer stage (Fig. 2*a*, lane 7). Moreover, ROP-1 is also mainly unprocessed in postdauer L4 animals, although worms have resumed feeding and normal development (Fig. 2*a*, lane 8). This interesting observation indicates that postdauer animals are different from L4 animals whose development did not transit through the dauer stage, although they appear morphologically indistinguishable. These results strongly suggest that the processing of ROP-1 is linked to dauer formation.

To test this further, we monitored the abundance and processing of ROP-1 in dauer formation mutant strains. All mutants

tested displayed no changes in overall levels of ROP-1 but showed a reduction in its processing (Fig. 2*a*, lanes 9–17, and data not shown). Indeed, partial processing was detected in all strains studied, with the exception of all *daf-2* alleles, in which no processing of ROP-1 was observed (Fig. 2*a*, lanes 9, 11, and 14–17). These results indicate that, although the activity responsible for the processing of ROP-1 is modulated by several components of the dauer pathway, it appears to be more tightly regulated by *daf-2.* The ROP-1 processing activity appears to be genetically upstream of *daf-16* because it was present in *daf-16* mutants but absent in *daf-16; daf-2* double mutants (Fig. 2*a*, lanes 10 and 11). Likewise, the activity was present but diminished in *age-1* animals (Fig. 2*a*, lane 12), which are mutant for the catalytic subunit of the phosphatidylinositol 3-kinase on which DAF-2 acts (31), suggesting that DAF-2 might not regulate this proteolytic activity solely through AGE-1 signaling but, at least in part, by an as yet unidentified branch of this signal transduction pathway. Based on the variety of *daf-2* phenotypes, other authors have already postulated the existence of an *age-1*-independent *daf-2* signaling pathway (21, 32–36).

An Aspartic Proteinase Is Involved in the Processing of ROP-1. The distinct pattern of ROP-1 mobility shift suggested that ROP-1 was processed by a developmentally regulated enzymatic activity. To characterize this activity, we performed *in vitro* reconstitution experiments by mixing and incubating L4 extracts, in which ROP-1 appears mostly as the faster-migrating form, with embryo extracts, in which ROP-1 appears solely as the slower-migrating form. By testing various conditions, we found that an acidic pH was necessary to reconstitute the activity (Fig. 2*b*, lane 5). Increasing the pH from 6.0 to 7.0 abolished the mobility shift of ROP-1 (Fig. 2*b*, lane 6), and the highest pH at which maximal processing of ROP-1 occurred was 6.1 (data not shown). Although a mixture of embryo and L4 extracts contains only the faster-migrating form when incubated together at room temperature, embryo extracts incubated alone contain only the slower-migrating form (Fig. 2*b*, lanes 3–5), indicating that the activity causing the mobility shift in ROP-1 is not present in embryo extracts but is found in L4 extracts. Similar results were obtained using *rop-1*(−) extracts (Fig. 2*b*, lanes 7 and 8), indicating that ROP-1 does not regulate the appearance of this enzymatic activity. These results demonstrate that our *in vitro* reconstitution appears to reproduce correctly the conditions observed *in vivo*. Furthermore, the fact that the presence or absence of ROP-1 did not modulate the activity suggests that ROP-1 does not participate in its regulation.

To identify the activity responsible for the processing of ROP-1, we tested inhibitors of phosphatases and several well-characterized proteases in *in vitro* reconstitution experiments. Pepstatin completely abolished the processing of ROP-1 *in vitro*, but the other enzyme-inhibiting compounds tested had no effect on the activity (Fig. 2*c*). Pepstatin has been shown to be a potent inhibitor of all members of the aspartic proteinase family (37). The enzymatic activity of this family of proteases is optimum in mostly acidic pH conditions (38), which is consistent with the complete inhibition at pH 7.00 that we observe (Fig. 2*b*, lane 6). Interestingly, it has previously been reported that there is an intracellular decrease in pH when worms exit the dauer stage to enter the postdauer stage (39). It is possible that such a pH fluctuation, on entry into and exit from the dauer stage, modulates the activity of the aspartic proteinase.

Discussion

We have shown that *rop-1*(−) animals are dauer-defective and that *rop-1* genetically interacts with genes in the dauer pathway. We have also demonstrated that ROP-1 is processed by an aspartic proteinase and the activity of this enzyme is regulated by the dauer pathway, mainly by *daf-2*(+) activity. Thus, these results suggest a model in which DAF-2 inhibits DAF-16 through AGE-1 but regulates the activity of an aspartic proteinase that controls the processing of ROP-1 by a partially independent pathway (Fig. 3). The effects of other dauer gene mutations on ROP-1 processing could be caused by their likely effect on DAF-2 signaling, because their protein products are all part of the same developmental process. Alternatively, because *rop-1* and *daf-7* interact genetically, it is possible that *daf-7* modulates the activity of the aspartic proteinase independently of *daf-2*. The processed form of ROP-1 is present in animals committed to normal development, and the complete absence of both forms of ROP-1 in the *rop-1(pk93)* mutant correlates with inhibition of dauer formation. This suggests that when DAF-2 activity is altered, by induction of dauer formation or by mutations, ROP-1 remains unprocessed and modulates the activity of dauer formation genes by affecting *daf-2, daf-7,* and possibly other genes. A disruption of *rop-1* abolishes this interaction and results in a deregulation of the dauer formation pathway. The exact molecular interactions at play will need to be defined to understand why the *rop-1* disruption has temperature-sensitive effects on *daf-7* and allele-specific effects on *daf-2*.

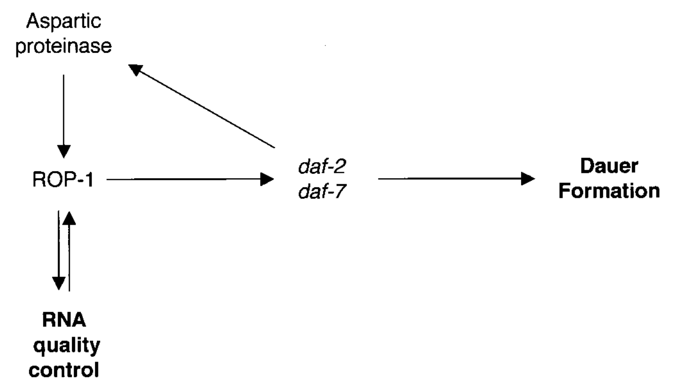

Fig. 3. Proposed model for the interaction of ROP-1 with components of the dauer formation pathway. ROP-1 modulates the activity of components in both the *daf-2* and *daf-7* pathways to influence dauer formation signaling. Favorable growth conditions allow a signal from *daf-2* (and maybe also *daf-7*) to activate an aspartic proteinase. This proteinase in turn catalyses the processing of ROP-1, which relieves its influence on both signaling pathways. This processing allows ROP-1 to perform another set of activities, possibly also related to RNA degradation as part of its RNA quality control function(s).

The fact that *rop-1*(−) displays differential dauer formation phenotypes (enhancement or suppression) in combination with various *daf-2* alleles is puzzling. *daf-2* alleles have been classified in two different groups according to their respective phenotypes (21). However, we do not observe any correlation between the phenotypes we observe and the class of *daf-2* allele that we used. Although the mutation in *e1370* has been characterized (conserved proline to serine change in the tyrosine kinase domain; ref. 6), the molecular lesions in the other three *daf-2* alleles that were used have not yet been published and thus cannot guide our interpretation. One very general model would suggest that DAF-2 interacts with its downstream effectors in more than one way, including activating and inhibiting interactions, with different *daf-2* alleles affecting one set of interactions more than the other. *rop-1(pk93)* might affect only a subset of these interactions and thus show allele-specific effects. This idea is supported by the observation that *daf-2* has allele-specific interactions with at least one other gene, *daf-12* (21, 40). Mapping of the molecular lesions on *daf-2* alleles onto the various sets of allele-specific effects should help clarify these questions.

It has previously been shown that Ro60, the vertebrate homologue of ROP-1, binds defective copies of 5S rRNA and that the disruption of *rop-1* leads to an increase in the number

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of mutant 5S rRNA incorporated into the ribosomes (16, 19), indicating that *rop-1* is involved in a 5S ribosomal RNA quality-control pathway. *rop-1* might be involved in other processes that could impinge on dauer formation, but there is yet no definite evidence for such function(s). Our observations therefore suggests that the dauer formation process might rely on a checkpoint mechanism that monitors the general quality of cellular components to determine whether a lengthy developmental arrest in the dauer stage is likely to be more beneficial to the animal than continuing normal development. In this view, *rop-1* would be part of a mechanism responsible for "sensing" internal cellular damage and relaying the information to components of the genetic pathway that regulates dauer formation. In the absence of *rop-1*, cellular damage could accumulate but not be sensed, which results in the dauer defectiveness phenotype of *rop-1*(−) animals. One corollary of this hypothesis is that entering the dauer stage might be a favorable developmental strategy to repair previously sustained damage before continuing development toward the adult stage. The latter possibility is suggested by the tendency of worms to enter the dauer stage at high temperature, even in the presence of food and the absence of externally added pheromone (41). The Ro complex might therefore be involved in this stress–response mechanism to regulate entry into dauer. This hypothesis is strengthened by the observation that the Ro particle participates in resistance to UV radiation in *D. radiodurans*, supporting the notion that this complex plays important role(s) in response to conditions that cause cellular damage (14).

The monitoring of 5S rRNA quality is likely to be also part of a more general control of ribosomal function. Interestingly, recent work has shown that an insulin-like signaling pathway also affects transcription factors of the Forkhead family in vertebrate cells and that this pathway affects developmental rates (42, 43) and programmed cell death (44–46). This raises the possibility that *rop-1* participates in a general conserved mechanism of developmental regulation that involves monitoring rRNA biogenesis and function.

We are grateful to Mark Edgley and Don Riddle for the gift of *daf-2* alleles *e1365*, *m579,* and *m596*. We also thank Bob Goldstein and Bernard Lakowski for comments on the manuscript, and Guillaume Lesage, Vasudevan Seshadri, Claire Bénard, Brent McCright, Bernard Lakowski, and all of the members of the Rokeach and Hekimi laboratories for helpful discussions and technical advice. Some strains used in this work were provided by the Caenorhabditis Genetics Center, which is funded by the National Center for Research Resources of the National Institute of Health. This work was supported by a studentship from the Fonds pour la Formation de Chercheurs et l'Aide à la Recherche (FCAR, Québec) to J.-C.L., a grant from the Canadian Arthritis Society to L.A.R., and a grant from the Medical Research Council of Canada to S.H.

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