Prolyl 4-hydroxylase is required for viability and morphogenesis in *Caenorhabditis elegans*

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The genome of *Caenorhabditis elegans* possesses two genes, *dpy-18* and *phy-2,* that encode α subunits of the enzyme prolyl 4-hydroxylase. We have generated deletions within each gene to eliminate prolyl 4-hydroxylase activity from the animal. The *dpy-18* mutant has an aberrant body morphology, consistent with a role of prolyl 4-hydroxylase in formation of the body cuticle. The *phy-2* mutant is phenotypically wild type. However, the *dpy-18; phy-2* double mutant is not viable, suggesting an essential role for prolyl 4-hydroxylase that is normally accomplished by either *dpy-18* or *phy-2*. The effects of the double mutation were mimicked by small-molecule inhibitors of prolyl 4-hydroxylase, validating the genetic results and suggesting that *C. elegans* can serve as a model system for the discovery of new inhibitors.

Collagen is the most abundant protein in animals. Each polypeptide chain of collagen is composed of repeats of the sequence: X-Y-Gly, where X is often an L-proline residue, and Y is often a 4(R)-hydroxy-L-proline (Hyp) residue. These chains are wound into tight triple helices, which are organized into fibrils of great tensile strength. The hydroxyl groups of the Hyp residues contribute greatly to the conformational stability of triple-helical collagen (1–3). Hyp residues are not incorporated into collagen by ribosomes. Rather, the hydroxylation of Pro residues in collagen strands is catalyzed by the enzyme prolyl 4-hydroxylase (EC 1.14.11.2). Prolyl 4-hydroxylase has been best characterized in vertebrates, including humans (4–7). The vertebrate enzyme is a tetramer, composed of two α subunits and two β subunits. The α subunit binds a Fe^{2+} divalent cation, α-ketoglutarate and ascorbate, and possesses the active site for hydroxylation. The β subunit is not only an essential component of the tetrameric prolyl 4-hydroxylase, but also has enzymatic activity of its own as protein disulfide isomerase (EC 5.3.4.1), an enzyme that catalyzes the unscrambling of nonnative disulfide bonds (8–10). A deficiency in prolyl 4-hydroxylase activity is observed in animals that lack dietary vitamin C and has severe consequences, resulting in the disease condition known as scurvy (11).

We have begun to investigate the biological role of prolyl 4-hydroxylase in *Caenorhabditis elegans*. This small nematode provides several important experimental advantages for *in vivo* studies: sophisticated forward and reverse genetics (12, 13), a simple body morphology that can be analyzed at the level of individual cells (14), and a virtually complete genome sequence (15). Collagen is a major component of *C. elegans*, comprising approximately 1% of the weight of an adult nematode (16). Most *C. elegans* collagen is in the cuticle and basement membranes (17).

One prolyl 4-hydroxylase α subunit and two potential β subunits were identified in the *C. elegans* genome and characterized biochemically (18, 19). The *C. elegans* α and β subunits are both homologous to their vertebrate counterparts. However, in contrast to the tetrameric vertebrate enzyme, the *C. elegans* α subunit formed an $\alpha\beta$ dimer with either the *C. elegans* β subunit or the human β subunit (protein disulfide isomerase). Like the vertebrate prolyl 4-hydroxylase, the *C. elegans* enzyme interacted

with the Fe^{2+} divalent cation, α-ketoglutarate and ascorbate, the prolyl 4-hydroxylase cofactors, and it also possessed prolyl 4-hydroxylase activity (18, 19).

Here, we report that the *C. elegans* genome possesses two genes that encode α subunits of prolyl 4-hydroxylase. We have generated a deletion mutant of each gene and explored their biological roles. One gene, *dpy-18,* is essential for wild-type body morphology; the other, *phy-2,* is required for *dpy-18* mutant viability, and vice versa. We have also shown that known small-molecule inhibitors of prolyl 4-hydroxylase can mimic the loss of *dpy-18* and *phy-2* gene activities.

Materials and Methods

Strains. Wild-type *C. elegans* is the N2 Bristol strain. Worms were cultured at 20°C under standard conditions (20) unless noted otherwise. The following mutations were used: linkage group (*LG*)*II*: *dpy-10(e128)*; *LGIII*: *dpy-17(e164)*, *dpy-18(e364am)*; *LGIV*: *dpy-13(e184)*, *dpy-20(e1282)*, *unc-22(e66)*; *LGV*: *dpy-11(e224)*; these have been described (21, 22).

Sequence Analysis. FASTA and BLAST programs were used to search the *C. elegans* genome for homologs of the α subunit of prolyl 4-hydroxylase. Sequence comparisons and analyses were performed by using the GCG-Wisconsin package version 10 for UNIX. Cladistic analysis was performed with the PAUP 4.0 beta version (23).

Isolation of Deletion Mutants. Deletion mutants were identified as described by Kraemer *et al.* (24). External primers for Y47D3B.10 (corresponding to *dpy-18*) were 5′-CACGACGAG-GAAGAGCGACTG-3′ and 5′-TACGATTTCCAGTTC-CCAAGC-3′; internal primers were 5′-GAAGAAGCTGTCG-GAGGAGTA-3′ and 5′-ACGGCTAGTGGGTTGAATCTC-3′; the expected PCR product from wild-type genomic DNA is 3.2 kb. External primers for F35G2.4 (corresponding to *phy-2*) were 5′-GCTCATGCAGATTTGTTCACT-3′ and 5′-GTCAG-CAGGAAGGCAGTAAAC-3′; internal primers were 5′-GAGCAGAGAAGGATGTAACAA-3′ and 5′-ATAGTGCG-CATTTCCGTTTCA-3′; the expected PCR product from wild-type genomic DNA is 2.8 kb. Each deletion mutant was outcrossed to wild-type N2 at least three times before further characterization. Deletion end points were determined by sequencing PCR products that spanned the deleted region.

Genetics. Complementation tests were performed by crossing *ok162/+* males into *dpy-18(e364)* homozygotes, and scoring

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male cross progeny. *dpy-18(ok162), dpy-18(e364)*, and *phy-2(ok177)* mutants were tested for temperature sensitivity at 15, 20, and 25°C. To construct the double mutant, we crossed *phy-2(ok177)* homozygous males into *dpy-18(ok162); unc-22(e66)* homozygous hermaphrodites and picked non-Dpy non-Unc cross-progeny of genotype *dpy-18(ok162)/+; phy-2(ok177)/unc-22(e66)*. From these heterozygotes, total broods were examined for phenotype and genotype at 15, 20, and 25°C.

Isolation of Cuticles. To isolate cuticles, L4s were washed in M9 and frozen at −80°C. Packed L4 larvae (2 ml) were thawed and washed with sonication buffer. Cuticle isolation was performed as described (25, 26) with the following modifications. Nematodes were suspended in 3 ml of sonication buffer [10 mM Tris·HCl (pH 7.4)/1 mM EDTA/1 mM PMSF] and given 10 20-s bursts with a Branson Sonifier 450 at 50% Duty Cycle and 5–7 output control. Cuticles were collected by centrifugation for 4 min at 2,000 rpm in a Sorvall Super T21 and washed several times with 10 ml of sonication buffer. Cuticles were then suspended in 1 ml of ST buffer [1% SDS/0.125 M Tris·HCl (pH 6.8)] and heated for 2 min in a boiling water bath. The sample was then incubated for 6 h, spun 60 s in an Eppendorf microcentrifuge, extracted with ST buffer as described, and shaken overnight. Disulfide cross-linked cuticle proteins were solubilized by heating for 2 min in a boiling water bath in 0.5 ml of ST buffer with 5% 2-mercaptoethanol. The sample was then rocked 6 h, and the solution reextracted; the sample was treated again and rocked overnight. The soluble fraction was resuspended to a final concentration of 0.1% SDS and concentrated to 100 μl with a Microcon 10 concentrator, diluted with 50 mM acetic acid, and concentrated three more times. The insoluble fraction was washed with distilled water, lyophilized, and stored at −20°C. Samples for amino acid analysis were hydrolyzed in 6 M HCl/0.1% phenol at 110°C for 22 h and assayed for the ratio of Hyp:Pro at the MIT Biopolymer Laboratory (Cambridge, MA).

Small-Molecule Inhibitors. 2,4-Diethylpyridine dicarboxylate (Inhibitor I) (27) was synthesized by refluxing pyridine 2,4-dicarboxylic acid in ethanol containing thionyl chloride. Dimethyloxalylglycine (Inhibitor II) was prepared as described (28). A concentrated stock solution was made by adding inhibitor to sterile-distilled water and filtering the solution through a 0.2-μm filter. The final concentration was determined by UV absorbance at 280 nm for inhibitor I and 350 nm for inhibitor II. Unseeded worm plates with 4 ml of agar were spread with 40 μl or 400 μl of a known stock solution of inhibitor and left overnight. Three to six L4 worms were then placed on the plates with OP50 bacteria. After 48–72 h, the plates were scored for embryonic and larval lethality as well as any other phenotypes.

Results and Discussion

Two *C. elegans* Prolyl 4-Hydroxylase α Subunits. Two genes in the *C. elegans* genome are predicted to encode proteins with sequence similarity to the human α subunit of prolyl 4-hydroxylase. One of these genes, Y47D3B.10, resides on *LGIII* (accession no. AL031635 Z98865); the other, F35G2.4, is located on *LGIV* (accession no. Z69637). Y47D3B.10 was identified and found to have prolyl 4-hydroxylase activity when expressed together with the human or *C. elegans* β subunit (18, 19). We show below that Y47D3B.10 corresponds to the *dpy-18* gene and F35G2.4 corresponds to *phy-2* (for *p*rolyl 4-*hy*droxylase). The predicted amino acid sequences of DPY-18 and PHY-2 proteins possess all residues defined as essential for catalytic activity (Fig. 1*A*; red for iron-binding residues and blue for interactions with α-ketoglutarate) as well as four Cys residues critical for α/β complex formation and enzymatic activity (Fig. 1*A*; yellow) (29–31). Sequence comparisons show that DPY-18 and PHY-2 bear

striking similarity throughout their lengths, both to each other and to their human counterparts (Figs. 1 *A* and *B*).

Phylogenetic analysis of prolyl 4-hydroxylase α subunits from multiple organisms separates DPY-18 and PHY-2 from the vertebrate enzymes, suggesting that they may have arisen as a gene duplication (Fig. 1*C*). The exon/intron structure of the two *C. elegans* genes reveals little conservation of splice sites (Fig. 2). The only intron with conserved splice sites occurs in *dpy-18* between exons 5 and 6 and in *phy-2* between exons 9 and 10. Therefore, the duplication event generating these two genes may be ancient.
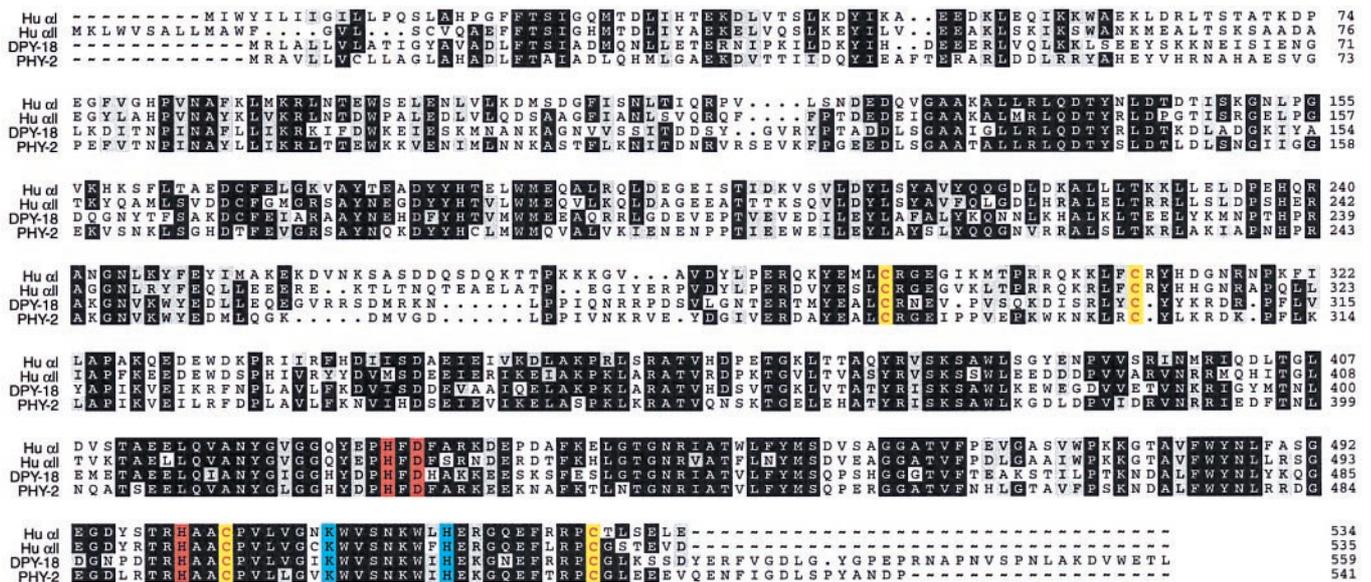
Generation of Mutants in Each Gene Encoding a Prolyl 4-Hydroxylase α Subunit. To examine the biological roles of the two *C. elegans* prolyl 4-hydroxylase α subunits, we generated a deletion mutant of each gene by PCR-based sib selection (see *Materials and Methods*). The *ok162* mutation is a deletion within Y47D3B.10 and *ok177* is a deletion in F35G2.4. Sequence analysis of *ok162* revealed a 2,315-bp deletion removing exons 2–4 and part of exon 5 followed by a small inversion of 194 bp (Fig. 2*A*); the OK162 protein is predicted to lack amino acids 89–308. Similar analysis of the *ok177* mutant allele revealed a 1,335-bp deletion removing most of exons 4–7 (Fig. 2*B*); the OK177 protein is predicted to lack amino acids 199–408 as well as the rest of the protein C terminally because of a stop codon shortly after the deletion end point. Both deletions eliminate the first two essential Cys (Fig. 1*A*; yellow). In addition, transcripts bearing premature stop codons are subject to nonsense-mediated mRNA decay and therefore may be less abundant than wild-type mRNAs (32). Therefore, each deletion is likely to eliminate the prolyl 4-hydroxylase activity of its corresponding mutant protein.

Y47D3B.10 Is Required for Body Morphology and Corresponds to the *dpy-18* Gene. Wild-type *C. elegans* has a characteristically long and sinuous body form (Fig. 3*A*). In contrast, *ok162* homozygotes are shorter than normal, a Dumpy (Dpy) phenotype (Fig. 3*B*). This Dpy phenotype was observed among *ok162* homozygotes after several generations of growth at each of three different temperatures (15, 20, and 25°C). Therefore, no maternal effect or temperature sensitivity was associated with a loss of *dpy-18* activity. Consistent with results obtained with the mutant, the same phenotype was observed by using RNA-mediated interference directed against the Y47D3B.10 transcript (not shown). Therefore, the primary function of the DPY-18 prolyl 4-hydroxylase α subunit is to ensure the normal body morphology.

The *dpy-18* gene resides on *LGIII* in the vicinity of Y47D3B.10 (12). Given the Dpy phenotype of *ok162* homozygotes, which is indistinguishable from that of *dpy-18(e364)* homozygotes, we tested *ok162* for complementation with *dpy-18(e364)*. We found animals of genotype *dpy-18(e364)/ok162* to be Dpy. Therefore, *ok162* fails to complement *dpy-18* and is most likely to reside in the *dpy-18* locus. To confirm this idea, we sequenced *dpy-18(e364am)*, an amber suppressible *dpy-18* allele (33), and found an amber mutation in the second exon at amino acid 92 (G → A; W to STOP) (Fig. 2*A*). We conclude that *dpy-18* encodes the Y47D3B.10 prolyl 4-hydroxylase α subunit.

***phy-2* Mutant Homozygotes Have No Apparent Phenotype.** Animals homozygous for the *phy-2(ok177)* deletion are phenotypically wild type (Fig. 3*C*). We examined *ok177* homozygotes for gross abnormalities, such as a change in body shape or movement, sterility, lethality, and failure in egg laying or male mating. None of these defects was observed at any of three different growth temperatures (15, 20, and 25°C). Furthermore, RNA-mediated interference directed against the F35G2.4 transcript had no obvious phenotypic effect (not shown). We suggest that the *phy-2* prolyl 4-hydroxylase is not essential.
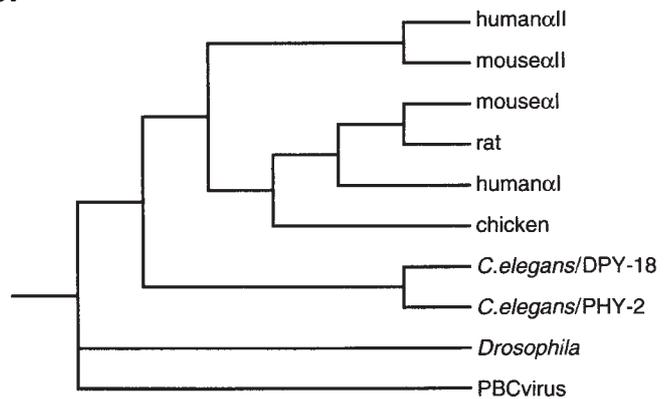
A.

Hu αI
Hu αII
DPY-18
PHY-2

(amino acid sequence alignment, residues numbered to 74, 76, 71, 73 ... 534, 535, 559, 541)

B.

% Amino Acid Identity

	Human		C. elegans	
	αI	αII	DPY-18	PHY-2
αI	100	64.2	43.9	47.2
αII		100	43.0	46.4
DPY-18			100	56.5
PHY-2				100

C.

humanαII
mouseαII
mouseαI
rat
humanαI
chicken
C.elegans/DPY-18
C.elegans/PHY-2
Drosophila
PBCvirus

Fig. 1. Sequence analysis of prolyl 4-hydroxylase α subunits. (*A*) Alignment of amino acid sequences of human αI (accession no. M24486), human αII (accession no. U90441), *C. elegans* DPY-18 (accession no. AL031635), and *C. elegans* PHY-2 (accession no. Z69637), by using the PILEUP program of the GCG-Wisconsin package. The human αI sequence is the form encoded by the more 5′ of the two alternatively spliced exons (43, 44). The DPY-18 sequence differs at amino acids 308–312 from that reported (18). Our sequence was confirmed by sequencing cDNA yk339d8 (provided by Y. Kohara, National Institute of Genetics, Mishiwa, Japan) and N2 genomic DNA. Amino acids conserved among at least three of the four proteins are shaded: identity, black; similarity, gray ({I, L, V}; {F, W, Y}; {D, E}; {K, R}; {S, T}). Amino acids essential for enzymatic activity are shown in color: Fe^{2+}-binding residues, red; amino acids involved in decarboxylation of α-ketoglutarate, blue; cysteine residues critical for α/β complex formation and enzymatic activity, yellow. (*B*) Amino acid identity among the human and *C. elegans* prolyl 4-hydroxylase α subunits as calculated by the DISTANCES program of GCG-Wisconsin package. (*C*) Phylogenetic tree of prolyl 4-hydroxylase α subunits (45–50). Phylogenetic analysis was performed by using the programs PAIRWISE of GCG and PAUP. The tree was generated with the PAUP parsimony analysis by using an exhaustive tree search rooted with the *Paramecium Borella Chlorella* (PBC) viral sequence. This tree is the same as that generated with a distance algorithm (data not shown).

The Two Prolyl 4-Hydroxylase α Subunits Share a Common Vital Function. We next constructed *dpy-18; phy-2* double mutants. To this end, we mated *dpy-18(ok162); unc-22* homozygous hermaphrodites with *phy-2* homozygous males to generate cross-progeny of genotype *dpy-18/+; phy-2/unc-22*. These cross-progeny were viable and morphologically normal. In contrast, some of the self-progeny from parents of genotype *dpy-18/+; phy-2/unc-22* did not survive. Specifically, dead embryos and dead larvae were observed among the progeny (Fig. 3E), along with surviving progeny that were either wild type, Uncoordinated (Unc), or Dpy Unc phenotypically. We initially suspected that the dead progeny might represent *dpy-18; phy-2* double mutants. Consistent with this idea, a reduction in both *dpy-18* and *phy-2* gene activities by RNA-mediated interference led to embryonic lethality (data not shown). However, further analysis showed that the dead progeny included three genotypes, including that of the double mutant (see below). The dead embryos elongated initially, usually to the twofold stage, but were unable to maintain their shape and often exploded (Fig. 3 *D* and *E*). Before the embryos exploded, they appeared similar to *emb-9* mutant embryos (34). EMB-9 is a type IV collagen and a major component of basement membranes (35). Therefore, the *dpy-18; phy-2* lethality may reflect a requirement for prolyl 4-hydroxylase activity in both basement membranes and cuticle formation. The twofold arrest is likely caused by a reduction in type IV collagen, whereas explosion of the embryos is likely caused by a reduction in cuticle collagens. We conclude that the *dpy-18* and *phy-2* genes serve a common function that is required for viability, and suggest that this shared function involves both formation of the basement membrane and embryonic cuticle.

A. *dpy-18*

5'   3'

ok162

B. *phy-2*

5'   3'

ok177

⊢⊣ 200 bp
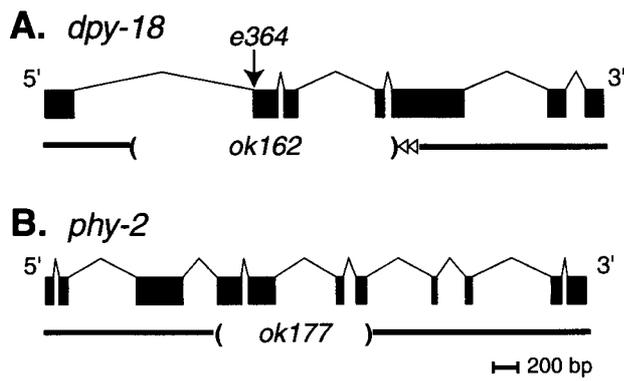
Fig. 2. Schematic of *dpy-18* and *phy-2* mRNAs and extent of deletions. Black boxes, exons; thin lines, introns; thick line, genomic sequence; parentheses, extent of deletion; white triangles, inversion. (*A*) The *dpy-18* gene encodes transcript Y47D3B.10. The *dpy-18(ok162)* allele possesses a deletion removing exons 2–4 and a small inversion in exon 5. The *dpy-18(e364)* nonsense mutation resides in the second exon. (*B*) The *phy-2* gene encodes transcript F35G2.4. The *phy-2(ok177)* mutation possesses a deletion removing exons 4–7.

Dosage Sensitivity of the Two Prolyl 4-Hydroxylase α Subunits. Fig. 3*E* shows that *dpy-18/+; phy-2/unc-22* heterozygotes segregate 32% dead progeny. This percentage is far greater than the 6.25% predicted for the *dpy-18; phy-2* double homozygote. We therefore analyzed the genotypes of all surviving progeny. Although *dpy-18; unc-22* homozygotes (Dpy Unc) were observed at the expected number of approximately 6.25%, no Dpy non-Unc progeny were observed (Fig. 3*E*). This lack of Dpy non-Unc progeny suggested that the dead embryos included both *dpy-18/dpy-18; phy-2/unc-22* and *dpy-18/dpy-18; phy-2/phy-2* genotypes. However, a third genotype was not found among the surviving progeny: none of the phenotypically wild-type animals segre-

gated Dpy but no Unc progeny, as would be expected for animals of genotype *dpy-18/+; phy-2/phy-2*. We therefore suggest that the 32% dead progeny comprised three genotypes: *dpy-18/dpy-18; phy-2/unc-22*, *dpy-18/+; phy-2/phy-2* and *dpy-18/dpy-18; phy-2/phy-2*. The presence of these three genotypes among dead embryos was then confirmed by single-embryo PCR (data not shown). Therefore, embryos homozygous for either *dpy-18* or *phy-2* could not tolerate the loss of one copy of the other gene. We conclude that both *dpy-18* and *phy-2* are dose sensitive in the absence of the other gene. This dose sensitivity may reflect the requirement for a high level of prolyl 4-hydroxylase activity at a specific stage of development, for example, when the cuticle is being formed. Such a dose sensitivity may also explain the need for two prolyl 4-hydroxylase α subunit genes in the nematode.

Temperature Sensitivity of Prolyl 4-Hydroxylase Mutants. The *dpy-18* and *phy-2* single mutants had no obvious difference in phenotype at different growth temperatures (see above). Similarly, the percentage of dead progeny produced by *dpy-18/+; phy-2/unc-22* parents was the same at three growth temperatures (Fig. 3*E*). However, at 15°C, death occurred later during larval development, whereas at 20°C and 25°C, death of embryos was more common, which is the only temperature sensitivity detected in animals lacking prolyl 4-hydroxylase. This result is surprising, because the integrity of triple-helical collagen is affected by temperature in a manner that depends strongly on the extent of its hydroxylation (1–3). We suspect that the conformational transition of collagen in the *dpy-18* and *phy-2* single mutants occurs at a temperature greater than 25°C, the highest growth temperature in our study, and that the extent of hydroxylation may play a more dominant role than temperature *in vivo*.

Reduced Hyp Content in *dpy-18* and *phy-2* Mutants. To compare the content of Hyp in wild-type animals to that of the *dpy-18* and *phy-2* mutants, cuticles were prepared from fourth larval-stage

A. wild type

B. *dpy-18(ok162)*

C. *phy-2(ok177)*

D. dead embryos

E. Progeny of *dpy-18(ok162)/+; phy-2(ok177)/unc-22(e66)*

T°C	DpyUnc (%)	DpynonUnc (%)	Dead (%)	Larval lethal (%)	Embryonic lethal (%)	n
15	4	0	32	20	12	219
20	5	0	32	9	23	275
25	8	0	32	11	21	179

Fig. 3. The *dpy-18* and *phy-2* phenotypes. (*A–D*) Nomarski differential interference contrast images, lateral view; (*A–C*) young adult hermaphrodites that have not begun to lay embryos. All three animals shown at same magnification. (*A*) Wild type; (*B*) *dpy-18(ok162)* homozygote; and (*C*) *phy-2(ok177)* homozygote. (*D*) Dead embryos from *dpy-18(ok162)/+; phy-2(ok177)/unc-22(e66)* parent. From left to right: twofold embryo, embryo starting to explode, and embryo after explosion. (*E*) Quantitation of self-progeny produced by *dpy-18(ok162)/+; phy-2(ok177)/unc-22(e66)* parents. T°C, temperature. Dpy Unc progeny are of genotype *dpy-18; unc-22*; Dpy non-Unc progeny were not observed; total dead progeny (dead) include larval lethal and embryonic lethal progeny. The wild-type and Unc classes of progeny (omitted from Table 1) were progeny-tested to determine genotype, and it was found that each genotype was present at the expected percentage.

Table 1. 4-Hyp in L4 cuticles

Genotype	Gly, %	Hyp + Pro, %*	Hyp:Pro
Wild type	27	26	1.297
dpy-18[†]	31	32	0.523
phy-2[‡]	30	21	0.829

*Total Hyp and Pro. Both 3- and 4-Hyp were assayed, but only 4-Hyp was detected.
[†]dpy-18(ok162) homozygotes.
[‡]phy-2(ok177) homozygotes.

(L4) animals (see *Materials and Methods*). For these experiments, we focused on the soluble fraction, which contains the most collagen (25). Table 1 summarizes our results. The content of collagen is indicated by percent Gly; percent Hyp + Pro was similar to percent Gly in all three preparations, suggesting that the quality of the cuticle preparations was good. Most important is the ratio of Hyp:Pro. Whereas the Hyp:Pro ratio in wild-type animals was 1.3, it was only 0.5 in *dpy-18* homozygotes and 0.8 in *phy-2* homozygotes. Therefore, Pro hydroxylation appears to be reduced in both mutants. We conclude that both enzymes have prolyl 4-hydroxylase activity, but that the DPY-18 enzyme may be more effective, at least at this stage, consistent with its severe Dpy phenotype.

Biological Role of Prolyl 4-Hydroxylase Activity in *C. elegans*. The *C. elegans* genome sequencing project has revealed 170 predicted collagen genes (15). Furthermore, genetic analyses have shown that certain collagens are particularly crucial for body form. For example, the *dpy-2, dpy-5, dpy-7, dpy-10, dpy-13*, and *dpy-17* genes all encode collagens (36–38), and mutants in any one of these genes possess a Dpy phenotype similar to that of *dpy-18* (12). Prolyl 4-hydroxylase activity affects collagen stability *in vitro* (3, 39). Here, we have shown that prolyl 4-hydroxylase activity affects body shape, which is most simply interpreted as an effect on collagen stability *in vivo*. Moreover, we show that the *dpy-18* and *phy-2* genes appear to have overlapping roles in the embryo, but that *dpy-18* is more important than *phy-2* during larval development.

Small Molecule Inhibition of Prolyl 4-Hydroxylase Activity. Small molecules that inhibit protein function can be used to confirm and extend results from genetic experiments (40, 41). We tested two known prolyl 4-hydroxylase inhibitors for their effects on *C. elegans*. Fig. 4*A* shows the structures of these inhibitors: 2,4-diethylpyridine dicarboxylate and dimethyloxalylglycine (inhibitor I and inhibitor II, respectively). Both inhibitors limit prolyl 4-hydroxylase activity in cells, where their esters are hydrolyzed to form competitors of α-ketoglutarate (27, 28). We exposed adult hermaphrodites that were genotypically wild-type, *dpy-18(ok162)* or *phy-2(ok177)* to varying concentrations of inhibitors. The animal placed in inhibitor was apparently unaffected, but dramatic effects were observed among their progeny. Indeed, when exposed to a high level of inhibitor I or II (2.7 μM and 1.3 μM, respectively), all progeny died, regardless of genotype (Fig. 4*B*). The dead embryos arrested at the twofold stage and then exploded (data not shown), a phenotype reminiscent of the *dpy-18; phy-2* dead embryos. This suggests that exposure to the inhibitors results in a lowered prolyl 4-hydroxylase activity.

At a 10-fold lower concentration, the inhibitors affected *dpy-18(ok162)*, but not *phy-2(ok177)* progeny (Fig. 4*B*). To ask whether animals with a Dpy phenotype were unusually sensitive

A. α–KG   Inhibitor I   Inhibitor II
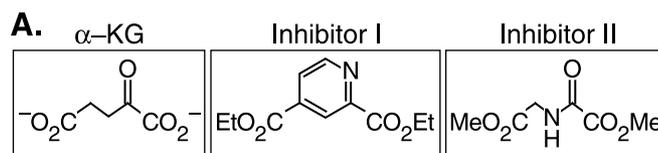
B.

Genotype	Inhibitor I		Inhibitor II	
	μM	Lethality (%)	μM	Lethality (%)
wild type	0	0	0	0
	0.27	0	0.128	0
	2.7	100[b]	1.28	100[b]
dpy-18 (ok162)	0	0	0	0
	0.27	100[a]	0.128	100[a]
	2.7	100[a]	1.28	100[a]
phy-2 (ok177)	0	0	0	0
	0.27	0	0.128	0
	2.7	100[b]	1.28	100[b]

[a] only dead embryos
[b] dead embryos and dead larvae

Fig. 4. Chemical inhibition of prolyl 4-hydroxylase. (*A*) Chemical structures of α-ketoglutarate, a cofactor for prolyl 4-hydroxylase (*Left*), and two inhibitors of prolyl 4-hydroxylase activity that are related chemically to α-ketoglutarate (*Center* and *Right*). (*B*) Effect of prolyl 4-hydroxylase inhibitors on wild-type *C. elegans* and *dpy-18* and *phy-2* homozygotes. a, Only dead embryos; b, dead embryos and dead larvae.

to inhibitor, we tested *dpy-10(e128)*, *dpy-11(e224)*, *dpy-13(e184)*, *dpy-17(e364)*, and *dpy-20(e1282)* mutants for inhibitor effects. However, these other *dpy* mutants were comparable to wild-type animals in their response to both inhibitors (not shown). Therefore, the sensitivity of *dpy-18* mutants to inhibitor is not caused by its Dpy phenotype. In *dpy-18* mutants, the only prolyl 4-hydroxylase activity remaining is PHY-2, and conversely, in *phy-2* mutants, the only remaining activity is DPY-18. We suggest that the effect of the inhibitor on *dpy-18* mutants reflects inhibition of the remaining PHY-2, and vice versa. Because *dpy-18*, but not *phy-2*, progeny were affected by inhibitor at low concentration, we suggest that PHY-2 is either less abundant or more sensitive than DPY-18.

Inhibitors of prolyl 4-hydroxylase have received much interest as potential antifibrotic agents (42). The nematode *C. elegans* may provide an excellent *in vivo* model organism for the discovery of new prolyl 4-hydroxylase inhibitors. In addition to being potential chemotherapeutics, such inhibitors may be useful in elaborating the physiological consequences of prolyl 4-hydroxylase activity as well as in identifying mutations that make animals resistant to prolyl 4-hydroxylase inhibition.

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