The Exoskeleton Collagens in *Caenorhabditis elegans* are Modified by Prolyl 4-Hydroxylases with Unique Combinations of Subunits*

Johanna Myllyharju¹, Liisa Kukkola¹, Alan D. Winter² and Antony P. Page²§

From the ¹Collagen Research Unit, Biocenter Oulu and the Department of Medical Biochemistry and Molecular Biology, University of Oulu, FIN-90014 Oulu, Finland and the ²Wellcome Centre for Molecular Parasitology, Anderson College, the University of Glasgow, Glasgow G11 6NU, United Kingdom

§Correspondence to: Dr. A. P. Page, Wellcome Centre for Molecular Parasitology, Anderson College, the University of Glasgow, Glasgow G11 6NU, United Kingdom.
Tel. (0044) 141 330 3650, Fax (0044) 141 330 5422
Email: a.page@udcf.gla.ac.uk

Running title: A novel ECM-specific prolyl 4-hydroxylase in *C. elegans.*
SUMMARY

The collagen prolyl 4-hydroxylases (P4Hs, EC 1.14.11.2) play a critical role in the synthesis of the extracellular matrix. The enzymes characterized from vertebrates and Drosophila are $\alpha_2\beta_2$ tetramers, in which protein disulfide isomerase (PDI) serves as the $\beta$ subunit. Two conserved $\alpha$ subunit isoforms, PHY-1 and PHY-2, have been identified in Caenorhabditis elegans. We report here that three unique P4H forms are assembled from these polypeptides and the single $\beta$ subunit PDI-2, both in a recombinant expression system and in vivo, namely a PHY-1/PHY-2/(PDI-2)$_2$ mixed tetramer and PHY-1/PDI-2 and PHY-2/PDI-2 dimers. The mixed tetramer is the main P4H form in wild-type C. elegans but phy-2$^{-/}$ and phy-1$^{-/}$ (dpy-18) mutant nematodes can compensate for its absence by increasing the assembly of the PHY-1/PDI-2 and PHY-2/PDI-2 dimers, respectively. All three of the mixed tetramer-forming polypeptides PHY-1, PHY-2 and PDI-2 are coexpressed in the cuticle collagen synthesizing hypodermal cells. The catalytic properties of the mixed tetramer are similar to those of other P4Hs, and analogues of 2-oxoglutarate were found to produce severe temperature-dependent effects on P4H mutant strains. Formation of the novel mixed tetramer was species-specific, and studies with hybrid recombinant PHY polypeptides showed that residues Gln121-Ala271 and Asp1-Leu122 in PHY-1 and PHY-2, respectively, are critical for its assembly.
INTRODUCTION

The free-living nematode Caenorhabditis elegans represents an excellent model system for studying the extracellular matrix (ECM\(^1\)) and the enzymes involved in its biosynthesis and modification (1-3). The major ECM formed in C. elegans is the collagenous cuticle or exoskeleton, a protective structure that is synthesized repeatedly during development. Over 150 small collagen genes are involved in the formation of this structure (2), producing stage-specific cuticles that are both structurally and chemically distinct (3).

Collagen prolyl 4-hydroxylases (P4Hs) are enzymes resident in the endoplasmic reticulum (ER) (4, 5) that play a critical role in the synthesis and processing of all collagens (6). Recently, an additional family of cytoplasmic P4Hs has been shown to play an essential role in O\(_2\) sensing and the hypoxia response (7, 8). The collagen P4Hs present in vertebrates (4, 5) and in Drosophila (9) are \(\alpha\beta_2\) tetramers in which the multifunctional chaperone protein disulfide isomerase (PDI) serves as the \(\beta\) subunit. The \(\alpha\) subunit binds Fe\(^{2+}\), 2-oxoglutarate and ascorbate (4, 5) and possesses the peptide substrate-binding site (10). The main role of PDI is to retain the \(\alpha\) subunits in a catalytically active, nonaggregated conformation within the ER (11-13). Two conserved P4H \(\alpha\) subunit isoforms have been described in human and mouse tissues, that form \([\alpha(I)]_2\beta_2\) or \([\alpha(II)]_2\beta_2\) tetramers (14-16). Initial characterization of a recombinant C. elegans \(\alpha\) subunit isoform (PHY-1) revealed a unique association, in that it formed an active \(\alpha\beta\) dimer with both human PDI and its C. elegans orthologue PDI-2, but not with C. elegans PDI-1 (17, 18). Since this initial PHY-1 characterization, C. elegans has become the first multicellular organism to have its genome completely sequenced (19), thereby permitting a detailed evaluation of this enzyme class in this metazoan.

In addition to the PHY-1 encoding gene, a second highly conserved P4H \(\alpha\) subunit encoding gene, phy-2, has been identified (20, 21). Detailed analyses of gene function employing RNA interference and/or homologous gene deletion techniques have recently been performed on phy-1 (20-22), phy-2 (20, 21) and pdi-2 (21). In addition, detailed temporal and spatial expression pattern analyses and embryonic phenotype time-lapse examinations have been performed on all three genes (21). These studies indicated that phy-1 is important for post-embryonic body morphology, since its deletion resulted in a dumpy (short and fat) phenotype with an associated decrease in the 4-hydroxyproline content of the fourth larval cuticle (20). The phy-1 gene was found
to correspond to the previously uncharacterized dumpy mutant, *dpy-18* (20-22). In contrast, *phy-2* was found to be dispensable or functionally redundant, since its deletion resulted in no visible phenotype, although a significant reduction in cuticular 4-hydroxyproline content was noted (20, 21). The combined disruption of *phy-1* and *phy-2* did however demonstrate an essential role for both genes in embryonic viability (20, 21). Nematodes lacking both genes developed normally during early embryogenesis, until the first collagenous ECM or cuticle was formed, after which the normal elongated body morphology was lost and death followed (21). Significantly, deletion of the characterized P4H β subunit gene *pdi-2* gave an identical time-lapse phenotype to the *phy-1/phy-2* combined disruption (21). Taken together with the shared hypodermal spatial expression patterns and the molting cycle-related temporal expression patterns of all three genes (21), the genetic analysis suggests a biochemical association of all three encoded polypeptides and supports a cuticle collagen-specific role for these P4H subunits.

We describe here a detailed *in vitro* and *in vivo* biochemical characterization of the second conserved *C. elegans* P4H α subunit isoform PHY-2, demonstrating the presence of a novel mixed PHY-1/PHY-2/(PDI-2)₂ tetramer formed by the three polypeptides both in recombinant insect cell coexpression experiments and *in vivo*. This mixed tetramer formation is species-specific, and the regions of PHY-1 and PHY-2 required for the association were identified. We also show that this mixed tetramer is far more abundant than the previously characterized recombinant PHY-1/PDI-2 dimer, and describe an additional PHY-2-containing dimer that can partially substitute for the mixed tetramer in a P4H mutant. A nuclear-excluded co-localization of all three polypeptides together with the newly synthesized chains of a cuticle collagen is consistent with their role in the ER modification of collagens synthesized by hypodermal cells and destined for the cuticular ECM.
MATERIALS AND METHODS

Nematode Strains - Wild-type (N2), dpy-18(e364), and phy-2(ok177) strains were received from the C. elegans Genetics Center. The deletion strain phy-3(ok199) was generated by the C. elegans Genome Deletion Consortium.

Generation of Recombinant Baculoviruses Encoding PHY-2 and Hybrid PHY Polypeptides - PHY-2 was expressed in insect cells by cloning the full-length phy-2 coding sequence into the vector pVL1392 (Invitrogen). PCR was performed on N2 C. elegans mixed stage cDNA (23) using Pfu polymerase (Stratagene) with the primers Phy-2BVF (NotI, underlined) gacgggcccgaTGAGAGCAGTTTTGCTAGTC and Phy-2BVR (XbaI, underlined) gcgtctagatAGAGCGAGTTTTGCTAGTC. The PCR product was cloned into the pCRScript vector (Stratagene), digested with NotI and XbaI and ligated into similarly digested pVL1392 (PharMingen). Baculovirus transfer vectors coding for hybrid PHY polypeptides with no linker between the PHY-1 and PHY-2 sequences were constructed by preparing two PCR fragments using kinase-treated primers and Pfu DNA polymerase (Promega) with pVL1392-phy-1 (17) and pVL1392-phy-2 as templates. To generate constructs coding for hybrid PHY polypeptides in which the N-terminal half consists of PHY-1 amino acids, the phy-1 PCR-fragments were amplified from the pVL1392 NotI site to the codons for PHY-1 Leu120 or Ala271, and the phy-2 PCR fragments from the codons for PHY-2 Gln123 or Leu268 to the stop codon followed by an XbaI site. To generate constructs coding for hybrid PHY polypeptides in which the N-terminal half consists of PHY-2 amino acids, the phy-2 PCR fragments were amplified from the pVL1392 NotI site to the codons for PHY-2 Leu122 or Ala267, and the phy-1 PCR fragments from the codons for PHY-1 Gln121 or Leu272 to the stop codon followed by an XbaI site. The phy-1 and phy-2 fragment pairs were coligated into NotI-XbaI-digested pVL1392. Hybrid A consists of the PHY-1 signal peptide, PHY-1 amino acids 1-120 and PHY-2 amino acids 123-523; hybrid B of the PHY-1 signal peptide, PHY-1 amino acids 1-271 and PHY-2 amino acids 268-523; hybrid C of the PHY-2 signal peptide, PHY-2 amino acids 1-122 and PHY-1 amino acids 121-543; and hybrid D of the PHY-2 signal peptide, PHY-2 amino acids 1-267 and PHY-1 amino acids 272-543 (Fig. 7). The recombinant baculovirus constructs were cotransfected into Spodoptera frugiperda Sf9 cells with a modified
Autographa californica nuclear polyhedrosis virus DNA using the BaculoGold transfection kit (Pharmingen), and the resultant virus pools were collected and amplified (24).

Expression and Analysis of Recombinant Proteins in Insect Cells - Insect cells (Sf9 or High Five, Invitrogen) were cultured in TNM-FH medium (Sigma) supplemented with 10% insect cell qualified fetal bovine serum (Gibco) as monolayers at 27°C. Cells seeded at a density of 5x10⁶ per 100-mm plate were infected at a multiplicity of 5 with different combinations of the viruses coding for the C. elegans PHY-1 (17), PHY-2, hybrid PHYs, PDI-1, or PDI-2 (18), human a(I) subunit (11), human a(II) subunit (15) or human PDI (12), and Drosophila a subunit and PDI (9). The cells were harvested 72 h after infection, washed with a solution of 0.15 M NaCl and 0.02 M phosphate, pH 7.4, homogenized in a solution of 0.1 M glycine, 0.1 M NaCl, 10 µM dithiothreitol, 0.1% Triton X-100, and 0.01 M Tris, pH 7.8, and centrifuged at 10,000 x g for 20 min. Samples of the resulting supernatants (50 µg total protein) were analyzed by 8% reducing SDS-PAGE (25) or 8% nondenaturating PAGE by leaving out the SDS and reducing agent from the standard SDS-PAGE protocol (25) and assayed for enzyme activity by a method based on the hydroxylation-coupled decarboxylation of 2-oxo[1-14C]glutarate (26). Km and Ki values were determined as described previously (26).

Antibodies - Polyclonal antibodies were generated against synthetic peptides representing the C-terminal residues of PHY-1 (CEPRNAPNVSPNLAKDVWETL), PHY-2 (CLEEEVQENFIGDLSPYANDP) and PDI-2 (CGASEEKEAAEDEEGHTEL). The peptides were conjugated to keyhole limpet hemocyanin (Sigma Genosys) via a cysteine residue (added to PHY-1 and PDI-2 sequences, C) and used to immunize two rabbits.

Western blot analysis of baculovirus-generated recombinant proteins was performed by transferring samples from SDS-PAGE or nondenaturing PAGE (25) to an Immobilon-P membrane (Millipore), followed by incubation with the antibodies described above and a subsequent incubation with an alkaline phosphatase-conjugated secondary antibody. No crossreactivity was noted between the three antisera, and specific bands of the correct estimated size were observed following Western blotting of reduced denatured worm extracts (data not shown).

The cuticle collagen-specific DPY-7 monoclonal antibody used in colocalization experiments was a gift from Dr. Iain Johnstone (The University of Glasgow).
Characterization of P4Hs from C. elegans Lysates - Native worm extracts were prepared by washing mixed stage nematodes from 4 x 100-mm plates in ice-cold PBS with a protease inhibitor mixture (1 mM PMSF, 1 mM EDTA, 1 mM EGTA, 2 µM E64 and 0.1 µM pepstatin). After several washes, the worms were pelleted by gentle centrifugation at 2000 x g and frozen at –70°C. Pellets (0.5-1ml of packed worms) were resuspended on ice in an equal volume of P4H buffer (0.1 M NaCl, 0.1 M glycine, 0.1% Triton X-100, 10 µM DTT, protease inhibitor mixture in 10 mM Tris, pH 7.8, at 4°C) and homogenized in a 3 ml Dounce tissue homogenizer. Insoluble material was removed by centrifugation and the supernatants (14 µg total protein/well) were resolved on 4-12% gradient nondenaturing PAGE gels (Novex, NuPage BisTris) following the manufacturers recommendations. Western blotting was performed with antibodies to PHY-1, PHY-2 and PDI-2 as described above. The P4H activity of the nematode lysates was assayed by measuring the formation of 4-hydroxy[14C]proline in a nonhydroxylated [14C]proline-labeled procollagen substrate (26).

Antibody Immunolocalization - Wild-type embryos were collected from agar plates and washed extensively in ice-cold PBS. They were then pipetted onto poly-L-Lysine coated slides and permeabilized by freeze-cracking (27). Samples were blocked in PBST (0.1% Tween-20) containing 5% dried skimmed milk and probed with combinations of monoclonal anti-DPY-7 (1/50) and polyclonal antisera to PHY-1, PHY-2 and PDI-2 (all at 1/100) in PBST. Samples were then washed extensively, and incubated in a mixture of 1/500 Alexa Fluor 594 goat anti-rabbit IgG and Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes). Samples were washed, and viewed under epifluorescence on a Zeiss Axioskop 2 microscope. Images captured with a CCD Hamamatsu digital camera were pseudocoloured and processed using Improvision Openlab and Adobe Photoshop software.

Cosubstrate Analogue Inhibition Assays in Vivo - Small (4 cm) Petri dishes were used for the inhibitor studies with wild-type, dpy-18(e364), phy-2(ok177) and phy-3(ok199) nematodes. Plates were prepared with NGM agarose (minus salts) and seeded with OP50 washed with S medium (100 mM NaCl, 50 mM KPO₄, pH 6, with 5 mg/l cholesterol). Plates were then inoculated with 400 µl of pyridine 2,4-dicarboxylate or pyridine 2,5-dicarboxylate diluted in S medium to final concentrations ranging from 60 µM to 10 mM. Fifteen 2-fold embryos of each strain were added to each plate, incubated at 20 or 25°C and viewed twice daily over several days. Phenotypes of inoculated nematodes and their progeny were viewed and images captured as above.
RNA Interference - Additional effects of phy-3 deletion were examined using standard RNAi injection protocols (21). The phy-3 coding sequence (28) was cloned from C. elegans mixed stage cDNA by PCR using the primers T20B3.7cLF(XbaI) gcctctagaATGATTTCTGTCACTTTCCG and T20B3.7NR(XhoI) gcgctcgagCGGTATAAGCCACGAAGCATG. The PCR product was cloned into the XbaI- XhoI-digested vector pPD129:36 (29). Double-stranded RNA for injection was produced in vitro as described previously (21). Ten young adults of wild-type, dpy-18(e364) and phy-2(ok177) strains were microinjected, allowed to recover overnight and transferred singly to fresh plates, and their progenies scored. To perform bacterially mediated RNAi, the construct pPD129:36-phy-3 was transformed according to published methods (29). Ten L4 animals of the strains N2, dpy-18(e364) and phy-2(ok177) were transferred to feeding plates and incubated for 2 days at 25°C, after which the adults were then transferred to fresh feeding plates and allowed to lay eggs for 24 h and the progeny were scored.

RESULTS

Expression of a Recombinant C. elegans PHY-2 Polypeptide and an Active C. elegans PHY-1/PHY-2/(PDI-2)2 Mixed Tetramer in Insect Cells - A recombinant baculovirus coding for the C. elegans PHY-2 polypeptide was generated and used to infect insect cells. The cells were harvested 72 h after infection, homogenized in a buffer containing 0.1% Triton X-100, and centrifuged. The cell pellets were further solubilized in 1% SDS, and the Triton X-100 and SDS-soluble proteins were analyzed by SDS-PAGE under reducing conditions (Fig. 1A). In agreement with data previously reported for the human P4H α(I) and α(II) subunits and the C. elegans PHY-1 (11, 15, 17), the PHY-2 polypeptide formed insoluble aggregates. And likewise, the efficient extraction of the recombinant PHY-2 from the cell homogenates required the use of 1% SDS (Fig. 1A, lane 2).

In order to study whether an association could be achieved between the C. elegans PHY-2 and the C. elegans PDI-1, PDI-2 or human PDI, insect cells were coinfectected with recombinant viruses coding for PHY-2 and one of the PDI subunits. In control experiments a recombinant virus coding for the C. elegans PHY-1 was used together with a virus coding for one of the PDI subunits. Triton X-100-soluble extracts of the cell homogenates were analyzed by nondenaturing PAGE followed by Coomassie Blue staining (Fig. 1B) and Western blotting (Fig. 1C). PHY-2 did not become associated with any of the PDI subunits (Figs. 1B and C, lanes 1-3), whereas...
in the control experiments, PHY-1 formed a dimer with the *C. elegans* PDI-2 or human PDI (Fig. 1B lane 6; and *C. elegans* lanes 4 and 6). The assembly of a recombinant PHY-1/PDI-2 dimer was fairly inefficient, however, as reported previously (18). Also this dimer could be detected only in Western blots and not in Coomassie-stained native gels (Figs. 1B and *C. elegans* lanes 4).

P4H activity in the Triton X-100 soluble extracts was analyzed with an assay based on the hydroxylation-coupled decarboxylation of 2-oxo-[1-14C]glutarate using (Pro-Pro-Gly)₁₀ as a substrate. As reported previously (18), the level of enzyme activity in extracts from cells coexpressing PHY-1 and PDI-2 was about 3% of that in extracts from cells coexpressing PHY-1 and human PDI (Table I). A very low amount of P4H activity was also detected in extracts from cells coexpressing PHY-2 and PDI-2, the level of this activity being about half of that in extracts from cells coexpressing PHY-1 and PDI-2 (Table I), and thus probably below the detection limit of the Western blot analysis used here. No significant P4H activity was present in extracts from cells coexpressing PHY-2 or PHY-1 and *C. elegans* PDI-1, or PHY-2 and human PDI (Table I).

To study whether PHY-2 can form mixed tetramers with PHY-1 and PDI-1, PDI-2 or human PDI, both types of PHY polypeptide were coexpressed in insect cells together with one of the PDI subunits. In control experiments, insect cells were coincfected with viruses coding for the human α(I) subunit and human PDI. The cells were harvested 72 h after infection, and Triton X-100-soluble proteins were analyzed by PAGE performed under nondenaturing conditions, followed either by Coomassie staining (Fig. 2A) or Western blotting (Figs. 2B and C). Coexpression of PHY-2 and PHY-1 with PDI-2 led to the formation of an active recombinant P4H (Table I) with a mobility similar to that of the human enzyme, thus indicating that a tetramer was formed when these three *C. elegans* polypeptides were coexpressed (Fig. 2A, *lanes 1 and 4*). Immunostaining with polyclonal antibodies against PHY-2 and PHY-1 showed that both polypeptides were present in this tetramer (Figs. 2B and *C. elegans* lanes 1). A small amount of a dimer formed by PHY-1 and PDI-2 was also seen in the same sample in Western blots (Fig. 2C, *lane 1*), whereas no association of PHY-1 and PHY-2 with *C. elegans* PDI-1 was observed (Fig. 2, *lanes 2*). Thus the Coomassie-stained native gels indicate that the mixed PHY-1/PHY-2/(PDI-2)₂ tetramer is the predominant P4H form produced by recombinant *C. elegans* P4H subunits in insect cells. When PHY-1 and PHY-2 were coexpressed with human PDI, PHY-1 efficiently associated with the latter to
form a dimer (Figs. 2A and C, lanes 3). In addition, a faint band corresponding to a tetramer could be observed in these samples when immunostained with antibodies against PHY-1 and PHY-2 (Figs. 2B and C, lanes 3).

The enzyme activity assay showed that the PHY-1/PHY-2/(PDI-2)₂ mixed tetramer was a fully active P4H (Table I). The level of enzymatic activity in these Triton X-100 extracts was nevertheless only 50-60% of those in extracts of cells coexpressing PHY-1 or human α(I) and human PDI (Table I). This observation is most likely due to the lower probability of an insect cell being infected simultaneously with three viruses than with two viruses, an observation also reported previously (30).

Catalytic Properties of the Recombinant C. elegans Mixed P4H Tetramer - The $K_m$ values for the cosubstrates Fe$^{2+}$ and ascorbate and the substrate (Pro-Pro-Gly)$_{10}$ of the mixed PHY-1/PHY-2/(PDI-2)$_2$ tetramer (Table II) were very similar to those of the dimer formed by PHY-1 and human PDI (17) and the human type I P4H (31). The $K_m$ value for 2-oxoglutarate of the mixed tetramer was slightly higher, being about 80 µM. The mixed tetramer was not inhibited by poly(L-proline) (data not shown), and thus it resembles the dimer formed by C. elegans PHY-1 and human PDI (17), and the human type II P4H (15). The $K_i$ values of the mixed tetramer for the structural analogues of 2-oxoglutarate, pyridine 2,4-dicarboxylate and pyridine 2,5-dicarboxylate, were comparable to those of the C. elegans PHY-1/human PDI dimer and the human type I P4H (Table II).

The Mixed Tetramer is the Major P4H Form in Wild-type C. elegans - The baculovirus coexpression studies demonstrated that the mixed tetramer is the major form of recombinant C. elegans P4H produced in insect cells, with assembly of the PHY-1/PDI-2 dimer being far less efficient. Previous studies indicate that deletion of PHY-2 does not cause a phenotype in C. elegans, while deletion of the phy-1 gene resulted in a dumpy phenotype because phy-1 is the dpy-18 gene (20-22). Combined disruption of the phy-1 and phy-2 genes led to embryonic lethality (20, 21) so the recombinant expression studies and the genetic data agree and were extended by the following in vivo observations. Native extracts were prepared from wild-type and phy null-mutant nematodes and analyzed by nondenaturing PAGE followed by immunostaining with subunit-specific antibodies (Fig. 3). As described for the baculovirus extracts, the wild-type nematodes were found to produce the mixed tetramer PHY-1/PHY-2/(PDI-2)$_2$ as their main P4H form, and the PHY-1/PDI-2 dimer was detected at a lower
abundance when probed with the PHY-1 antibody (Fig. 3A, lane 1). When wild-type extracts were probed with the PHY-2 specific antibody, only the mixed tetramer band was detected (Fig. 3B, lane 1).

Two phy-1 mutant strains, dpy-18(e364) (Figs. 3A and B, lanes 2) and dpy-18(e1096) (not shown), were similarly examined, both of which gave identical immunoblot profiles. No reactive bands were noted when these strains were probed with the PHY-1 antibody (Fig. 3A, lane 2), thus confirming that both strains are true nulls and that PHY-1 is involved in both tetramer and dimer formation. When the same extracts were probed with the PHY-2 antibody, no tetramer was seen (Fig. 3B, lane 2). A new PHY-2 immunoreactive band was however detected in the dpy-18 extracts, that migrated slightly higher than the PHY-1/PDI-2 dimer, indicating the presence of a novel form containing PHY-2, evidently a PHY-2/PDI-2 dimer (Fig. 3B, lane 2). The finding that coexpression of PHY-2 and PDI-2 in insect cells, but not PHY-2 and PDI-1, produced a small amount of enzyme activity (Table I) provides further evidence to support the existence of a dimer containing PHY-2. The variation in mobility observed between the PHY-1/PDI-2 and PHY-2/PDI-2 dimers (Figs. 3A, lane 3 and B, lane 2) can be accounted for by differences in the predicted pI values of PHY-1 (pI 5.83) and PHY-2 (pI 6.33).

Nematodes having a deletion in the phy-2 gene, strain ok177, have been predicted to be true null mutants (20), an observation confirmed here by the absence of any PHY-2 immunoreactive bands in the extracts prepared from this strain (Fig. 3B, lane 3). Immunoblotting of this extract with PHY-1 antibody showed the mixed tetramer to be absent, while assembly of the PHY-1/PDI-2 dimer was significantly increased (Fig. 3A, lane 3). This compensation may therefore account for the wild-type appearance of the deletion mutants (20, 21).

A deletion mutant has also been obtained for a third divergent P4H α subunit gene, phy-3(ok199), which displays no visible phenotype (28). The possible role of this polypeptide in the assembly of P4H forms from the PHY-1, PHY-2 and PDI-2 polypeptides was analyzed by immunoblotting extracts from the phy-3(ok199) strain with PHY-1 (Fig. 3A, lane 4) and PHY-2 antibodies (Fig. 3B, lane 4). This mutant was found to be identical to wild-type nematodes, in that it contained the mixed PHY-1/PHY-2/(PDI-2) tetramer and the PHY-1/PDI-2 dimer. PHY-3 is thus not expected to interact with the cuticular ECM-specific P4H subunits, an observation further supported by the RNAi experiments described below (Table III).
RNAi analysis of the phy-3 transcript by injection and feeding in a wild-type background confirmed the lack of any visible phenotype (Table III) noted for the deletion mutant (28). Likewise, no supplementary effect was noted for phy-3 RNAi in dpy-18(e364) and phy-2(ok177) mutant backgrounds, as the progenies either remained dumpy due to the phy-1 deletion or wild-type, respectively (Table III). These results provide further evidence that PHY-3 has no role in the assembly of the P4H forms that are involved in synthesis of the cuticular ECM.

**P4H Activities of Wild-type, dpy-18(e364) and phy-2(ok177) C. elegans Extracts** - The P4H activities of the wild-type, dpy-18(e364) and phy-2(ok177) strains were assayed from two independent nematode lysates by measuring the formation of 4-hydroxy[^4C]proline in an unhydroxylated[^4C]proline-labeled protocollagen substrate (26). The enzyme activities in the dpy-18(e364) lysates were found to be reduced to 0.7% and 2.5%, and those in the phy-2(ok177) lysates to 54% and 57% relative to the corresponding lysates from wild-type nematodes. These data support the phenotypes observed in these strains (20, 21) and validate the P4H subunit associations detected in these deletion mutants (Fig. 3).

**PHY-1, PHY-2 and PDI-2 are Coexpressed with the Cuticle Collagen DPY-7** - The spatial expression patterns of the phy-1, phy-2 and pdi-2 genes were characterized initially by examining promoter-LacZ reporter gene constructs, and all three transcripts were found to be exclusively coexpressed in the hypodermal cells that synthesize cuticle collagens (21). The expression patterns of these genes at the levels of the encoded polypeptides were studied here by applying subunit specific antibodies to fixed wild-type embryos. In the pre-elongated 1.5-fold embryo the collagenous cuticle has not been formed (32), and identical immunostaining patterns in the hypodermal cells were observed at this stage when probed with PDI-2, PHY-1 and PHY-2 antibodies (Fig. 4A). Pre-immune control sera gave no discernible signal when analyzed against any stage of C. elegans (data not shown). The nuclear-excluded pattern observed probably represents an endoplasmic reticulum (ER) location, as PDIs are known to be located in the ER (5), and as double labeling with a monoclonal antibody to the cuticle collagen, DPY-7, gave an identical staining pattern (Fig. 4A, merge). In elongated embryos the first larval cuticle has been synthesized (32). At this stage, the hypodermal ER location was maintained for all three P4H subunits (Fig. 4B), while the cuticle collagen DPY-7 has already been secreted from the ER and has been fully incorporated into the developing cuticle (Fig. 4B).
Effects of 2-Oxoglutarate Analogues on Wild-type and phy Mutant Nematodes - Since the structural analogues of 2-oxoglutarate, pyridine 2,4-dicarboxylate and pyridine 2,5-dicarboxylate were shown to be efficient competitive inhibitors of the *C. elegans* recombinant P4Hs *in vitro* (Table II), the *in vivo* effects of these compounds were examined in a plate assay performed on live nematodes. A limited range of effects were noted in the wild-type, *phy-2(ok177)* and *phy-3(ok199)* nematodes, including mild molting defects at 5-10 mM concentrations of the compounds (data not shown). Severe effects were however noted in the *dpy-18(e364)* strain for both compounds at concentrations of 0.5 to 4 mM, and included embryonic lethality, sterility, severe dumpiness and molting defects (as shown in Figs. 5B-D for pyridine 2,5-dicarboxylate) as compared with untreated wild-type and *dpy-18(e364)* nematodes (Fig. 5A). These phenotypes are identical to the previously described *phy-1/phy-2* combined RNAi phenotypes (21). The effects of these compounds were found to be more potent at elevated culture temperatures (25°C compared with 20°C), and extended the severe dumpy effects from 500 µM down to 60 µM. This observation is most probably due to the decrease in the 4-hydroxyproline content affecting the ultimate thermal stability of the cuticular ECM.

*Assembly of the Mixed Tetramer is Species-Specific* - To study whether PHY-1 and PHY-2 can form a mixed tetramer with P4H subunits from other species, insect cells were coinfected with viruses coding for PHY-1 or PHY-2, together with viruses coding for human P4H α(I) or α(II), or the *Drosophila* α subunit in combination with *C. elegans* PDI-1, PDI-2, human PDI or *Drosophila* PDI. The cells were harvested as described above, and Triton X-100-soluble proteins were analyzed by nondenaturing PAGE followed by Western blotting with PHY-2 (Fig. 6) or PHY-1 antibodies (not shown). Involvement of PHY-2 in a mixed enzyme tetramer was found to be very specific, as a band corresponding to an enzyme tetramer was seen only when PHY-2 was coexpressed with PHY-1 and *C. elegans* PDI-2 (Fig. 6, lane 2). Likewise, PHY-1 did not become assembled into a mixed enzyme tetramer with any other α subunit (data not shown).

*Identification of Regions in the PHY-1 and PHY-2 Polypeptides that are Critical for the Assembly of the Mixed P4H Tetramer* - In order to identify regions in the PHY-1 and PHY-2 polypeptides that are required for mixed tetramer assembly, four recombinant baculoviruses coding for hybrid PHY polypeptides A-D were generated (Fig. 7) and coexpressed in insect cells with the PHY-1 or PHY-2 and PDI-2 polypeptides. The cells
were harvested and homogenized as above and analyzed by non-denaturing PAGE followed by Western blotting with the PHY-1 or PHY-2 antibody. When the four hybrid PHY polypeptides A-D were coexpressed with PHY-1 and PDI-2 (Fig. 7, panel I), tetramer assembly was obtained with hybrids C and D, indicating that the PHY-2 residues Asp1-Leu122 are sufficient for a hybrid polypeptide to act as a PHY-2-like unit in the mixed tetramer assembly (Figs. 7, panel I, and Fig. 8, lanes 2 and 3). When the four hybrid polypeptides were coexpressed with PHY-2 and PDI-2 (Fig. 7, panel II), only hybrids B and C formed mixed tetramers, indicating that a critical region for a hybrid polypeptide to act as a PHY-1-like unit in mixed tetramer assembly is located between the PHY-1 residues Gln121 and Ala271 (Figs. 7, panel II, and 8, lanes 4 and 5). As expected on the basis of these results, when the four hybrid PHY polypeptides were coexpressed alone with PDI-2 (Fig. 7, panel III), only hybrid C, which contained both the critical PHY-2 Asp1-Leu122 and PHY-1 Gln121-Ala271 regions, formed a P4H tetramer (Figs. 7, panel III, and 8, lane 6). To further confirm the identification of the critical PHY-1 and PHY-2 regions, various combinations of two hybrids were coexpressed with PDI-2 (Fig. 7, panel IV) and analyzed by non-denaturing PAGE followed by Western blotting with the PHY-1 and PHY-2 antibodies. As expected, hybrid B, containing the critical PHY-1 region, assembled into a mixed tetramer with hybrids C and D (Figs. 7, panel IV, and 8, lanes 7 and 8) that possess the critical PHY-2 region, whereas hybrid A, which is lacking both critical regions, did not become associated with any of the other hybrid polypeptides (Fig. 7, panel IV). Assembly of a mixed tetramer from hybrids C and D (Fig. 7, panel IV) could not be studied because hybrid C alone formed an enzyme tetramer, and because both polypeptides in this combination would have had the C-terminal ends of PHY-1, and therefore a mixed tetramer could not be identified in Western blots.

DISCUSSION

We describe here a detailed characterization of the unique P4H forms that are involved in the synthesis of the cuticular ECM collagens in C. elegans. Three P4H forms are assembled from a unique combination of α and β subunits, namely a PHY-1/PHY-2/(PDI-2)2 mixed tetramer, and PHY-1/PDI-2 and PHY-2/PDI-2 dimers. All three forms are novel, as vertebrate α(I) and α(II) subunits do not form a mixed tetramer with PDI (15), and to date, no αβ dimers have been reported in any other species (4, 5). Although formation of recombinant PHY-
1/PDI-2 and PHY-1/human PDI dimers in insect cells has been described previously (17, 18), the present results indicate that the PHY-1/PHY-2/(PDI-2)₂ mixed tetramer represents the predominant form in wild-type nematodes in vivo. The in vivo and in vitro data presented here corroborate the findings obtained in previous genetic analyses of the subunit-encoding genes (20, 21).

Assembly of the mixed tetramer was found to be exclusively dependent on the PHY-2 polypeptide, since phy-2(ok177) deletion mutant nematodes produced no tetramers, nor were any tetramers formed in insect cell coexpression studies when this polypeptide was omitted. The phy-2(ok177) mutants do however compensate for the lack of the mixed tetramer, by increasing assembly of the PHY-1/PDI-2 dimer, and are consequently wild-type in appearance (20, 21). Assembly of the PHY-1/PDI-2 dimer is however much less effective than that of the mixed tetramer, and accordingly insect cells expressing the recombinant dimer possess much less P4H activity, only about 7% of that shown by cells expressing the mixed tetramer. Likewise, the levels of P4H activity measured with protocollagen assays in extracts from the phy-2(ok177) mutant nematodes were only about half of those from their wild-type counterparts, and correspondingly the cuticle collagens of the fourth larval stages from this strain also contain significantly less 4-hydroxyproline residues than their wild-type equivalents (20).

Although the mixed tetramer and the PHY-1/PDI-2 dimer were the only P4H forms detected in the wild-type nematodes in vivo, our data from dpy-18 nematodes demonstrated that an additional enzyme form, the PHY-2/PDI-2 dimer, partially compensates for the lack of the two other forms. The existence of this novel dimer provides an explanation as to why the dpy-18 nematodes still possess a low level of 4-hydroxyproline in their cuticle collagens when compared with the wild-type, and thus survive, albeit in a morphologically mutant form (20, 21). The PHY-2/PDI-2 dimer was detected in native extracts of the dpy-18 strains and in accordance with this, protocollagen assays revealed low but detectable P4H activities in these extracts (0.7% to 2.5% of wild-type extract activity). The involvement of PDI-2 in this novel dimer was supported by PHY-2/PDI-2 insect cell coinfection studies, as the P4H activity detected was in the same range as the protocollagen-based activity found in the native dpy-18 extracts. It is intriguing to note, that simultaneous inactivation of the phy-1 and phy-2 genes resulted in an identical phenotype to that of the pdi-2 deletion, manifesting in a loss of all the enzyme
forms listed above, and involving a consequent induction of embryonic death within minutes of the synthesis of the first larval cuticle (21). It is exactly at this time that the cuticular ECM serves to maintain worm morphology, and therefore performs a critical role for the subsequent development of the animal (33). Previous studies have also indicated a dose sensitivity for phy-1 and phy-2, in that embryonic death resulted from both double homozygous deletion phy-1⁻/⁻;phy-2⁻/⁻ and from mixed homozygous/heterozygous deletions phy-1⁻/⁻;phy-2⁺/⁻ and phy-1⁺/⁻;phy-2⁻/⁻ (20). This dose sensitivity can now be fully explained by the unique and specific associations identified here between the PHY-1, PHY-2 and PDI-2 polypeptides.

The recombinant mixed tetramer was shown to have similar catalytic properties to the human enzyme tetramer and PHY-1/human PDI dimer. Likewise, the $K_i$ values for the structural analogues of 2-oxoglutarate, pyridine 2,5-dicarboxylate and pyridine 2,4-dicarboxylate, were comparable to those of the human tetramer and PHY-1/human PDI dimer. Significantly, both of these structural analogues produced temperature-dependent effects on dpy-18 strains that were consistent with the findings from the previous combined phy-1;phy-2 deletion studies (21), namely embryonic lethality, severe dumpiness and molting defects. All of these features are in accord with PHY-1 and PHY-2 representing the sole P4H subunits critical for the modification of the cuticular ECM.

The cuticle is synthesized and secreted from a specialized group of nematode cells called the hypodermis. The exclusive role of the PHY-1, PHY-2 and PDI-2 polypeptides in the formation of this structure was confirmed in immunolocalization studies with subunit-specific antibodies. All three polypeptides are coexpressed in the hypodermal cell lineage, as evidenced by the complete overlay with the DPY-7 cuticle collagen expression pattern. Previous spatial coexpression analysis of the phy-1, phy-2 and pdi-2 translational reporter fusions and a temporal expression study (21), also indicate that all three transcripts are co-expressed in the hypodermis in a cyclic fashion that mirrors the cuticle molting cycle. An additional divergent P4H gene, phy-3, also examined here, was found not to be involved in any genetic or biochemical association with either PHY-1 or PHY-2.

Assembly of the PHY-1/PHY-2/(PDI-2)₂ mixed tetramer was found to be highly specific, as none of the C. elegans polypeptides could be replaced by a P4H α or β subunit from any of the other species examined. Experiments with hybrid PHY polypeptides localized the critical regions required for mixed tetramer assembly to
PHY-1 residues Gln121-Ala271 and PHY-2 residues Asp1-Leu122. The amino acid sequence identity between the 122 N-terminal residues of PHY-2 and the corresponding residues of PHY-1 is 45%, and that between residues 121-271 of PHY-1 and the corresponding residues of PHY-2 is 47%, whereas that between the 271C-terminal residues of PHY-1 and the corresponding 256 residues of PHY-2 is 60%. The most obvious difference between the PHY-1 and PHY-2 polypeptides is an 18-amino-acid extension in the C terminus of the PHY-1 polypeptide. This region clearly has no role in determining mixed tetramer formation, as mixed tetramers were assembled from PHY polypeptides that had either a PHY-1 (e.g. PHY-1 and hybrid D, Fig. 7) or PHY-2 (e.g. PHY-2 and hybrid B, Fig. 7) C-terminal region.

The present data reveal an unexpected level of complexity in the interaction between P4H subunits in this organism and the subsequent modification of the collagens that compose its cuticular ECM.

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FOOTNOTES

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§Correspondence to: Dr. A. P. Page, Wellcome Centre for Molecular Parasitology, Anderson College, The University of Glasgow, 56 Dumbarton Road, Glasgow G11 6NU, United Kingdom. Tel. (0044) 141 330 3650; Fax (0044) 141 330 5422; Email: a.page@udcf.gla.ac.uk.

1 The abbreviations used are: P4H, prolyl 4-hydroxylase; PHY, C. elegans prolyl 4-hydroxylase α subunit; PDI, protein disulfide isomerase; ER, endoplasmic reticulum; ECM, extracellular matrix.

2 I. L. Johnstone, University of Glasgow, unpublished observations.
FIG. 1. **Analysis of the expression of the C. elegans PHY-2 polypeptide in insect cells (A)** and **P4H formation from PHY-2 or PHY-1 with various PDIs (B and C).**

_A_, Insect cells were infected with a virus coding for the PHY-2 polypeptide, harvested 72 h after infection, homogenized in a Triton X-100-containing buffer, and centrifuged. The remaining pellets were resuspended in 1% SDS. The Triton X-100 (lane 1) and 1% SDS-soluble (lane 2) fractions were analyzed by 8% reducing SDS-PAGE and Coomassie Blue staining. _B_ and _C_, Insect cells were coinfectd with viruses coding for PHY-2 and PDI-2 (lanes 1), PDI-1 (lanes 2), or human PDI (lanes 3), and with PHY-1 and PDI-2 (lanes 4), PDI-1 (lanes 5), or human PDI (lanes 6). Cells were harvested and homogenized as above, and the Triton X-100-soluble fractions were analyzed by nondenaturing PAGE followed by Coomassie Blue staining (B) or by Western blotting (C) using antibodies against PHY-2 (lanes 1-3) and PHY-1 (lanes 4-6). The positions of the PHY-2 polypeptide, and the PHY-1/PDI-2 and PHY-1/human PDI dimers (D) are indicated by arrows.

FIG. 2. **Assembly of a recombinant C. elegans PHY-1/PHY-2/(PDI-2)_2 mixed tetramer.**

Recombinant PHY-1 and PHY-2 polypeptides were coexpressed in insect cells together with PDI-2 (lanes 1), PDI-1 (lanes 2) or human PDI (lanes 3). In control experiments, the human P4H α(I) subunit was coexpressed with human PDI in panel _A_ (lane 4). The cells were harvested and homogenized as described in the legend to Fig. 1. Triton X-100-soluble fractions were analyzed by nondenaturing PAGE followed by Coomassie staining (A) or by Western blotting using antibodies against PHY-2 (B) and PHY-1 (C). The positions of the P4H tetramers (T) and dimers (D) are indicated by arrows.

FIG. 3. **Analysis of P4H forms in C. elegans in vivo.** Nematodes from the wild-type N2 (lanes 1), _dpy-18(e364) _lanes 2), _phy-2(ok177) _lanes 3), and _phy-3(ok199) _lanes 4) strains were homogenized in a buffer containing Triton X-100 and the soluble fractions were analyzed by nondenaturing PAGE followed by Western blotting using antibodies against PHY-1 (A) and PHY-2 (B). Triton X-100-soluble proteins from insect cells
coexpressing PHY-1, PHY-2 and PDI-2 were used as a control in panel A (lane 5). The positions of the P4H tetramers (T) and dimers (D) are indicated by arrows.

FIG. 4. **Immunofluorescent localization of PHY-1, PHY-2, PDI-2 and DPY-7 in *C. elegans* embryos.** 1.5-fold (A) and elongated (B) wild-type embryos were incubated with rabbit polyclonal antibodies against the PHY-1, PHY-2, or PDI-2 polypeptides, and with a mouse monoclonal antibody against DPY-7 collagen polypeptide, and stained with AF594-labeled anti-rabbit IgG (red) and AF488-labeled anti-mouse IgG (green). A, In the 1.5-fold embryos all four polypeptides were colocalized in the hypodermal cells. B, In the elongated embryos the hypodermal location is maintained for the P4H subunits PHY-1, PHY-2 and PDI-2, but DPY-7 was incorporated into the developing cuticle.

FIG. 5. **Effect of pyridine 2,5-dicarboxylate on dpy-18(e364) nematodes.** A, Untreated adult wild-type and *dpy-18(e364)* (arrowed) nematodes at 20°C (magnification 100X). B, Treatment with 4 mM pyridine 2,5-dicarboxylate at 20°C leads to embryonic death in the *dpy-18(e364)* strain (magnification 1000X). C, Treatment with 500 µM pyridine 2,5-dicarboxylate at 25°C leads to severe dumpiness of the *dpy-18(e364)* larvae (magnification 400X). D, Treatment with 500 µM pyridine 2,5-dicarboxylate at 25°C leads to molting defects (unshed cuticle is indicated by an arrow) in adult *dpy-18(e364)* nematodes (magnification 400X).

FIG. 6. **Analysis of P4H formation by PHY-2 with P4H α subunits and PDI polypeptides from other species.** Recombinant PHY-2 was coexpressed in insect cells together with PHY-1 and PDI-1 (lane 1), PDI-2 (lane 2), human PDI (lane 3) or *Drosophila* PDI (lane 4), with the human P4H α(I) subunit and PDI-2 (lane 5), human PDI (lane 6), or *Drosophila* PDI (lane 7), with human P4H α(II) subunit and PDI-2 (lane 8), human PDI (lane 9) or *Drosophila* PDI (lane 10), and with the *Drosophila* α subunit and PDI-2 (lane 11), human PDI (lane 12) or *Drosophila* PDI (lane 13). The cells were harvested and homogenized as described in the legend to Fig. 1. Triton X-100-soluble fractions were analyzed by nondenaturing PAGE followed by Western blotting using an antibody against PHY-2. The position of the P4H tetramer (T) is indicated by an arrow.
**FIG. 7. Schematic representation of P4H tetramer formation by hybrid PHY polypeptides.**

Hybrids A-D were coexpressed in insect cells together with PHY-1 and PDI-2 (panel I), PHY-2 and PDI-2 (panel II), with PDI-2 only (panel III), and in combination with other hybrid PHY polypeptides and PDI-2 (panel IV). The cells were harvested and homogenized as described in the legend to Fig. 1. Triton X-100-soluble fractions were analyzed by nondenaturing PAGE followed by Western blotting using antibodies against the C-terminal residues of PHY-1 or PHY-2 (see Fig. 8). The PHY-1 and PHY-2 amino acids of the polypeptides are shown in black and white, respectively. Due to gaps in alignment (21) the numbers shown for the PHY-1 and PHY-2 amino acids in fact represent consecutive residues. Two PDI-2 polypeptides are also involved in tetramer assembly, but for clarity these are not drawn in the schematic representation. Formation of an active P4H tetramer is indicated by ‘+’ and a lack of tetramer assembly by ‘-’. Assembly of a mixed tetramer from hybrids C and D (indicated by ‘?’ in panel IV) could not be studied because hybrid C alone formed an enzyme tetramer with PDI-2 (panel III), and because both PHY polypeptides in C + D combination would have the C-terminal ends of PHY-1, so that a mixed tetramer could not be distinguished in Western blots. The critical regions in the PHY-1 and PHY-2 polypeptides required for mixed P4H tetramer assembly are indicated in panel V.

**FIG. 8. Nondenaturing PAGE analysis of P4H tetramer formation by hybrid PHY polypeptides.** Triton X-100-soluble proteins from the insect cell coexpression experiments described in Fig. 7 were analyzed by nondenaturing PAGE followed by Western blotting using antibodies against PHY-1 (lanes 1-3 and 6) or PHY-2 (lanes 4, 5, 7 and 8). Only samples from the coexpression experiments leading to tetramer formation are shown, while several examples of coexpression experiments in which no tetramer is formed can be seen in Fig. 6. Hybrid PHY polypeptides are indicated by letters as in Fig. 7. PDI-2 was always present in the coexpression experiments. The arrow indicates the position of the tetramer (T).
**TABLE I**

*P4H activity in Triton X-100 extracts of insect cells coexpressing C. elegans (Cel) or human (Hu) α subunits with various PDI subunits*

Values are given in dpm/10 µl of extractable cell protein, mean ± SD for at least four experiments.

| Polypeptides expressed                     | P4H activity  |
|--------------------------------------------|---------------|
|                                            | dpm/10 µl     |
| CelPHY-2 and CelPDI-2                      | 310 ± 160     |
| CelPHY-2 and CelPDI-1                      | < 50          |
| CelPHY-2 and HuPDI                         | < 50          |
| CelPHY-1 and CelPDI-2                      | 750 ± 460     |
| CelPHY-1 and CelPDI-1                      | < 50          |
| CelPHY-1 and HuPDI                         | 22120 ± 3610  |
| CelPHY-1 and CelPHY-2 and CelPDI-2         | 10280 ± 1540  |
| Huα(I) and HuPDI                           | 17390 ± 4560  |
TABLE II

**Kₘ values of the C. elegans mixed tetramer, C. elegans PHY-1/human PDI dimer and human type I P4H for cosubstrates and the peptide substrate and Kᵢ values for certain competitive inhibitors with respect to 2-oxoglutarate**

| Cosubstrate, substrate, or inhibitor | Kₘ or Kᵢ | PHY-1/PHY-2/(PDI-2)² | PHY-1/HuPDI<sup>a</sup> | HuP4H-I |
|-------------------------------------|-----------|-----------------------|---------------------|---------|
|                                     | Constant  | µM                    | µM                 | µM      |
| 2-Oxoglutarate                      | Kₘ        | 80                    | 20                 | 20<sup>b</sup> |
| Fe<sup>2+</sup>                      | Kₘ        | 2                     | 5                  | 2<sup>b</sup> |
| Ascorbate                           | Kₘ        | 350                   | 300                | 300<sup>b</sup> |
| (Pro-Pro-Gly)<sub>10</sub>          | Kₘ        | 20                    | 15                 | 15<sup>b</sup> |
| Pyridine-2,4-dicarboxylate          | Kᵢ        | 1                     | 2                  | 2<sup>b</sup> |
| Pyridine-2,5-dicarboxylate          | Kᵢ        | 1                     |                    | 0.8<sup>c</sup> |

<sup>a</sup>Ref. 17.

<sup>b</sup>Ref. 31.

<sup>c</sup>Ref. 5.
### TABLE III

**Double-stranded RNA interference of phy-1, phy-2 and phy-3**

| Injection construct | Strains and phenotypes |  |
|---------------------|------------------------|--|
|                     | Wild type              | *phy-1*<sup>-/-</sup> (*dpy-18e364*) | *phy-2*<sup>-/-</sup> (*ok177*) | *phy-3*<sup>-/-</sup> (*ok199*) |
| None                | Wild-type              | Dumpy                        | Wild-type              | Wild-type              |
| *phy-1*             | Dumpy<sup>a</sup>      | nd<sup>b</sup>              | Dumpy                  |
| *phy-2*             | Wild-type<sup>a</sup>  | Embryonic lethal<sup>a</sup> | Wild-type              |
| *phy-3*             | Wild-type              | Dumpy                        | Wild-type              |

<sup>a</sup> Ref 21.

<sup>b</sup> nd, not determined.
Figure 1

A  Triton  SDS

|   | Triton | SDS |
|---|--------|-----|
| 1 |        |     |
| 2 |        |     |

Coomassie Blue

|   | PHY-2+PDI-2 | PHY-2+PDI-1 | PHY-1+PDI-2 | PHY-1+PDI-1 | PHY-1+HuPDI |
|---|-------------|-------------|-------------|-------------|-------------|
| 1 |             |             |             |             |             |
| 2 |             |             |             |             |             |
| 3 |             |             |             |             |             |
| 4 |             |             |             |             |             |
| 5 |             |             |             |             |             |
| 6 |             |             |             |             |             |

Coomassie Blue

|   | PHY-2+PDI-2 | PHY-2+PDI-1 | PHY-1+PDI-2 | PHY-1+PDI-1 | PHY-1+HuPDI |
|---|-------------|-------------|-------------|-------------|-------------|
| 1 |             |             |             |             |             |
| 2 |             |             |             |             |             |
| 3 |             |             |             |             |             |
| 4 |             |             |             |             |             |
| 5 |             |             |             |             |             |
| 6 |             |             |             |             |             |

anti-PHY-2  anti-PHY-1
Figure 8

|   | PHY-1 + PHY-2 | PHY-1 + C | PHY-1 + D | PHY-2 + B | PHY-2 + C | C + C | B + C | B + D |
|---|---------------|-----------|-----------|-----------|-----------|-------|-------|-------|
| 1 |               |           |           |           |           |       |       |       |
| 2 |               |           |           |           |           |       |       |       |
| 3 |               |           |           |           |           |       |       |       |
| 4 |               |           |           |           |           |       |       |       |
| 5 |               |           |           |           |           |       |       |       |
| 6 |               |           |           |           |           |       |       |       |
| 7 |               |           |           |           |           |       |       |       |
| 8 |               |           |           |           |           |       |       |       |
The exoskeleton collagens in caenorhabditis elegans are modified by prolyl 4-hydroxylases with unique combinations of subunits
Johanna Myllyharju, Liisa Kukkola, Alan D. Winter and Antony P. Page

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