Characterization of a Novel Caenorhabditis elegans Prolyl 4-Hydroxylase with a Unique Substrate Specificity and Restricted Expression in the Pharynx and Excretory Duct*

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Collagen prolyl 4-hydroxylases (C-P4Hs) have a critical role in collagen synthesis, since 4-hydroxyproline residues are necessary for folding of the triple-helical molecules. Vertebrate C-P4Hs are \( \alpha_2\beta_2 \) tetramers in which the \( \beta \) subunit is identical to protein-disulfide isomerase (PDI). Three isoforms of the catalytic \( \alpha \) subunit, PHY-1, PHY-2, and PHY-3, have been characterized from Caenorhabditis elegans, PHY-1 and PHY-2 being responsible for the hydroxylation of cuticle collagens, whereas PHY-3 is predicted to be involved in collagen synthesis in early embryos. We have characterized transcripts of two additional C. elegans \( \alpha \) subunit-like genes, Y43F8B.4 and C14E2.4. Three transcripts were generated from Y43F8B.4, and a polypeptide encoded by one of them, named PHY-4.1, assembled into active \((\text{PHY-4.1})_2/(\text{PDI-2})_2\) tetramers and PHY-4.1/PDI-2 dimers when coexpressed with C. elegans PDI-2 in insect cells. The C14E2.4 transcript was found to have a frameshift leading to the absence of codons for two residues critical for P4H catalytic activity. Thus, C. elegans has altogether four functional C-P4H \( \alpha \) subunits, PHY-1, PHY-2, PHY-3, and PHY-4.1. The tetramers and dimers containing recombinant PHY-4.1 had a distinct substrate specificity from the other C-P4Hs in that they hydroxylated poly(l-proline) and certain other proline-rich peptides, including ones that are expressed in the pharynx, in addition to collagen-like peptides. These data and the observed restricted expression of the phy-4.1 transcript and PHY-4.1 polypeptide in the pharyngeal gland cells and the excretory duct suggest that in addition to collagens, PHY-4.1 may hydroxylate additional proline-rich proteins in vivo.

Two animal prolyl 4-hydroxylase (P4H) \( ^2 \) families are currently known that are functionally distinct, although they both belong to the 2-oxoglutarate-dependent dioxygenases and require \( \text{Fe}^{2+} \), 2-oxoglutarate, and molecular oxygen for catalysis (1–6). The collagen P4Hs (C-P4Hs), enzymes residing within the endoplasmic reticulum, catalyze the hydroxylation of proline residues in the -Pro-Gly- triplets in collagens (1–3). Vertebrates have at least 28 collagen types, encoded by 43 distinct genes (3, 7), and C-P4Hs have a vital role in the synthesis of all of them, since the 4-hydroxyproline residues formed are required for folding of the newly synthesized collagen polypeptide chains into stable triple-helical molecules (1–3). The other P4H family acts as an oxygen sensor by regulating the stability of the hypoxia-inducible transcription factor (HIF), an \( \alpha \beta \) dimer (2, 4–6). Under normoxic conditions, two specific proline residues in the HIF-\( \alpha \) subunit are subject to hydroxylation by the HIF-P4Hs, which are located in the cytoplasm and nucleus, resulting in binding of the von Hippel-Lindau E3 ubiquitin ligase complex and rapid targeting of HIF-\( \alpha \) for proteasomal degradation (2, 4–6). In hypoxia, the activity of the HIF-P4Hs is inhibited, so that HIF-\( \alpha \) accumulates and forms an active dimer with HIF-\( \beta \), which induces the expression of various hypoxia-inducible genes, such as those for vascular endothelial growth factor, erythropoietin, glucose transporters, and glycolytic enzymes (2, 4–6).

The vertebrate C-P4Hs are \( \alpha_2\beta_2 \) tetramers, the \( \beta \) subunits of which are identical to the enzyme and chaperone protein-disulfide isomerase (PDI) (1–3). The \( \alpha \) subunits contain the catalytic site for proline 4-hydroxylation, with conserved residues for the binding of \( \text{Fe}^{2+} \) and 2-oxoglutarate and a separate peptide-substrate-binding domain (8–12). Three vertebrate C-P4H isoenzymes differing in their catalytic \( \alpha \) subunit, i.e. the \([\alpha(\text{I})_2]\beta_2, [\alpha(\text{II})_2]\beta_2, \) and \([\alpha(\text{III})_2]\beta_2\) tetramers, called the type I, II, and III C-P4Hs, respectively, have been identified and characterized (13–17). C-P4H-I is the major form in most tissues, and lack of its function leads to early embryonic death in mice, which is consistent with the role of the gene in early embryonic development in mice.

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§ Two abbreviations used are: P4H, prolyl 4-hydroxylase; C-P4H, collagen prolyl 4-hydroxylase; HIF, hypoxia-inducible transcription factor; HIF-P4H, HIF prolyl 4-hydroxylase; PHY, \( \alpha \) subunit of C. elegans P4H; PDI, protein-disulfide isomerase; RNAi, RNA interference; PBS, phosphate-buffered saline; PBST, PBS-Tween; PBCV-1, Paramecium bursaria Chlorella virus-1; E3, ubiquitin-protein isopeptide ligase; RACE, rapid amplification of cDNA ends; HSP, heat shock protein.
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whereas C-P4H-II is the main form in chondrocytes, osteoblasts, endothelial cells, and some other cell types, and C-P4H-III is expressed in many tissues but at lower levels than the other two isoenzymes (16, 18–20). The vertebrate HIF-P4Hs, which are most probably monomers in solution, also have three isoenzymes (21–24), of which HIF-P4H-2 is currently regarded as the most important (25–27).

Animal C-P4Hs and HIF-P4Hs have also been characterized from the fly Drosophila melanogaster and the nematode Caenorhabditis elegans. Both species probably have several C-P4H isoenzymes but only a single HIF-P4H (2, 3, 21, 22, 28). The D. melanogaster genome contains a large gene family of 20 members that encode C-P4H α-subunit-like polypeptides (29). One of these has been characterized as a recombinant protein and shown to assemble into an α2β2 tetramer with PDI and to hydroxylate collagen-like peptides (30). Expression of two other D. melanogaster C-P4H α-subunit-like genes has been shown to be restricted to the salivary glands and to be necessary for the maintenance of an open salivary gland lumen and proper secretory function (29, 31). Since D. melanogaster has only three genes coding for collagens, all of these polypeptides being found in its basement membranes (3), and since salivary glands do not produce these collagens, it is highly likely that these α-subunit-like polypeptides hydroxylate some other, yet unknown, collagen-related proteins with proline-rich sequences (29, 31).

In contrast to D. melanogaster, C. elegans has a very large collagen family consisting of cuticle collagens with close to 180 different polypeptides and basement membrane collagens with three different polypeptides (3). Three C-P4H α subunits, PHY-1, PHY-2, and PHY-3, have been characterized in C. elegans (32–37), two of which, PHY-1 and PHY-2, are expressed in the cuticle collagen-synthesizing hypodermal cells in a cyclical fashion that corresponds the molting cycle and the times of maximal cuticle collagen synthesis (34, 35). Analyses of genetically mutant nematodes or nematodes subjected to RNA interference (RNAi) have shown that PHY-1 and PHY-2 are involved in the synthesis of cuticle collagens in C. elegans and its close homologue Caenorhabditis briggsae (33–36, 38). C. elegans PHY-3 is expressed in the embryos, late larval stages, and adult nematodes, its expression in the larval and adult stages being restricted to the spermatheca, a specialized region of the gonad where fertilization of the oocytes occurs (37). Nematodes lacking PHY-3 have no obvious phenotypic abnormalities, but the 4-hydroxyproline content of their embryos is markedly reduced, suggesting that PHY-3 is involved in the synthesis of collagens in early embryos, most likely the collagens present in the egg shell (37). C. elegans has three PDI isoforms, PDI-1, PDI-2, and PDI-3, of which PDI-2 functions as the C-P4H β subunit (38, 39).

The Caenorhabditis C-P4Hs assembled from the PHY-1 and PHY-2 polypeptides have unique molecular compositions by comparison with the vertebrate C-P4Hs and the one D. melanogaster C-P4H that has been characterized in detail. The main C-P4H form in C. elegans is a PHY-1/PHY-2/(PDI-2)2 mixed tetramer, but both C. elegans PHY polypeptides can also assemble into an active dimer with PDI-2, albeit much less effectively (36). The assembly properties of the highly homologous C. briggsae PHY-1 and PHY-2 are similar to those of the corresponding C. elegans polypeptides with the exception that in the absence of PHY-1, C. briggsae PHY-2 assembles into an active (PHY-2)2/(PDI-2)2 tetramer instead of a PHY-2/PDI-2 dimer (40). Combined disruption of phy-1 and phy-2 gene function leads to the absence of all cuticle C-P4H forms in the two Caenorhabditis species and results in an embryonic lethal phenotype (33, 35, 36, 40). Disruption of the phy-2 gene alone produces no visible phenotype, since the enhanced assembly of the PHY-1/PDI-2 dimer can fully compensate for the lack of the mixed tetramer (33, 35, 36, 40). Inactivation of the C. elegans and C. briggsae phy-1 genes alone leads to abnormal cuticle formation and dumpy and small phenotypes, respectively (33–36, 40). Although enhanced assembly of the PHY-2/PDI-2 dimer and (PHY-2)2/(PDI-2)2 tetramer occurs in phy-1 null C. elegans and C. briggsae, respectively, these C-P4H forms cannot fully compensate for the lack of the mixed tetramer and the PHY-1/PDI-2 dimer (36, 40). The recombinant C. elegans PHY-3 differs from PHY-1 and PHY-2 in that C-P4H activity is only generated when it is coexpressed with another C. elegans PDI isoform, PDI-1, but not with PDI-2 (37, 38). Whether PHY-3 assembles into a complex with PDI-1 or whether PDI-1 just assists in its folding is as yet unknown, however (37).

In the present study we characterized transcripts, generated from the C. elegans genes Y43F8B.4 and C14E2.4, which were predicted to code for polypeptides with similarity to the catalytic α subunits of the human and C. elegans C-P4Hs. Three alternative transcripts were generated from the Y43F8B.4 gene, one of which, named phy-4.1, coded for a polypeptide that, when coexpressed in insect cells, assembled into an active C-P4H dimer and tetramer with C. elegans PDI-2. The spatial expression of the phy-4.1 transcript and PHY-4.1 polypeptide was found to be restricted to the pharyngeal gland cells and the excretory duct. Down-regulation of the phy-4.1 expression by RNAi did not lead to any obvious phenotypic abnormalities, however. A frameshift in the C14E2.4 transcript led to an omission of two of the four catalytically critical residues in the encoded polypeptide, which thus cannot possess P4H activity. These data indicate that C. elegans possesses four functional C-P4H α subunits, namely PHY-1, PHY-2, PHY-3, and PHY-4.1. The recombinant (PHY-4.1)2/(PDI-2)2 tetramer and PHY-4.1/PDI-2 dimer had a distinct substrate specificity from other C-P4Hs, in that they hydroxylated polypeptide and certain other proline-rich peptides in addition to collagen-like peptides. These data taken together with the unique expression pattern suggest that in addition to collagens, PHY-4.1 may be involved in the hydroxylation of some other proline-rich proteins in vivo.

EXPERIMENTAL PROCEDURES

C. elegans Strains and cDNA Pools Used in the Cloning of phy-4—The wild-type Bristol (N2), daf-2(e1370), RNAi-sensitive rrf-3(NL2099), dpy-18(e3664), and phy-2(ok177) strains were obtained from the Caenorhabditis Genetics Center and maintained at 20°C by standard methods (41). The phy-3(ok199) strain (37) was generated from nematodes carrying a deletion in phy-3 that were obtained from the C. elegans Genome Deletion Consortium. Heat shock treatment of N2
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nematodes was performed at 37 °C for 1 h, and dauer formation was induced in the daf-2(e1370) strain by maintaining it at 25 °C for 1 week with a low food supply according to instructions in the C. elegans data base (available on the World Wide Web). Total RNA and mRNA were isolated from normal N2 and dauer daf-2(e1370) mixed stage cultures with the TRIzol LS reagent (Invitrogen) and a Poly(A) quick mRNA isolation kit (Stratagene), respectively. Using the cDNA pools generated above; and PCR with primer pairs Phy4PRT10C-TTGAATTCT-TTGATTTTGTTGCATTAC (the artificial restriction sites are set in lower-case type and underlined, and an additional cytotoxic following the restriction site was introduced to obtain in-frame cloning with the GP67 signal sequence) and the longer variant phy-4.2 with the primers Phy4ABamHI5’-FNFL and Phy4BNo13’Stop (5’-taaagcggccgctcAAGGTGATCGTGT-TGTGGTTGTTGTG-3’) that is located in exon 5 in the prediction Y43F8B.4. 30 PCR cycles with Pfu polymerase were performed as follows: denaturation for 1 min at 94 °C, annealing for 2 min at 55 °C and extension for 3 min at 72 °C, the final cycle including an additional extension for 10 min at 72 °C. The sequences of the expression constructs were verified using an automated DNA sequencer. The recombinant baculovirus vectors were cotransfected into Spodoptera frugiperda Insect cells (Sf9; Invitrogen) with a modified Autographa californica nuclear polyhedrosis virus DNA using the BaculoGold transfection kit (Pharmingen), and the resultant viral pools were collected and amplified (42).

Insect cells (Sf9 or High Five; Invitrogen) were cultured in TNN-FH medium (Sigma) supplemented with 10% insect cell qualified fetal bovine serum (Invitrogen) as monolayers at 27 °C. Cells seeded at a density of 5 x 10^6 cells/100-mm plate were infected at a multiplicity of 5 with the viruses coding for the PHY-4.1 or PHY-4.2 polypeptide with or without a virus coding for C. elegans PDI-1 or PDI-2 (38). In control experiments, the cells were infected with the virus coding for C. elegans PDI-2 alone or coinfected with those coding for C. elegans PHY-1, PHY-2, and PDI-2 (32, 36, 38). 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 (phosphate-buffered saline, PBS), 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 × g for 20 min. The remaining pellets were further solubilized in 1% SDS. Aliquots of the samples were analyzed by 8% reducing SDS-PAGE and nondenaturing PAGE followed by Coomassie Blue staining or Western blotting with polyclonal antibodies against C. elegans PHY-4.1 (see below), PDI-1, and PDI-2 (36, 38). N-Glycosidase F treatment was performed according to the manufacturer’s instructions (Roche Applied Science). P4H activity in the Triton X-100-soluble fractions was assayed by a method based on the hydroxylation-coupled decarboxylation of 2-oxo-[1-14C]glutarate (43). The synthetic collagen-like peptide substrate (Pro-Pro-Gly)_10 was purchased from the Peptide Institute, other synthetic peptides were from Innovagen, and poly(l-proline) was from Sigma. The peptides, except for poly(l-proline), were denatured by heating to 100 °C for 10 min, followed by rapid cooling before the addition to the enzyme reaction mixture. K_m values were determined as described previously (9). The molecular weight of the P4H assembled from PHY-4.1 and PDI-2 was analyzed by gel filtration on a calibrated Superdex S-200 column (GE Healthcare). N-terminal sequencing was performed using AccQ-Tag chemistry (Waters) in a Procise protein sequencer (Applied Biosystems).

Expression and Analysis of Recombinant PHY-4 Variants in Insect Cells—The cDNAs for the transcripts phy-4.1 and phy-4.2 (Fig. 1) without the sequences for the predicted transmembrane helix and signal peptide, respectively, were amplified by PCR and cloned into a BamHI-NotI-digested baculovirus expression vector pACGP67-A (Invitrogen) in frame with the baculovirus GP67 signal sequence. The phy-4.1 cDNA was amplified from the normal N2 mixed stage cDNA pool by PCR with the primers Phy4ABamHI5’-FNFL (5’-cgggatccccTTCAATTTCCTCACAACCTGTT-3’) and Phy4ANotI3’Stop 5’-cgggatccccTTATGAGAATGTGT-TGTGGTTGTTGTA-3’ (the artificial restriction sites are set in lowercase type and underlined, and an additional cytotoxic following the restriction site was introduced to obtain in-frame cloning with the GP67 signal sequence) and the longer variant phy-4.2 with the primers Phy4ABamHI5’-FNFL and Phy4BNo13’Stop (5’-taaagcggccgctcAAGGTGATCGTGT-TGTGGTTGTTGTG-3’) that is located in exon 5 in the prediction Y43F8B.4. 30 PCR cycles with Pfu polymerase were performed as follows: denaturation for 1 min at 94 °C, annealing for 2 min at 55 °C and extension for 3 min at 72 °C, the final cycle including an additional extension for 10 min at 72 °C. The sequences of the expression constructs were verified using an automated DNA sequencer. The recombinant baculovirus vectors were cotransfected into Spodoptera frugiperda Insect cells (Sf9; Invitrogen) with a modified Autographa californica nuclear polyhedrosis virus DNA using the BaculoGold transfection kit (Pharmingen), and the resultant viral pools were collected and amplified (42).
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case type and underlined). The fragment was digested with XbaI-Smal and ligated into the promoterless nucleus-localized vector pPD96.04 (Addgene), allowing in-frame fusion of the phy-4 promoter sequence and green fluorescent protein/lacZ reporter genes.

Transgenic strains were generated by microinjecting the reporter plasmid (20 μg/ml) together with the marker plasmid pRF-4 rol-6(su1006) (44) (100 μg/ml) into the syncytial gonad of wild-type nematodes. Three independent F2-F3 lines were maintained and examined for reporter gene expression as of wild-type nematodes. Three independent F2-F3 lines were viewed and photographed under Nomarski optics using a Zeiss Axioscop 2 microscope.

**Immunofluorescence Staining**—A polyclonal rabbit antibody was generated against a synthetic PHY-4.1 peptide TLWVEHHKKQEFSLP (Innovagen) and purified using a HiTrap Protein A HP column (GE Healthcare) followed by desalting in a PDI-10 column (GE Healthcare) according to the manufacturer’s instructions. Wild-type and heat-shocked nematodes were washed from the plates with ice-cold PBS. The washing step was repeated 3–10 times, and the animals were pipetted onto poly-l-lysine-coated slides, permeabilized by freeze-cracking (46) and fixed in methanol for 10 min followed by acetone for 10 min, both at −20 °C. In an alternative method, the nematodes were fixed in 49% Bowin fixative (75% saturated picric acid, 25% formaldehyde, and 5% glacial acetic acid), 49% methanol, and 1.2% 2-mercaptoethanol for 30 min at room temperature, frozen in liquid N2, and quickly thawed under hot water (47). The slides were blocked in PBS containing 0.1% Tween 20 (PBST) and 5% dried skimmed milk and incubated with the PHY-4.1 antibody at 1:100 in PBST, followed by extensive washing and incubation in Alexa Fluor 594 goat anti-rabbit IgG (Molecular Probes), and the signal was amplified with biotinylated streptavidin-phycocerythrin (Vector Laboratories) and a second staining with streptavidin-phycoerythrin, followed by scanning on an HP GeneArray Scanner. The expression data were analyzed using the Affymetrix MicroArray Suite version 5.0. The signal intensities of all of the probe sets were scaled to the target value of 500.

**RNA Interference**—The effects of down-regulation of phy-4.1 were examined using standard RNAi injection protocols (35). A fragment covering the nucleotides 162–723 of the coding region of the phy-4.1 cDNA was amplified from C. elegans mixed stage cDNA by PCR using the primers 5’TTCGAGGCCTTCACTCCTACAG-3’ and 3’RNAi-Phy4-5’-ggcgcgcgctTTCTTCTCGAGACCATTGAAGTCT-3’ (artificial restriction sites set in lowercase type and underlined). The PCR product was cloned into the BamHI-NotI-digested vector pPD129:36 (48), and a double-stranded RNA was produced in vitro as described previously (35). Fifty young adults of the wild-type N2 strain were microinjected, allowed to recover overnight, and transferred singly to fresh plates, and their progenies were scored. Bacterially mediated RNAi was performed as described previously (48), by transferring several L4 animals of the wild-type N2, RNAi-sensitive NL2099, dpy-18(c364), phy-2(ok177), and phy-3(ok199) strains to feeding plates containing E. coli transformed with the pPD129:36-phy-4 construct. The plates were incubated for 2 days at 15, 20, and 25 °C, after which the adults were transferred to fresh feeding plates and allowed to lay eggs for 24 h, and the progenies were scored.

**Analysis of the Ultrastructure of the Pharynx and Pharyngeal Pumping**—Bacterially mediated RNAi with the pPD129:36-phy-4 construct was performed on L1 larvae of the wild-type N2 strain. The L1 larvae were generated using the basic bleaching method (41). The bleached eggs were transferred to an unseeded plate and incubated at 20 °C overnight. The hatched L1 larvae were transferred at 20 °C to fresh feeding plates containing E. coli transformed with the pPD129:36-phy-4 construct. The pharyngeal pumping rate of the phy-4 RNAi and wild-type L1, L2, L3, L4, and adult nematodes was analyzed under an Olympus BX51 light microscope. For high pressure freezing, the phy-4 RNAi and wild-type L2 nematodes were mixed into a thick paste made from dry bakers’ yeast and 10% methanol. The paste was placed to flat specimen carriers (Leica Microsystems) and cryofixed in a Leica EM Pact high pressure freezer. The specimens were further freeze-substituted for Epon embedding in a Leica AFS freeze-substitution system. The specimens were infiltrated in 1% osmiumtetroxide and 0.1% uranyl acetate in acetone at −90 °C for 48 h and gradually warmed to 20 °C in 5 °C steps. The specimens were removed from the carriers and infiltrated to Epon LX112 (Ladd Research Industries) at room temperature and polymerized at 60 °C for 48 h. Thin sections were cut with a Leica Ultratric UCT ultramicrotome, followed by staining in uranyl acetate and lead citrate, and examined in a Philips CM100 transmission electron microscope. Images were captured by a CCD camera equipped with TCL-EM-Menu version 3 from Tietz Video and Image Processing Systems GmbH.
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RESULTS

Analysis of Transcripts and Polypeptides Generated from the Predicted Genes Y43F8B.4 and C14E2.4—A sequence homology search of the C. elegans genome indicated the presence of two novel predicted genes Y43F8B.4 and C14E2.4 coding for polypeptides that show sequence similarity to the C-terminal regions of the catalytic subunits of the human and C. elegans C-P4Hs (i.e. human α(I), α(II), and α(III), and C. elegans PHY-1, PHY-2, and PHY-3) (Fig. 1). The predicted Y43F8B.4 and C14E2.4 genes have seven and six exons, respectively (Fig. 1).

The putative Y43F8B.4 gene was predicted to code for a 533-amino acid polypeptide that included two sets of the catalytically critical conserved residues, two histidines and one aspartate that bind the Fe²⁺ atom and a lysine that binds the C-5 carboxyl group of 2-oxoglutarate (9), are indicated above the respective exons by H, D, and K. The phy-4.1 RNAi construct is also shown schematically in A.

Three transcripts, termed phy-4.1, phy-4.2, and phy-4.3, were identified (Fig. 1A). Transcript phy-4.1 contains the sequences of the predicted exons 1–4, but instead of exon 4 being spliced to exon 5, the transcript continues to intron 4, where an in-frame translation stop codon occurs 65 nucleotides downstream from the 3’-end of exon 4 (Fig. 1A). The phy-4.1 transcript thus codes for a 278-residue polypeptide (Fig. 2). The transcript phy-4.2 also contains the exon 1–3 sequences, exon 4 now being spliced into exon 5, but so that the last nucleotide of the predicted exon 4 is lost, which leads to an in-frame stop codon 78 nucleotides downstream in exon 5 (Fig. 1A). The phy-4.2 transcript codes for a 282-residue polypeptide (Fig. 2). The PHY-4.1 and PHY-4.2 polypeptides thus only differ from each other at their C-terminal ends starting from residue 257 (Fig. 2). The third cDNA product identified, termed phy-4.3, contains the last 83 nucleotides of the predicted exon 5, which is spliced into exon 6, but the latter is spliced into exon 7 in such a manner that the last two nucleotides of the predicted exon 6 and the first two of exon 7 are lost, which leads to a frameshift and an in-frame stop codon 73 nucleotides downstream in exon 7 (Fig. 1A). As a result of the frameshift, the codons for the second histidine binding the Fe²⁺ and the lysine binding the C-5 carboxyl group of 2-oxoglutarate are not present in the phy-4.3 transcript (Fig. 1A). These catalytically critical residues are thus lacking in the PHY-4.3 polypeptide, which therefore was not analyzed further.

Sequencing of the PCR and RACE products obtained with the C14E2.4-specific primers showed that a single transcript is generated from this gene that contains exons 1–3 as predicted, but 4 nucleotides in the data base sequence of the 3’ end of exon 4 are missing, which leads to a frameshift when spliced into exon 5, omission of codons for two catalytically critical residues, and a premature stop codon (Fig. 1B). Since the encoded polypeptide cannot function as a P4H, it was not studied further.

The PHY-4.1 amino acid sequence starting from Leu¹⁸ after the N-terminal transmembrane helix is 23% identical to residues 214–523 of the 543-amino acid C. elegans PHY-1, 25% identical to the corresponding residues of PHY-2, and 27% identical to the 295-residue PHY-3 (Fig. 2). The corresponding identities of the PHY-4.2 polypeptide with the C. elegans PHY-1, PHY-2 and PHY-3 are 21, 22, and 26%, respectively (Fig. 2). The sequence conservation is highest within the C-terminal regions where the catalytically critical residues are located, the PHY-4.1 and PHY-4.2 amino acids 138–256 being 35–37% identical to the corresponding regions of PHY-1, PHY-2, and PHY-3 (Fig. 2). The two histidines and one aspartate that bind...
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The amino acid sequences of PHY-1 and PHY-2 that precede the alignment region and the signal peptide of PHY-3 are not shown. Gaps were introduced for maximal alignment. Residues of PHY-4.1 or PHY-4.2 that are identical with any of the other PHY polypeptides are given in white letters on a black background. The catalytically critical residues (9) are indicated by asterisks, and the potential N-glycosylation site in PHY-4.1 and PHY-4.2 is shown (o).

![Alignment of the amino acid sequence of PHY-4.1 and PHY-4.2 with those of PHY-1, PHY-2, and PHY-3.](image)

FIGURE 2. Expression of recombinant PHY-4.1 and PHY-4.2 polypeptides in insect cells and analysis of their assembly with PDI-2. A, insect cells were infected with viruses coding for PHY-4.1 (lanes 1–3) or PHY-4.2 (lanes 4 and 5) polypeptides, harvested 72 h after infection, homogenized in a Triton X-100-containing buffer, and centrifuged. The remaining pellets were solubilized in 1% SDS; and the Triton X-100-soluble fractions were analyzed by 8% SDS-PAGE followed by Western blotting using antibodies against PHY-4.1, PDI-1, and PDI-2 (Fig. 3, lanes 1, 2, 4, and 5). Like the human C-P4H α subunits and the C. elegans PHY-1, PHY-2, and PHY-3 polypeptides (14, 32, 36, 37), the PHY-4.1 and PHY-4.2 polypeptides formed insoluble aggregates, and 1% SDS was required for their efficient solubilization (Fig. 3A, lanes 2 and 5). To study whether the potential N-glycosylation site present in PHY-4.1 and PHY-4.2 is utilized in insect cells, recombinant PHY-4.1 was digested with N-glycosidase F (Fig. 3A, lane 3). Two forms were seen in the nondigested sample, whereas only the lower band remained after the digestion, indicating that the nondigested sample contained both monoglycosylated and nonglycosylated forms of the PHY-4.1 polypeptide (Fig. 3A, lanes 2 and 5). N-terminal sequencing showed that the GP67 signal peptide was correctly cleaved from the PHY-4.1 polypeptide (data not shown). Taken together, these two results indicate that the recombinant PHY-4.1 polypeptide was correctly translocated into the lumen of the endoplasmic reticulum in the insect cells. Since the N terminus of the recombinant PHY-4.2 polypeptide is identical to that of PHY-4.1, it can be expected to have the same subcellular location.

To study the association of PHY-4.1 and PHY-4.2 with PDI, insect cells were infected with recombinant viruses coding for PHY-4.1 or PHY-4.2 with or without those coding for C. elegans PDI-1 or PDI-2. In control experiments, insect cells were infected with the virus coding for PDI-2 alone or coinfected with those coding for PHY-1, PHY-2, and PDI-2. Triton X-100-soluble extracts of the cell homogenates were analyzed by non-denaturing PAGE followed by Western blotting with antibodies against PHY-4.1, PDI-1, and PDI-2 (Fig. 3, B and C). When

the Fe$^{2+}$ atom and the lysine that binds the C-5 carboxyl group of 2-oxoglutarate (8, 9) are all conserved (Fig. 2). The human C-P4H α subunits and the C. elegans PHY-1 and PHY-2 have four cysteine residues in conserved positions that have been shown by site-directed mutagenesis of the human α(1) subunit to be involved in the formation of an essential intrachain disulfide bond (8, 49). The PHY-4.1 polypeptide has cysteine residues in the corresponding positions, whereas the fourth one is missing in PHY-4.2 (Fig. 2). The PHY-4.1 and PHY-4.2 polypeptides contain one potential attachment site for N-linked oligosaccharide units, which is located in the same position as one of the five potential glycosylation sites of PHY-3 (37) (Fig. 2).
PHY-4.1 was coexpressed with PDI-2, two bands with mobilities intermediate between those of the PHY-1/PHY-2/(PDI-2)2 tetramer and the free PDI-2 polypeptide were stained by the PHY-4.1 and PDI-2 antibodies, indicating that these two polypeptides had become associated into soluble complexes (Fig. 3, B and C, lanes 3). In contrast, PHY-4.2 did not associate with PDI-2 (Fig. 3C, lane 5), and neither PHY-4.1 nor PHY-4.2 associated with PDI-1 (data not shown). To study the molecular composition of the soluble complexes formed by PHY-4.1 and PDI-2, Triton X-100 extracts from cells coexpressing PHY-4.1 and PDI-2 were applied to a calibrated Superdex-200 gel filtration column, and the fractions were analyzed by nondenaturing PAGE followed by Western blotting. The two bands stained by both PHY-4.1 and PDI-2 antibodies eluted in positions corresponding to molecular weights of ~180,000 and 90,000 (data not shown), indicating that PHY-4.1 and PDI-2 had assembled into a (PHY-4.1)2/(PDI-2)2 tetramer and a PHY-4.1/PDI-2 dimer. The nondenaturing PAGE analysis indicated that the assembly of the PHY-4.1/PDI-2 dimer was much more efficient than that of the (PHY-4.1)2/(PDI-2)2 tetramer (Fig. 3, B and C, lanes 3).

P4H activity in the Triton X-100-soluble extracts from cells coexpressing PHY-4.1 and PDI-2 was analyzed using an assay based on the hydroxylation-coupled decarboxylation of 2-oxo-[1-14C]glutarate with a collagen-like (Pro-Pro-Gly)10 peptide as a substrate. Define C-P4H activity was generated in these extracts but only when relatively high (Pro-Pro-Gly)10 concentrations were used (data not shown). However, even when (Pro-Pro-Gly)10 was used at a concentration of up to 4 mM, the amount of C-P4H activity generated in these samples was typically 10–30-fold lower than that in extracts from cells expressing the PHY-1/PHY-2/(PDI-2)2 tetramer with an 80 μM concentration of (Pro-Pro-Gly)10 as a substrate (data not shown). No C-P4H activity was generated in the samples from cells expressing PHY-4.1 alone or coexpressing PHY-4.1 and PDI-1, PHY-4.2 and PDI-2, or PHY-4.2 and PDI-1 (data not shown).

C. elegans P4H Tetramers and Dimers Containing PHY-4.1 as the Catalytic Subunit Have a Unique Substrate Specificity—Since the Triton X-100-soluble extracts from insect cells expressing the PHY-4.1/PDI-2 dimers and (PHY-4.1)2/(PDI-2)2 tetramers seemed to hydroxylate the collagen-like (Pro-Pro-Gly)10 peptide relatively poorly, we studied whether they would hydroxylate other proline-rich sequences (Table 1). Recombinant P4Hs from the eukaryotic algal virus *Paramecium bursaria* Chlorella virus-1 (PBCV-1), the monocellular green alga *Chlamydomonas reinhardtii*, and the plant *Arabidopsis thaliana* have been shown to hydroxylate various proline-rich peptides, including poly(1-proline), whereas they mostly hydroxylate collagen-like peptides only very inefficiently (50–53). In contrast, the vertebrate and *C. elegans* C-P4Hs characterized so far have a strict specificity for -X-Pro-Gly- sequences, and they do not hydroxylate poly(1-proline), which, in the case of the vertebrate C-P4H-I, acts as a powerful competitive inhibitor with respect to the collagen substrate (10, 14–16). The peptides were studied at 200–400 μM concentrations as substrates for soluble extracts of insect cells coexpressing *C. elegans* PHY-4.1 and PDI-2, whereas extracts of insect cells expressing PDI-2 alone served as a negative control. About double the amount of P4H activity was found in the extracts of cells coexpressing PHY-4.1 and PDI-2 when the peptide (Pro-Pro-Glu-Pro-Pro-Ala)5 was used as a substrate instead of (Pro-Pro-Gly)10 (Table 1). Proteins containing Pro-Glu-Pro-Pro-Ala repeats are encoded in the PBCV-1 genome, and the (Pro-Glu-Pro-Pro-Ala)5 peptide is efficiently hydroxylated by the PBCV-1 P4H and the *A. thaliana* P4H isoenzyme 1 (50, 51). Extracts of cells coexpressing PHY-4.1 and PDI-2 also hydroxylated poly(1-proline), but the amount of P4H activity generated was 4–6-fold lower than that obtained with (Pro-Pro-Gly)10 (Table 1). We also tested various other synthetic peptides representing collagen-like sequences (*i.e.* (Pro-Ala-Gly)5 and (Ala-Ala-Gly)5), proline-rich sequences of PBCV-1 (*i.e.* (Pro-Ala-Lys)5), or *C. reinhardtii* proteins (*i.e.* (Ser-Pro-Glu/Lys-Pro-Pro)5, (Pro-Ser)3-Pro-Ile-(Pro-Ser)2-Pro-Lys-(Pro-Ser)-2-Pro); two proline-rich repeats encoded by open reading frames of the *C. elegans* genome (*i.e.* (Pro-Glu-Pro-Gln)5 and (Glu-Pro-Pro-Lys-Thr)5); and a peptide representing the C-terminal hydroxylation site in the human HIF-1α (*i.e.* Asp-Leu-Asp-Leu-Glu-Met-Leu-Asa-Pro-Tyr-Ile-Pro-Met-Asp-Asp-Phe-Gln-Leu) as substrates. None of these peptides was hydroxylated by the extracts of cells coexpressing PHY-4.1 and PDI-2 (data not shown).

The values are means ± S.D. from 5–9 independent experiments. Triton X-100-soluble extracts from insect cells expressing *C. elegans* PDI-2 alone were used as controls and gave <40 dpm/100 μg of protein extract with all of the peptides tested.

### TABLE 2

| Substrate | (PHY-4.1)2/(PDI-2)2 + PHY-4.1/PDI-2 | PHY-1/PHY-2/(PDI-2)2 | PBCV-1 P4H
|-----------|----------------------------------|---------------------|----------|
| C-P4H activity | 2,500 | 20 | 2,900 |
| (Pro-Pro-Gly)10 | 250 | ND | 1,000 |
| Poly(1-proline) | 220 | 500 | 110 |
| M | 30,000–40,000 | 200 | 200 |

a Ref. 36.
b Ref. 50.
c Not determined.
d Does not serve as a substrate for PHY-1/PHY-2/(PDI-2)2.

### TABLE 1

| Peptide substrate | Peptide concentration | Activity* |
|------------------|-----------------------|-----------|
| (Pro-Pro-Gly)10 | 400 | 1,840 ± 460 |
| (Pro-Glu-Pro-Pro-Ala) | 400 | 4,100 ± 830 |
| Poly(1-proline), M | 5,000–10,000 | 980 ± 130 |
| Poly(1-proline), M | 30,000–40,000 | 630 ± 160 |

* The values are means ± S.D. from 5–9 independent experiments. Triton X-100-soluble extracts from insect cells expressing *C. elegans* PDI-2 alone were used as controls and gave <40 dpm/100 μg of protein extract with all of the peptides tested.

* Sequence encoded by the PBCV-1 genome.

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**A Novel *C. elegans* Prolyl 4-Hydroxylase**

**TABLE 2**

| Km (μM) | Km (μM) | Km (μM) |
|---------|---------|---------|
| (Pro-Pro-Gly)10 | >2,500 | 20 | 2,900 |
| (Pro-Glu-Pro-Pro-Ala) | 250 | ND | 1,000 |
| Poly(1-proline) | 220 | 500 | 110 |
| M | 30,000–40,000 | 200 | 200 |

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A Novel C. elegans Prolyl 4-Hydroxylase

TABLE 3

| Cosubstrate | (PHY-4.1)/ (PDI-2) | PHY-1/(PDI-2) | Human C-P4H-1a | C. reinhardtii P4H-1b |
|-------------|-----------------|---------------|----------------|-------------------|
|             | µM              | µM            | µM             | µM                |
| Fe2⁺        | 10              | 2             | 2              | 30                |
| 2-Oxoglutarate | 30             | 80            | 20             | 250               |
| Ascorbate   | 40              | 350           | 300            | 20                |

a Ref. 36.

b Ref. 9.

c Ref. 53.

Pro-Ala)10, 250 µm, was at least 10-fold lower than that for (Pro-Pro-Gly)10 and 4-fold lower than the Km of PBCV-1 P4H for the same peptide (Table 2). The Km values for the M, 5000–10,000 and 30,000–40,000 poly(L-proline) peptides were 220 and 110 µM, respectively, about 2-fold lower than those of the PBCV-1 P4H (Table 2).

Km Values of C. elegans P4H Tetramers and Dimers Containing PHY-4.1 as the Catalytic Subunit for Cosubstrates—Km values were determined for the P4H reaction cosubstrates Fe2⁺, 2-oxoglutarate, and ascorbate (Table 3). The Km for Fe2⁺ was 10 µM, 5-fold higher than the values of the C. elegans PHY-1/(PDI-2)2 tetramer and the human C-P4H-1 and one-third of that of the C. reinhardtii P4H-1 (Table 3). The Km value for 2-oxoglutarate, 30 µM, was about 2.5-fold lower than that reported for C. elegans PHY-1/(PDI-2)2 and similar to that of human C-P4H-1, whereas the C. reinhardtii P4H-1 had an approximately 8-fold higher value (Table 3). The Km for ascorbate was unusually low, 40 µM, about 8-fold lower than those of the C. elegans PHY-1/(PDI-2)2 and the human C-P4H-1 and similar to that of the C. reinhardtii P4H-1 (Table 3).

Spatial Expression of phy-4.1 Is Restricted to the Pharyngeal Gland Cells and Excretory Duct—A DNA fragment spanning the region from -953 to +6 relative to the translation initiation codon of phy-4.1 was ligated in frame to a lacZ reporter gene, and the construct was microinjected into the germ line with a marker plasmid containing a rol-6(su1006) gene. Three independent lines were generated, and a large number of individual nematodes from each line representing all of the different life cycle stages were examined by staining for β-galactosidase activity. Expression of phy-4.1::lacZ was detected in the nuclei of three pharyngeal gland cells g1 AL, g1 AR, and g1 P, of all of the larval stages, adults, and late embryos (54) (Fig. 4A). These gland cells occupy the space in the terminal bulb of the pharynx, and each g1 cell extends three cuticle-lined ducts anteriorly from the terminal bulb (54). Two of these ducts pass through the isthmus before emptying into the pharyngeal lumen near the anterior bulb, and one extends much further and empties near the anterior limit of the pharynx (54).

Expression of the PHY-4.1 polypeptide in the pharynx was confirmed by immunofluorescence staining with a purified polyclonal antibody against PHY-4.1 (Fig. 4B). Staining was seen in the pharynx lumen and at the boundary between the pharynx and the gut (Fig. 4B). In addition, staining for the PHY-4.1 polypeptide was found in the excretory duct of the excretory system (Fig. 4B). This cuticle-lined tube lies next to the terminal bulb of the pharynx and is involved in the secretion of metabolites from the excretory system (55).

C. elegans P4H Tetramers and Dimers Containing PHY-4.1 as the Catalytic Subunit Hydroxylate Proline-rich Sequences Expressed in the Pharynx—Based on the restricted expression pattern of the PHY-4.1 polypeptide, we searched for potential new substrates for the C. elegans P4H tetramers and dimers that contain PHY-4.1 as the catalytic subunit by screening the C. elegans data base (available on the World Wide Web) for proline-rich sequences that were expressed in the pharynx or excretory duct. The search identified two genes, D1007.7 and frl-1 (Y489G4.4) that are expressed in various regions of the nematode, including the pharynx or pharyngeal gland cells, and code for polypeptides with proline-rich regions. Based on the amino acid sequences, two synthetic peptides Pro-Ile-Pro-Leu-Pro-Gln-Asn-Leu-Ser-Gly-Ala-Pro8 and Ala-Pro4-Gly-Ile-Pro-Gly-Tyr-Pro2-Ala-Pro4-Gly-Val-Gly-Pro4-Gln-Gly, representing the polypeptides coded by the frl-1 and D1007.7 genes, respectively, were ordered and tested as substrates for soluble extracts of insect cells coexpressing C. elegans PHY-4.1 and PDI-2 when measured with two peptide substrates representing proline-rich proteins expressed in the pharyngeal gland cells.
PHY-4.1 as the catalytic subunit thus hydroxylate the novel substrates with much higher maximal reaction velocity than the (Pro-Glu-Pro-Pro-Ala)\textsubscript{5} peptide despite their lower affinity.

Analysis of the Effect of Heat Shock on the Expression Level of phy-4.1—It had seemed during the analysis of the phy-4 transcripts that phy-4.1 cDNA was more abundant in the cDNA pools generated from the heat-shocked nematodes than from those maintained at the normal culture temperature. To study the effect of heat shock on the expression level of the phy-4 gene, a microarray hybridization experiment was performed on an Affymetrix C. elegans genome array, which contains ~22,500 C. elegans genes, with probes isolated from N2 nematodes cultured at normal temperature or subjected to heat shock. Previous studies have identified 28 and 32 genes of 11,917 and 17,661 studied, respectively, to be up-regulated by about 2–110-fold by heat shock, several of these coding for members of the heat shock protein (HSP) families 70 and 16 (56, 57). In accordance with the previous data (56, 57) the genes C12C8.1, F44E5.4, and T27E4.8 coding for the HSP-70 and HSP-16 proteins had the highest induction levels, 24–120-fold in our analysis (data not shown). The expression level of the Y43F8B.4 gene coding for PHY-4.1 was not markedly induced by heat shock (1.13-fold) (data not shown). Likewise, the expression levels of the genes coding for PHY-1, PHY-2, PHY-3, and PDI-2 did not change significantly upon heat shock treatment.

phy-4.1 RNAi Does Not Lead to Any Obvious Phenotypic Abnormalities—RNAi analysis of the phy-4.1 transcript was performed using an RNAi construct that also targets the phy-4.2 transcript by injection and feeding in wild-type, RNAi-sensitive N2 nematodes (data not shown). The cosubstrate requirements of the (PHY-4.1)\textsubscript{2}/(PDI-2)\textsubscript{2} tetramer and PHY-4.1/PDI-2 dimer were largely similar to those of the PHY-1/PHY-2/(PDI-2)\textsubscript{2} mixed tetramer and PHY-4.1/PDI-2 dimers. Probably an extreme end of the range of nematode P4H assembly versatility is represented by the PHY-1 polypeptide of the filarial nematode Brugia malayi, which does not require PDI as the β subunit but assembles into an active (PHY-1)\textsubscript{4} homotetramer (58).

The substrate specificity of the two C. elegans P4H forms that contain PHY-4.1 as the catalytic subunit indicates that they form a separate class of P4Hs in C. elegans that is distinct from the C-P4Hs and HIF-P4Hs. The P4H tetramers and dimers containing PHY-4.1 are capable of hydroxylating other proline-rich sequences, including poly(ω-proline), in addition to collagen-like sequences. Since (Pro-Pro-Gly)\textsubscript{10} was hydroxylated less effectively than (Pro-Glu-Pro-Pro-Ala)\textsubscript{5} by the (PHY-4.1)\textsubscript{2}/(PDI-2)\textsubscript{2} tetramer and PHY-4.1/PDI-2 dimer, it is highly likely that their main in vivo substrates are not collagens but some other proline-rich proteins. Two putative new substrates are the proline-rich sequences encoded by the C. elegans D1007.7 and frl-1 genes. These genes are expressed in many organs of the nematode, including the pharynx or pharyngeal gland cells (59). The D1007.7 codes for a putative RNA-binding protein, whereas frl-1 encodes a homologue of human leukocyte formins (available on the World Wide Web). As the functions of these genes are yet unknown, it remains to be established whether they are hydroxylated in vivo.

The cosubstrate requirements of the (PHY-4.1)\textsubscript{2}/(PDI-2)\textsubscript{2} tetramer and PHY-4.1/PDI-2 dimer were largely similar to those of the PHY-1/PHY-2/(PDI-2)\textsubscript{2} mixed tetramer and the human C-P4H-I, a notable exception being the markedly lower Km for ascorbate, 20 μM, whereas PHY-1 and PHY-2 are expressed in the hypodermal cells that synthesize cuticle collagens (35, 36), and PHY-3 is expressed in the spermatheca (37). The distinct expression pattern of PHY-4.1 and its unusual substrate specificity suggest that it is not involved in the hydroxylation of cuti-
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