Egg Shell Collagen Formation in Caenorhabditis elegans Involves a Novel Prolyl 4-Hydroxylase Expressed in Spermatheca and Embryos and Possessing Many Unique Properties*

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The prolyl 4-hydroxylases (P4Hs), enzymes residing within the lumen of the endoplasmic reticulum, catalyze the formation of 4-hydroxyproline in collagens and more than 15 other proteins (1) by the hydroxylation of proline in X-Pro-Gly repeats (2, 3). P4Hs have a central role in the synthesis of all collagens, as 4-hydroxyproline residues are essential for the folding of the collagen triple helix. In addition, a family of three cytoplasmic P4Hs plays a critical role in the regulation of the hypoxia-inducible transcription factor HIFα (4, 5). The P4Hs require Fe²⁺, 2-oxoglutarate, O₂, and ascorbate (2, 3). The vertebrate enzymes, and also a P4H from Drosophila melanogaster (6), are αβ₂ tetramers in which the β subunits are identical to protein disulfide isomerase (PDI) (2, 3). At least two isotypes of the catalytic α subunit are found in human (7) and mouse (8) tissues, the α(I)β₂ tetramer being the main form in many cell types, whereas [α(II)]β₂ is found especially in chondrocytes and vascular endothelial cells (9, 10). The properties of the two isoenzymes are very similar, but differences are found between them in the binding of peptide substrates and peptide inhibitors (7, 11).

Two P4H α subunits, PHY-1 (also known as DPY-18) and PHY-2, have previously been characterized from Caenorhabditis elegans (12–16). Both are 43–46% identical to the human α(I) and α(II) subunits, the highest degree of identity being found within the catalytically important C-terminal regions (12, 14, 16). Unlike the vertebrate α subunits, the C. elegans PHY-1 formed an αβ dimer in insect cell coexpression experiments with human PDI or its corresponding C. elegans isoform, PDI-2 (12, 13). The phy-1 and phy-2 genes are expressed in collagen-synthesizing hypodermal cells at times of maximal collagen synthesis, suggesting an important role in cuticle formation at all developmental stages (14). Deletion of the phy-1 gene or elimination of its expression by RNA interference caused a dumpy (short and fat) phenotype, whereas elimination of the phy-2 gene function produced no visible phenotype (14, 15). The phy-1/phy-2 double mutant was embryonic lethal, however, suggesting that phy-2 is required for phy-1 mutant viability (14, 15).

Our sequence homology search of the C. elegans genome (17) indicated the presence of three additional less well conserved P4H α subunit-like genes. We report here on the cloning and characterization of one of these, termed phy-3. Nematodes carrying homozygous deletion of the phy-3 gene were morphologically normal at both the adult and larval stages, but their early embryos had egg shells with a markedly reduced 4-hydroxyproline content. Our data thus indicate that PHY-3 is involved in the hydroxylation of proline residues in the early embryo, most probably in the collagens of the egg shell (18).

MATERIALS AND METHODS

C. elegans Strains and Cloning of the phy-3 cDNA—The wild-type Bristol N2 strain was provided by the Caenorhabditis Genetic Center and was cultured by standard methods (19). A GeneBank™ data base search indicated the presence of an open reading frame T20B3.7 showing sequence similarity to the C-terminal region of the human and

1 The abbreviations used are: P4H, prolyl 4-hydroxylase; PHY, prolyl 4-hydroxylase; PDI, protein disulfide isomerase (PDI).

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5 The abbreviations used are: P4H, prolyl 4-hydroxylase; PHY, C. elegans prollyl 4-hydroxylase a subunit; PDI, protein disulfide isomerase; PBCV-1, P. bursaria Chlorella virus-1; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.
C. elegans P4H α subunits (Fig. 1). The T20B3.7 gene has four exons, but the encoded polypeptide has no signal peptide (20). Only one 208-bp open reading frame, including a signal sequence (20), was found within an 8-kb region upstream of T20B3.7.

The full-length phy-3 cDNA was cloned by amplifying two fragments from the genomic clone cDNA library UNIZAP 930706 (Stratagene). The first fragment corresponding to the T20B3.7 open reading frame (Fig. 2C, PCR1) was amplified with the primers 5′-ATTTCCGAT-TTCATCTGACATCACCTACG-3′ and 5′-AACATTCAAAATGTTCTCAAATATCAATTGGG-3′, while the second corresponding to a sequence beginning from the start codon in the upstream 208-bp exon and extending to the BamHI site in the fourth exon of T20B3.7 (Fig. 2C, PCR2) was amplified with 5′-GAAAACCATGATTTCTGTCACTT-CATTTGG-3′ and 5′-ATTTAGAACCGGATCAAATGTCGCGATC-3′, while ama-1 was amplified with the primers described previously (21). The PCR products were electrophoresed on 2% agarose gels, Southern blotted, and hybridized under stringent conditions with 32P-labelled PCR products corresponding to the phy-3 and ama-1 genes. The hybridized bands were excised and counted by scintillation.

In a previous study using the reporter gene lacZ under the control of the phy-3 promoter (21), the PCR products were electrophoresed on 2% agarose gels, Southern blotted, and hybridized in rabbits to a synthetic PHY-3 peptide CPSLSNRFR-C-3′, while 32P-labelled PCR products corresponding to the phy-3 and ama-1 genes. The hybridized bands were excised and counted by scintillation.

The phy-3 vector was cotransfected into Spodoptera frugiperda insect cells (Sf9, Invitrogen) with BaculoGold™ DNA (PharMingen) by calcium-phosphate transfection according to the manufacturer’s instructions. The resultant viral pool was collected, amplified, and plaque-purified (22). The plasmids from recombinant baculoviruses used were those coding for C. elegans PHY-1 (12), PDI-1 and PDI-2 (12, 13), and human α(I) and PDI (28).

Sf9 cells were cultured as monolayers (27) and infected with viruses coding for PHY-3, PHY-1, or the human α(I) alone or together with viruses coding for C. elegans PDI-1, PDI-2, or human PDI. The recombinant proteins were analyzed by 12% SDS-PAGE (27) and assayed for PDI activity by a method based on the decarboxylation of 2-oxo-[1-14C]glutarate (29). N-Glycosidase F treatment was performed according to the instructions of the manufacturer (Roche Molecular Biochemicals) and Western analysis with the antibody 2681.

Results

Amino Acid Sequence of PHY-3 and Its Comparison with Those of Other P4H α Subunits—A sequence homology search indicated that the C. elegans genome contains an open reading frame T20B3.7 consisting of four exons that encodes a 239-amino acid polypeptide showing sequence similarity to the conserved C-terminal region of the vertebrate P4H α(I) and α(II) subunits (7, 8, 30) and C. elegans PHY-1 and PHY-2 (12, 14, 15) (Figs. 1 and 2B). However, this polypeptide did not contain a signal peptide, and analysis of the genome suggested the presence of an additional 208-bp exon 4151 bp upstream from T20B3.7, coding for 69 amino acids, including a signal peptide (Fig. 2A). A cDNA containing the exon sequences from the start codon in this upstream exon to an internal BamHI site in the last exon of T20B3.7 was obtained by PCR from a mixed-stage cDNA library (Fig. 2C, PCR2), and sequencing of this product showed that in addition to exon 1, the predicted T20B3.7 gene lacked 29 bp from the 5′ end of exon 2 (Fig. 2B). A full-length phy-3 cDNA was subsequently obtained by PCR from mixed stage cDNA. The cDNA encodes a 318-amino acid polypeptide (Fig. 1), the most likely cleavage site of the signal peptide being located between Ser23 and Gin24 (20). The processed PHY-3 is thus 295 amino acids, much shorter than the vertebrate P4H α subunits and C. elegans PHY-1 and PHY-2, with a size range from 514 to 542 residues.
The sequence of the processed PHY-3 is 17% identical to residues 256–542 in PHY-1 (12) and 18–20% identical to the corresponding residues in PHY-2 (14, 15) and the human α(I) and α(II) subunits (8, 30) (Fig. 1). The sequence conservation is highest within the C-terminal regions, the PHY-3 amino acids 150–295 being 23–30% identical to the corresponding residues of PHY-1 and PHY-2 and human α(I) and α(II). The two histidines and one aspartate that bind the Fe²⁺ atom and the lysine that binds the C5 carboxyl group of 2-oxoglutarate (27) are all conserved (marked by * in Fig. 1).

**Physical Structure of the phy-3 Gene**—The sequence of phy-3 is found on cosmid T20B3 (GeneBank™ accession number Z81593), which maps to chromosome V (17). The nearest genes to T20B3.2, T20B3.13, and T20B3.1 are at a distance of several kilobases, it seems unlikely that phy-3 belongs to a gene cluster or operon. The open reading frame T20B3.7 in phy-3, which codes for the large subunit of RNA polymerase II (21), was microinjected into the germ line with a marker plasmid containing a rol-6(su1006) gene (22, 23). A large number of nematodes from two independent lines, selected on the basis of their roller phenotype, were stained for β-galactosidase activity.

**Expression of phy-3::lacZ in Late Larvae and Adult Nematodes**—Is Exclusive to the Spermaphera—A putative promoter fragment, 1480 bp upstream of the translation initiation codon, was ligated in-frame to a lacZ reporter gene. The construct, phy-3::lacZ, was microinjected into the germ line with a marker plasmid containing a rol-6(su1006) gene (22, 23). A large number of nematodes from two independent lines, selected on the basis of their roller phenotype, were stained for β-galactosidase activity.

**Expression of phy-3::lacZ was consistently detected in the spermatheca of L4 larvae and adult nematodes (Fig. 4, A and B), this specialized region of the gonad being the site of oocyte fertilization (18). Some additional staining of gonadal cell nuclei was observed when a sensitive staining method was used (Fig. 4A), whereas no β-galactosidase expression was observed in the hypodermal cells. Expression of the PHY-3 polypeptide in spermatheca was confirmed by immunofluorescence staining with a polyclonal antibody against PHY-3 (Fig. 4, C and D).

**Homozygous Deletion of phy-3 Leads to a Marked Reduction in the 4-Hydroxproline Content**—Homozygous deletion mutants in the phy-3 locus were backcrossed six times to remove non-related mutations. This mutant, phy-3⁻⁻ ok199, contained a 1241-bp deletion that corresponded to position 25,891–27,132 in T20B3 (Fig. 2D) and removes exons 2, 3, and 4 and part of exon 5. The homozygous nematodes were phenotypically similar to the wild type, and no defects were found in their gross morphology, fertility, or behavior. As phy-3 was expressed exclusively in embryos and in the spermatheca of L4 larvae and
adult nematodes, we carefully examined the early embryos. Those of the phy-3<sup>−/−</sup> strain were morphologically of the wild-type when viewed with Nomarski optics (data not shown). The 4-hydroxyproline content of the phy-3<sup>−/−</sup> early embryos was dramatically reduced by about 90% (p < 0.0005) relative to their wild-type counterparts, and a small decrease (p < 0.05) was also seen in the proline content (Table I). In contrast, the 4-hydroxyproline content of the whole phy-3<sup>−/−</sup> nematodes was not decreased (data not shown).

Expression of Recombinant PHY-3 in Insect Cells—Recombinant PHY-3 was produced in insect cells, and the cell lysate was analyzed by 12% SDS-PAGE followed by Coomassie staining and Western blotting (Fig. 5). In agreement with data previously reported for PHY-1 (12) and the human P4H<sub>x</sub> subunits (7, 28), the majority of PHY-3 formed insoluble aggregates, and its efficient extraction required 1% SDS (Fig. 5). To study whether any of the five potential N-glycosylation sites present in PHY-3 (Fig. 1) are utilized in insect cells, samples were digested with N-glycosidase F. Several forms of PHY-3 were seen in SDS-PAGE of the nondigested sample, whereas only one major band and two minor bands, probably representing degradation products, were present after the treatment (Fig. 5).

To study the association of PHY-3 with various PDI isoforms, insect cells were coinfectected with viruses coding for PHY-3 and human PDI (28) or C. elegans PDI-1 or PDI-2 (13), and Triton X-100 extracts of cell homogenates were analyzed for P4H activity with an assay based on the hydroxylation-coupled decarboxylation of 2-oxo-[1-<sup>14</sup>C]-glutarate (29). The recombinant PHY-3 yielded P4H activity only when coexpressed with C. elegans PDI-1 (Table II). This activity level was about 22–27% of that in extracts from cells expressing the C. elegans PHY-1/human PDI dimer or the human type I P4H (Table II). The amount of PHY-3 polypeptide in the soluble fraction was...
blotting in B by 12% SDS-PAGE followed by Coomassie staining in lanes 1 and lanes 2 presence (and lane 2). Similar results were obtained in more than 10 additional experiments. Values are given in dpm/mg of Triton X-100 extractable cell protein. Statistical significance was analyzed by the Student’s t test, n.s. = not significant.

| Genotype     | n   | 4-Hyp/1000 | Pro/1000 | Gly/1000 |
|--------------|-----|------------|----------|----------|
| Wild-type    | 5   | 26 ± 13    | 66 ± 22  | 100 ± 10 |
| phy-3−/−     | 8   | 2.6 ± 2.1, p < 0.0005 | 42 ± 16, p < 0.05 | 13 ± 13, n.s. |

The size of the processed PHY-3 polypeptide, 295 amino acids, is markedly different from the more than 510 residues of collagen synthesized in this organ.

The proline content of the phy-3−/− early embryos was not increased, but rather slightly decreased. As 4-hydroxyproline residues are essential for the stability of the collagen triple helix (2, 3), it is probable that the markedly 4-hydroxyproline-deficient collagen chains synthesized in the phy-3−/− early embryos either formed no triple-helical molecules at all or formed molecules with unstable triple helices. Both possibilities would lead to a rapid degradation of the 4-hydroxyproline deficient protein, and thus there should be no accumulation of a protein with a corresponding increase in the proline content. The deficiency of collagen in the phy-3−/− embryos may also have caused structural changes in the egg shells that may have led to a secondary loss of some additional egg shell proteins either in vivo or during isolation of the eggs, and this may have contributed to the decrease in the proline content. Our findings differ from those that applied to the cuticle collagens in the phy-1 and phy-2 mutants, in which the deficiency in 4-hydroxyproline led to a corresponding increase in the proline content (14, 15). As the phy-1 and phy-2 mutations led only to a partial deficiency of 4-hydroxyproline, its remaining content may have contributed to proline hydroxylation of the type IV collagen synthesized in this organism.

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Furthermore, if phy-3 were involved in type IV collagen synthesis, elimination of its function could be expected to lead to embryonic lethality. We cannot, however, exclude the possibility that PHY-3 expressed in spermatheca may have contributed to proline hydroxylation of the type IV collagen synthesized in this organ.

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The size of the processed PHY-3 polypeptide, 295 amino acids, is markedly different from the more than 510 residues of PHY-1 and PHY-2 (12, 14, 15) and the vertebrate P4H α subunits (2, 3). An even shorter α subunit, of 210 residues, has been characterized from the Paramecium bursaria Chlorella.
No short collagen P4H α subunit form so far has been identified in vertebrates.

PHY-1 and the vertebrate α subunits form an active P4H with PDI (7, 8, 12, 28), whereas the viral (38) and A. thaliana2 α subunits are catalytically active monomers. Coexpression of recombinant PHY-3 in insect cells with C. elegans PDI-1 produces a relatively small but distinct amount of P4H activity in all experiments (n > 10), whereas coexpression of PHY-1 with PDI-1 produces no activity (13). Due to the small amounts of the soluble PHY-3 polypeptide produced and due to an aggregation tendency of the solubilized protein, it was not possible to determine whether PDI-1 formed with PHY-3 a tetramer or dimer or whether PHY-3, like the PBCV-1 and PDI-1 forms an active P4H.

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