A novel protein disulfide isomerase gene, pdi-3, was isolated from the nematode *Caenorhabditis elegans*. This gene encodes an enzyme related to the ERp60 class of thioredoxin proteins and was found to exhibit unusual enzymatic properties. Recombinant protein displayed both disulfide bond isomerase activity and calcium-dependent transglutaminase-like cross-linking activity. The pdi-3 transcript was developmentally constitutively expressed, and the encoded protein is present in many tissues including the gut and the hypodermis. The nematode hypodermis synthesizes the essential collage- nonextracellular matrix (ECM) called the cuticle. Transcript disruption via double-stranded RNA interference resulted in dramatic and specific synthetic phenotypes in several *C. elegans* mutant alleles with weak- ened cuticle, such as e364, ok162, e186364, ak162, and e2620. These nematodes displayed severe dumpy phenotypes and disrupted lateral alae, a destabilized cuticle and abnormal male and hermaphrodite tail morphologies. These defects were confirmed to be consistent with hypodermal seam cell abnormalities and corresponded with the severe disruption of a cuticle collagen. Wild type nematodes did not exhibit observable morphologi- cal defects; however, cuticle collagen localization was mildly disrupted following pdi-3 RNA interference. The unusual thioredoxin enzyme, protein disulfide isomerase-3, may therefore play a role in ECM assembly. This enzyme is required for the proper maintenance of post- embryonic body shape in strains with a weakened cuticle, perhaps through ECM stabilization via cross-linking activity, disulfide isomerase protein folding activity, protein disulfide isomerase chaperone activity, or via multifunctional events.

This nematode exoskeleton or cuticle is a true extracellular matrix (ECM) that is essential for viability, helps maintain the post-embryonic body shape of the animal, and protects it from adverse environmental factors (1, 2). This structure is involved in locomotion via the attachment of opposed muscles, and in *Caenorhabditis elegans*, the cuticle is initially synthesized in the embryo and then shed and replaced four times at the end of each larval stage (3), resulting in five structurally and chemically distinct stage-specific ECMs (4). This ECM is predominantly composed of small highly cross-linked collagens, and in *C. elegans*, over 150 genes encode cuticle collagens (2), representing 1% of the entire genome (4). The assembly of these collagens to form the cuticle is under tight temporal control (2) and involves numerous complex post-translational modifications (4). Mutations in *C. elegans* cuticle collagen genes can result in abnormal body shape (1). One such abnormal phenotype is the dumpy (Dpy) phenotype, a shortening of the body length and widening of the animal, and several Dpy loci have been assigned to mutations in individual collagen genes, namely dpy-7 (2), dpy-2 and dpy-10 (5), and dpy-13 (6). The Dpy phenotype has additionally been shown to be the result of mutations in genes coding for enzymes involved in collagen assembly or modification such as dpy-11 and dpy-18. The dpy-11 locus has been mapped to a gene encoding a thioredoxin-like enzyme (7). The thioredoxin enzymes are essen- tial redox cofactors in numerous diverse biochemical reactions and cell functions (7). A mouse thioredoxin gene knock-out resulted in embryonic lethality, the function of which however remains to be elucidated (8). Similarly, no function or substrate was identified for dpy-11, but its hypodermal expression pattern and phenotype suggest that it is involved in cuticular ECM assembly. The dpy-18 locus was mapped to the α-subunit (phy-1) of prolyl 4-hydroxylase (P4H) (9–11). As opposed to dpy-11, the biochemical function of dpy-18 has been well de- fined (9, 12), being involved in the hydroxylation of proline residues in cuticle collagens and thereby increasing the ther- mal stability of the collagen triple helix (13). Another key enzyme that is also a part of the P4H complex is the protein disulfide isomerase (PDI, EC 5.3.4.1) enzyme PDI-2 (9, 12). PDI-2 forms the sole β-subunit of the cuticle-specific P4H complexes and is essential for nematode development, a fact con- firmed by the embryonic lethal RNAi phenotype associated with this enzyme (9). PDIs are multifunctional endoplasmic reticulum (ER) resident proteins belonging to the thioredoxin superfamily (14). They can act as molecular chaperones, cata- lyze disulfide bond formation (14), and can have specialized functions as exemplified by the β-subunit of P4H (12). A second *C. elegans* PDI-encoding gene pdi-1 has been characterized. pdi-1 is expressed in an operon with cyp-9 (15), an arrangement that is conserved in the closely related species *Caenorhabditis briggsae* (16) with both PDI-1 and CYP-9 being expressed in...
the hypodermis in a similar temporal pattern (15). This operon is predicted to contribute to ECM assembly through the chaperone and disulfide bond formation activity of PDI-1 and the peptidyl-prolyl cis/trans-isomerase activity of CYP-9 (15).

An additional predicted pdi with homology to pdi-1 and pdi-2 was identified from the C. elegans genome (chromosome I, cosmid H06001). This third C. elegans pdi was named pdi-3. An ortholog of pdi-3 has been studied in the parasitic nematode *Dirofilaria immitis* and has been termed ERp60-like protein (17). ERp60 proteins are part of the PDI family; however, they do not substitute for PDI in the P4H4 complex (18). *D. immitis* pdi-3 was shown to be involved in many processes such as apoptosis (21) and pseudocolored using Improvision OpenLab software. Similarly, microtiter plates were coated with the synthetic peptide CREVKDFVSFISKHSTDGLKGFS. The cysteine residue (underlined) to allow directional subcloning into the *C. elegans* embryo, Larvae, and Adults—A PDI-3-specific anti-peptide antisemur was produced in rabbits using the synthetic peptide CREVKDFVSFISKHSTDGLKGFS. The cysteine residue (underlined) was added to permit conjugation to keyhole limpet hemocyanin prior to immunization. N2 embryos and larvae collected from NGM agar plates were placed on poly-L-lysine slides and freeze-cracked according to previously published methods (23). The slides were blocked and washed, and the pdi-3 antisemur and/or a monocular antibody anti-DPY-7 (a kind gift from Iain Johnstone, Glasgow University) were added (both at 1:50 dilutions). Following incubation, the slides were washed and the secondary anti-rabbit conjugate antibody Alexa Fluor 488 and/or anti-mouse conjugate Alexa Fluor 594 or 595 (both from Molecular Probes) were applied. Following incubation, the slides were washed and the solution (50% glycerol, 2.5% DABCO) containing antifadant and nuclear staining solution was added to the slides. Slides were viewed by epifluorescence on a Zeiss Axioskop 2 microscope, and images were taken with a Hamamatsu digital camera (Invitrogen).

**MATERIALS AND METHODS**

*C. elegans* Strains—Wild type (Bristol N2), CB27[dpy-9], CB364[dpy-5], CB884[dpy-7], M475[dpy-7], CB130[dpy-8], CB128[dpy-10], CB224[dpy-11], CB1180[dpy-11], CB458[dpy-13], CB364[dpy-18], EM767[dpy-18], JK2729[dpy-18], CB1350[ok199], BE63[dpy-19], CB61[dpy-5], CB4121[ok10], and JK667[unc-19(e2498):3’Tcs1w51] strains were obtained from a cosmid (H06o01.1, GenBank™ accession number Cab07480.1). Two constructs encoding 4075- and 1301-base pair 5′ regions of the pdi-3 gene were amplified from genomic DNA by the PCR with *Taq* polymerase (AB DNA). The primers contained an artificial restriction site (lowercase and underlined) to allow directional subcloning in the multiple cloning sites of the vector pPD96-04: pdi3p for 5′-gaattcc-**GAAACTGTACGTCGACC-3′* (sense, *Pst*I); pdi3pPreg for 5′-**gatt-**CCCTGTTGTAATATGCTTCG-3′ for the 4075-bp fragment; and 5′-gaggtc-**CCCGGGGCGACCTTAC-3′ for the 1301-bp fragment (antisense, *BamHI*). These constructs permitted a translational fusion to lacZ with the first six amino acids of the second exon of pdi-3 (pPDI-3-SPlacZ1) or the first five amino acids of the first exon of pdi-3 (pPDI-3-SPlacZ2). PCR products were initially cloned into pRTvector (Stratagene, digested with *Pst*I and *BamHI*, and ligated into similarly digested pPD96-04. Miniprep DNA was prepared using a Qiagen Miniprep kit, and sequencing was performed to confirm the identity and the transcriptional context of the pdi-3 operon. Transformation of the plasmid vector pDI-3 was performed by microinjection of plasmid DNA into the syncytial gonad and was carried out using standard methods as described previously (25). Transformed nematodes were fixed and stained with 0.3% X-gal solution to detect β-galactosidase activity using standard methods also described previously (25). The semi-quantitative RT-PCR method including the generation of synchronous nematode cultures for staged mRNA and subsequent RNA samples were digested in detail elsewhere (26). The gene combinations, pdi-3 and the control gene ama-1 (which encodes the large subunit of RNA polymerase II), were amplified from the staged cDNA samples and electrophoresed on 1% agarose gels stained with a 0.3% X-gal solution to detect β-galactosidase activity. Similarly, combinations were used as follows: pdi-3 and ama-1 for 5′-GACACCGGAGACTGGTTG-3′ and pdi3nRe for 5′-CTTCACTGTCCTCTTCTC-5′; and ama-1, ama1F for 5′-TGACGATGGTAGGTGAATGCTTC-3′ and ama1R for 5′-TACAGATTTCTCCACAGGACGACGGGA-3′. The PCR reaction samples were electrophoresed, Southern blotted, and probed with pdi-3 and ama-1 DNA fragments labeled with [32P]dCTP using a random priming kit (PrimeIt™II Random Primer labeling kit, Stratagene). The blots were autoradiographed and quantified using a Typhoon imager and software. The relative abundance of expression was determined by comparing its signal to *ama-1* following four independent RT-PCR reactions.

**Materials and Methods**

**Immunocytochemistry of C. elegans Embryos, Larvae, and Adults—A PDI-3-specific anti-peptide antisemur was produced in rabbits using the synthetic peptide CREVKDFVSFISKHSTDGLKGFS. The cysteine residue (underlined) was added to permit conjugation to keyhole limpet hemocyanin prior to immunization. N2 embryos and larvae collected from NGM agar plates were placed on poly-L-lysine slides and freeze-crack...**
control. The data were expressed as percentage of activity compared with that of native RNase A. Both assays were also performed with recombinant PDI-1 and PDI-2, which were expressed in a similar manner as PDI-3 described above.\(^2\)

Double-stranded RNA-mediated Interference—The RNA interference procedures employed in this study were as described previously (25). Full-length pdi-3 cDNA minus the signal peptide-encoding region was produced using the primers described above. The pdi-3 PCR product was ligated in pCRScript (Stratagene) linearized with appropriate restriction enzymes, ScaI for T7 reaction and EcoRV for T3 reaction. T7 or T3 Ribomax kits (Promega) were used to generate sense and antisense RNAs by following the manufacturer’s instructions. The injection and soaking RNAi methodology has been described elsewhere (25). For RNAi feeding, the pdi-3-pCRScript EcoRV and ScaI fragment was ligated into similarly digested L4440 feeding vector. The construct was then transformed into HT115(DE3) cells, which were used for RNAi feeding at 25°C unless otherwise stated. The nematode strains tested in the RNAi experiments are described above.

Scanning Electron Microscopy of pdi-3-specific RNAi on N2 and Mutant Alleles of dpy-18—N2 and CB384(dpy-18) hermaphrodites and EM76(dpy-18 males) were fed induced pdi-3-L4440 HT115(DE3) cells as described above, and nematodes were collected and washed with M9 buffer. Nematodes were fixed for 1.5 h on ice in 2.5% glutaraldehyde in phosphate-buffered saline pH 7.4, rinsed three times in phosphate-buffered saline, and then post-fixed in 1% osmium tetroxide in distilled water for 1 h followed by three 10-min rinses in distilled water. Samples were then incubated in the dark for 1 h in 0.5% aqueous uranyl acetate followed by a wash in distilled water. Nematodes were then dehydrated in acetone, critical point dried in CO\(_2\), mounted on stubs, coated with gold (Polaron SC515), and examined in a Phillips SEM500 scanning electron microscope.

RESULTS

C. elegans PDI-3 Belongs to the ERp60 Class of Thioredoxin Proteins—The gene pdi-3 is found within cosmid H06001 located on linkage group I. The predicted open reading frame for pdi-3 (H06001.1) has 8 exons (ranging from 77 to 300 bp). Six of the seven introns ranged between 46 and 362 bp, whereas the first intron was relatively large (2539 bp) for C. elegans (Fig. 1A). The alignment of the PDI-3 protein, PDI-1, PDI-2, human ERp60, and the D. immitis ERp60-like protein (GenBank\(^\text{TM}\) accession numbers Z92970, Q17567, Q10576, S68363, and AFO08300) confirms that the two thioredoxin active site domains (WCGHCK) are highly conserved (Fig. 1B). The percentage of identical amino acid residues between full-length PDI-3 compared with PDI-1, PDI-2, human ERp60, and D. immitis ERp60-like protein was 28, 30, 45, and 63%, respectively. PDI-3 was more similar to human ERp60 than to C. elegans PDI-1 or PDI-2, but it was most closely related to the ERp60-like protein from the parasitic nematode D. immitis. The alignment of human, rat, mouse, bovine, and hamster ERp60s (respectively GenBank\(^\text{TM}\) accession numbers S68363, A28807, P27773, P38657, and AAL18160) showed that these vertebrate ERp60s were highly conserved, having 85% of their amino acid residues in common (alignment not shown). The vertebrate ERp60s did not possess the standard ER retention signals of PDI (KDEL) but instead had the sequence QEDL. Preceding this sequence was a lysine rich stretch of amino acid residues (PKKKKKA), which may act as a nuclear localization signal. ER retention signals were present in PDI-1, PDI-2, PDI-3, and D. immitis ERp60-like protein, but they did diverge slightly from the more usual KDEL, being HEEL, HTEL, KTEL, and KEEL, respectively. Indeed a previous study (28) have shown that many non-KDEL sequences can act as effective protein ER retention signals (Prosite, www.expasy.org/prosite). In addition, both PDI-3 and D. immitis ERp60-like protein displayed the C-terminal ER membrane retention signals KKTE and KKEE, respectively (29). No conserved cluster or bipartite nuclear localization motifs (30) were detected in PDI-1, PDI-2, PDI-3, or the D. immitis ERp60-like protein. The alignment data suggested that despite their high protein sequence homology to human ERp60, the nematode PDIs may have a different cellular localization and hence a different function.

pdi-3 Is Constitutively Expressed in the Nematode Gut and Hypodermal Tissues—Wild type embryos and larvae were probed with a PDI-3-specific polyclonal antibody in combination with a DPY-7-specific monoclonal antibody (Fig. 2). DPY-7 is a cuticle collagen that is expressed solely in the hypodermis and is localized in the cuticular ECM from the elongated embryo stage onwards.\(^3\) In the 2-fold embryo (~500 min after fertilization), PDI-3 staining was observed in most tissues including the gut and the hypodermis (Fig. 2A). Conversely, DPY-7 localized exclusively in the hypodermal cells (Fig. 2B). In L1 larvae, PDI-3 localized in many tissues with predominant staining found in the hypodermal and the gut tissues (Fig. 2C). It can be noted that the pharynx, gut lumen, and body cavity were not stained with either antibody tested (Fig. 2, B and C). Gut nuclei did not stain and appeared as dark patches among the PDI-3-labeled gut tissue (Fig. 2C). DPY-7 antibody was localized solely to the annular furrows of the hypodermal derived cuticle (Fig. 2D).

The antibody expression pattern was confirmed following the examination of the nuclear-localized reporter constructs comprising the pdi-3 promoter region fused in frame with the lacZ gene. The initial four transgenic lines generated from the construct, comprising the promoter region and the first exon and first large intron of pdi-3 (Fig. 1A, pPDI-3-PLacZ21) did not produce a discernable β-galactosidase staining pattern in the four independent lines generated (data not shown). The second promoter reporter construct (Fig. 1A, pPDI-3-PLacZ22), which only contained a few amino acids of pdi-3 exon one, resulted in the isolation of three transgenic lines in which each produced distinct β-galactosidase staining patterns. Prominent β-galactosidase reporter expression was observed in many tissues including the lateral seam cell hypodermis (Fig. 2E), and additionally, strong staining was also observed in the gut cells (Fig. 2F).

The analysis of the pdi-3 temporal expression pattern via RT-PCR on staged mRNA samples demonstrated that it was constitutively expressed throughout the post-embryonic life cycle of C. elegans (Fig. 3). However, peaks of highest relative abundance were observed at final L4 larval stage and in the young adult stages (Fig. 3).

C. elegans PDI Recombinant Enzymes PDI-1, PDI-2, and PDI-3 Display Both Disulfide Isomerase and Transglutaminase-like Cross-linking Activity—The isomerase activity of recombinant PDI-3 was measured using an RNase A refolding assay (14). PDI-3 was able to refold denatured RNase A and therefore has disulfide isomerase activity (Fig. 4A). Following a 4–5-min lag phase, the RNase A activity increased when compared with denatured RNase A in the absence of enzyme (Fig. 4A). Similarly, the C. elegans hypodermally expressed PDIs, PDI-1 (15) and PDI-2 (9) were also found to refold denatured RNase A (Fig. 4A), and activity was dependent on the concentration of all three recombinant enzymes (Fig. 4B). PDI-1 had the highest refolding activity over a 20-min reaction (77% RNase A activity recovered) followed by PDI-2 (64%) (Fig. 4B), whereas PDI-3 was determined to be the least active, having a recovery rate of only 44% for RNase A over the same time interval (Fig. 4B).

In addition to the above disulfide isomerase activity, all three PDIs also displayed TGase-like cross-linking activity using a microplate-based assay (27) (Fig. 5A). However, the levels of TGase activity determined for PDI-3 were significantly higher.

\(^2\) S. C. P. Eschenlauer and A. P. Page, unpublished data.

\(^3\) I. L. Johnstone, personal communication.
**Fig. 1.** Gene structure and amino acid alignment of PDI-3. **A,** genomic organization of pdi-3 and schematic representation of the promoter reporter constructs applied in this study. **B,** amino acid alignment of C. elegans PDI-1, PDI-2, and PDI-3; human ERp60; and D. immitis ERp60. The alignment was performed using the AlignX program of Vector NTI (InforMax). The thioredoxin domains were indicated by asterisk. The N-terminal signal sequences of the protein (lowercase) were not considered in the alignment. The C-terminal ER membrane retention signals (29) were underlined uppercase. Nuclear localization signals are indicated as underlined lowercase.
transgenic lines also displayed gut cell staining (Fig. 2A and B) and the L1 larvae stage (C and D) with magnification ×630 are shown. A, PDI-3 antibody staining is observed in numerous tissues including the gut (g) and the hypodermis (h). B, DPY-7 is exclusively localized in the embryonic hypodermal cells. C, PDI-3 antibody staining is observed in the larval gut and the hypodermis; however, the pharynx (p), gut lumen (gl), gut nuclei (gn), and body cavity (bc) are not labeled. D, DPY-7 is exclusively localized in annular furrows (af) of the larval cuticle. E, β-galactosidase staining was observed in lateral seam cell nuclei of transgenic reporter lines containing an extra-chromosomal construct with lacZ gene under the control of the pdi-3 promoter (×200). The position of the pharynx (p) is denoted. F, transgenic lines also displayed gut cell staining (×100). The position of the pharynx (p) is denoted. The nuclear localization is maintained due to the presence of an nuclear localization signal in the reporter construct. Lateral seam cell nuclear staining (E) and gut cell nuclei staining (F) are indicated by black arrowheads.

Functional Characterization of pdi-3 by RNAi Experiments—

The disruption of pdi-3 was investigated in various C. elegans strains using a range of double-stranded RNAi techniques: injection, soaking, and feeding (Table I). Strains examined included wild type, cuticle collagen mutants, and mutants for enzymes involved in cuticle collagen biosynthesis such as the prolyl 4-hydroxylase α-subunits, CB364(dpy-18), EM76(dpy-118), JK2729(dpy-18), JK2757(phy-2); and thioredoxin-like enzymes, CB224(dpy-11), and CB1180(dpy-11) (Table I). The effective depletion of pdi-3 expression following RNAi in N2 and CB364(dpy-18) strains was confirmed by Western blotting with a PDI-3-specific antibody against nematode extracts from treated and control samples (data not shown).

Examination of the RNAi effects of pdi-3 in a wild type background did not produce a gross phenotype, because all

Fig. 3. Temporal expression of pdi-3 during post-embryonic development assayed by semi-quantitative RT-PCR. The pdi-3 transcript levels were compared with a constitutively expressed gene ama-1. The mRNA was isolated from synchronous C. elegans cultures. Hours post-L1 larval arrest are indicated. The data was expressed as the ratio of pdi-3 to ama-1 transcripts. The RT-PCR was performed four independent times and the error bars indicate the mean ± S.E.

Fig. 4. PDI-3 RNase A refolding activity. A, refolding assay of denatured RNase A by PDI performed over a 20-min time period: native RNase A (8 μM) (■), denatured RNase A (8 μM) (□), PDI-1 (2 μM) (▲), PDY-2 (2 μM) (●), and PDI-3 (2 μM) (●). B, refolding assay of denatured RNase A by varying amount of PDI as end point assay: native RNase A (8 μM) (filled bar), denatured RNase A (8 μM) (open bar), PDI-1 (▲), PDY-2 (●), and PDI-3 (●). The data were expressed as percentage of activity compared with that of native RNase A, and the error bars indicated the mean ± S.E.
stages remained wild type in appearance, developed normally, and were fertile (data not shown). Adult stage nematodes were indistinguishable at the light microscope level to wild type untreated adults (Fig. 6A). This result was obtained irrespective of the RNAi delivery method and the temperature under which the experiments were conducted (25, 20, or 15 °C). The CB364(dpy-18) mutant phenotype is medium Dpy with characteristically shorter and fatter worms (9) (Fig. 6B) compared with wild type animals (Fig. 6A). However, dpy-3 RNAi in a CB364(dpy-18) mutant background did result in more dramatic body shape defects in the F1 progeny (Fig. 6C). This synthetic phenotype was observed irrespective of the RNAi methods employed and was not dependent on temperature (Table I). Following RNAi, the progeny were ~50% shorter than CB364(dpy-18) animals with a bulbous severe Dpy phenotype at the mid to rear body of the animal. The internal organs were greatly compressed within the constricting exoskeleton and were commonly observed to protrude from the vulva. The treated animals also presented egg-laying defects (Egl) with several embryos within the uterus in an advanced stage of development (elongated 3-fold embryos). Embryos also developed very slowly, taking up to 2 days to hatch; however, an embryonic lethal phenotype was not observed. The Dpy morphology of the larval stages was likewise more severe than that noted in the CB364 background strain (data not shown). The structure of the hermaphrodite tail was also severely affected having a shortened and swollen abnormal tail phenotype instead of normal long whip-like appearance (Fig. 6, compare tails in B and C). The synthetic RNAi effect was specifically restricted to the dpy-18 mutant strains (CB364, EM76, and JK2729) and was neither observed in the other two prolyl 4-hydroxylase α-subunit mutant backgrounds tested, namely JK2757(phy-2) and TP7(phy-3) nor in the thioredoxin (dpy-11) mutant alleles, all of which maintained their original wild type or Dpy appearance following dpy-3 RNAi (Table I). From the 12 mutant cuticle collagen strains tested (Table I), only one mutant allele of sqt-3(e2117) displayed a synthetic phenotype upon dpy-3 RNAi. In the sqt-3(e2117) mutant strain CB4121, animals are medium Dpy at 15 °C but extreme Dpy, 70 embryonic and larval lethal, at 25 °C. This temperature-sensitive phenotype is caused by mutations in the COL-1 collagen gene that causes the cuticular ECM to be thermally unstable (31), and the cuticle of dead embryos at 25 °C lack a striated layer normally present at 15 °C (32). Following dpy-3 RNAi feeding at 25 °C, the characteristic severe Dpy, embryonic and larval lethal phenotypes, were observed. However, at 15 °C, the dpy-3 RNAi feeding caused a synthetic phenotype similar to that observed in all the dpy-18 strains examined (Fig. 6E), whereas the control RNAi feeding of an unrelated gene, cyp-5, in CB4121(sqt-3) worms did not produce this effect (Fig. 6D). The control gene cyp-5 is a gut-expressed cyclophilin with no discernable RNAi phenotype (25). The dpy-3 RNAi feeding in the CB4121(sqt-3) mutant background resulted in a shortening of the animal with a bulbous mid to rear body phenotype (Fig. 6, compare D with E). The hermaphrodite tail also displays a dominant abnormal tail phenotype (Fig. 6E) instead of the characteristic wild type whip-like shape (Fig. 6D).

The synthetic morphological effect of dpy-3 RNAi in the dpy-18 mutant background was characterized further using an SEM approach. In agreement with light microscopy observations, SE confirmed that no external morphological defects were induced by dpy-3 RNAi in the wild type background (data not shown), being equivalent to the normal wild type cuticle (Fig. 6F). However, the SE analysis of CB364(dpy-18) mutant worms following dpy-3-specific RNAi revealed that the alae were severely disrupted in the areas corresponding to the severely Dpy regions of these animals (Fig. 6G, black arrowheads). This bulbous region and the associated branched alae are characterized by the fact that the lateral seam cell cords are correspondingly highly thickened in this region (Fig. 6G, double arrow). In contrast, the ventral/dorsal hypodermal-derived cuticle morphology was relatively unaffected (Fig. 6G, an) and was comparable to the CB364(dpy-18) mutant strain (data not shown). As observed by light microscopy, the hermaphrodite abnormal tail phenotype was also evident following the SEM analysis (Fig. 6G). The effects of dpy-3 RNAi on the male tail morphology were likewise assessed at the SEM level. The male tail is a complex cuticular structure derived from the posterior seam cells of the fourth larval stage whose primary function is in mating (33). In wild type C. elegans, the percentage of males to hermaphrodites is relatively low (~1%), and therefore, the male-enriched strain EM76(dpy-18(bx26);hin-5(e1490)) was used in these SEM studies. Feeding experiments revealed that no phenotypic differences were noted in the wild type male tail structure (data not shown) and were comparable to the normal wild type tail structures (Fig. 6H). However, the male tail structure in the strain EM76 prior to RNAi was relatively abnormal compared with wild type; the ray and fan structures having a crumpled short appearance (Fig. 6D). However, after dpy-3-specific RNAi in the EM76 background, the specialized male tail structures were virtually eliminated (Fig. 6J). The fan and rays are completely absent being replaced by a smooth
rounded tail; however, minor spicule structures were still evident and the ventral surface was packed with adherent bacteria (Fig. 6J).

The characterization of the cuticular ECM-related phenotypes associated with pdi-3 RNAi were extended further by examining the effects of this treatment on the normal distribution of the cuticle collagen DPY-7 (Fig. 7). The DPY-7 expression pattern was examined using a specific monoclonal antibody raised to this collagen, which localizes specifically to the annular furrows of the dorso-ventral hypodermally derived cuticle of all larval and adult stages. The wild type DPY-7 expression pattern was confirmed in this study (Fig. 7A). The larval progeny of pdi-3 RNAi-treated wild type nematodes were phenotypically wild type in appearance; however, this treatment led to a mild disruption of the DPY-7 antibody staining pattern in the annular furrows (Fig. 7B) with regularly dispersed nuclei were observed in wild type untreated worms (data not shown). The regular band preceding the final L4 to adult molt (3). The MH27 and JR667 expression patterns in wild type worms following pdi-3 RNAi (Fig. 8, A and B) were similar to those observed in untreated wild type worms (data not shown).

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enzyme displayed unusual properties, namely dual disulfide coding protein from the free-living nematode *C. elegans* (4234 pdi-3 ERp60-like protein disulfide isomerase gene hermaphrodite CB364(CB364(dpy-3) adult after *pdi-3* RNAi at 25 °C (×100). The animals were up to 50% shorter than untreated CB364(dpy-18) with a severe Dpy phenotype, abnormal tail (t), and internal organs protruding out of the vulva (v). The head is indicated (h). D, differential interference contrast microscopy image of CB4121(sqt-3) adult following control cyp-3 RNAi feeding at 15 °C (×100). Head (h) is denoted. E, differential interference contrast microscopy picture of CB364(dpy-18) adult after *pdi-3* RNAi at 25 °C (×100). The animals were up to 50% shorter than untreated CB364(dpy-18) with a severe Dpy phenotype, abnormal tail (t), and internal organs protruding out of the vulva (v). The head is indicated (h). F, scanning electron microscopy image of the mid-body of an untreated N2 adult hermaphrodite with lateral alae (la) and annuli (an) highlighted (×800). G, SEM image of F1 adult CB364(dpy-18) after *pdi-3* RNAi at 25 °C (×800). Abnormal lateral alae (la) morphology can be observed in the region (double arrow) where the body of the animal is enlarged (bifurcations indicated by single black arrowheads). Internal material is also visualized bursting out of the vulva (v). The bulbous tail (t) is also indicated. H, SEM image of wild type N2 adult male tail (×1600) with tail rays (r) and spicule (s) highlighted. I, SEM picture of EM76(dpy-18) male tail (×1600). The spicule and rays are also present, but the male tail fan structure is crumpled. J, SEM picture of EM76(dpy-18) male tail after *pdi-3* RNAi at 25 °C (×1600). The male tail fan has completely disappeared, and the only remaining feature of the tail is a single spicule.

Protein sequence alignment of human ERp60, PDI-1, PDI-2, PDI-3, and *D. immitis* ERp60-like protein demonstrated that PDI-3 was more closely related to human ERp60 and the *D. immitis* ERp60-like protein than to the other *C. elegans* PDIs. *C. elegans* PDI-1, PDI-2, PDI-3, and *D. immitis* ERp60-like protein did not display the standard PDI ER retention signal (KDEL) but they had ER-like retention signals such as HEEL, HTEL, KTEL, and KEEL, respectively. Several non-KDEL sequences have been shown to be effective in protein ER retention: DKEEL, RDEL, and KNEL (28). It is also noteworthy that for all four divergent ER retention signals, the last two amino acid residues (Glu-Leu) were completely conserved. Additionally, it was reported that replacement of Glu or Leu by Gln or Ala, respectively, in the KDEL ER retention signal resulted in a loss of ER retention of the protein (28). The divergent sequence observed in PDI-1, PDI-2, PDI-3, and *D. immitis* ERp60-like protein may therefore act as ER retention signals. In contrast to the nematode proteins, a non-ER function for vertebrate ERp60s has been proposed. A lysine-rich stretch of amino acid residues (PKKKKKA) in human ERp60 that is similar to the nuclear localization signal described for SV40 large T-antigen (34) is evident, and this protein has been detected in the nuclear matrix (35). Furthermore, human ERp60 was found to interact with calreticulin (36), a nuclear-localized calcium-binding protein (37). Conversely, both PDI-3 and *D. immitis* ERp60-like protein lack nuclear localization signals despite their high sequence identity to human ERp60 (45 and 47%, respectively). Based on these sequence observations, it would

**DISCUSSION**

In this paper, we describe the detailed characterization of the ERp60-like protein disulfide isomerase gene *pdi-3* and its encoded protein from the free-living nematode *C. elegans*. This enzyme displayed unusual properties, namely dual disulfide isomerase and transglutaminase-like cross-linking activity. A potential role in the cross-linking and the ultimate stability of the collagenous cuticular ECM were supported by the observed synthetic phenotypes in specific ECM-related mutant *C. elegans* backgrounds.

**FIG. 6.** *pdi-3* RNA interference by feeding. **A,** differential interference contrast microscopy image of untreated N2 (wild type) adult hermaphrodite *C. elegans* (×100). The head end of nematode is denoted h. **B,** differential interference contrast microscopy image of untreated CB364(dpy-18) adult hermaphrodite (×100). Head (h) is denoted. **C,** differential interference contrast microscopy picture of CB364(dpy-18) adult after *pdi-3* RNAi at 25 °C (×100). The animals were up to 50% shorter than untreated CB364(dpy-18) with a severe Dpy phenotype, abnormal tail (t), and internal organs protruding out of the vulva (v). The head is indicated (h). **D,** differential interference contrast microscopy image of CB4121(sqt-3) adult following control cyp-3 RNAi feeding at 15 °C (×100). Head (h) is denoted. **E,** differential interference contrast microscopy picture of CB364(dpy-18) adult after *pdi-3* RNAi at 25 °C (×100). The animals were up to 50% shorter than untreated CB364(dpy-18) with a severe Dpy phenotype, abnormal tail (t), and internal organs protruding out of the vulva (v). The head is indicated (h). **F,** scanning electron microscopy image of the mid-body of an untreated N2 adult hermaphrodite with lateral alae (la) and annuli (an) highlighted (×800). **G,** SEM image of F1 adult CB364(dpy-18) after *pdi-3* RNAi at 25 °C (×800). Abnormal lateral alae (la) morphology can be observed in the region (double arrow) where the body of the animal is enlarged (bifurcations indicated by single black arrowheads). Internal material is also visualized bursting out of the vulva (v). The bulbous tail (t) is also indicated. **H,** SEM image of wild type N2 adult male tail (×1600) with tail rays (r) and spicule (s) highlighted. **I,** SEM picture of EM76(dpy-18) male tail (×1600). The spicule and rays are also present, but the male tail fan structure is crumpled. **J,** SEM picture of EM76(dpy-18) male tail after *pdi-3* RNAi at 25 °C (×1600). The male tail fan has completely disappeared, and the only remaining feature of the tail is a single spicule.

**Fig. 7.** Localization of DPY-7 cuticle collagen following *pdi-3* RNA interference. **A,** wild type larvae stained with DPY-7 antibody (×630). DPY-7 is localized in the annular furrows (af), which have a regular circumferential staining pattern. **B,** DPY-7 antibody staining in wild type larvae after *pdi-3* RNAi at 25 °C (×630). The annular furrows (af) staining pattern is disrupted in the lateral region of the cuticle (center of the worm) where dorsal and ventral hypodermal cells oppose. **C,** CB364(dpy-18) larvae stained with DPY-7 antibody (×630). The staining pattern is slightly disrupted compared with the wild type worm. The circumferential annular orientation is indicated by white double-headed arrow, and the interaction between ventral and dorsal annular furrows is not completely opposed. **D,** DPY-7 antibody staining in CB364(dpy-18) larvae after *pdi-3* RNAi at 25 °C (×630). In addition to the severe Dpy phenotype, there is severe disruption of the DPY-7 antibody staining pattern. The abnormal orientation of severely truncated annular furrows (af) is indicated by white double-headed arrows.
cells, the position of which correspond to the severely Dpy regions of the larvae of TP50 displayed misshapen and severely mislocalized seam magnification (of the severely disrupted alae. All pictures were taken at the same with severely misaligned nuclei. These defective cells are located at the transgenic lines studied. However, a second potential cuticle assembly, perhaps cross-linking a role for this bryonic life cycle with highest peaks of abundance in the L4 dpy-18 possession for the second intron of the prolyl 4-hydroxylase expression. A similar negative regulatory function has been pro-

Morphology of seam cell defects in the dpy-18 background following pdi-3 RNA interference. Composite images of the adherens junctions of hypodermal seam cells stained with MH27 antibody (red, denoted MH) and nuclei of seam cells highlighted with the gfp marker Jr667 (green, denoted JR). A, wild type L4 larvae following pdi-3 RNAi. B, wild type adults following pdi-3 RNAi. The seam cell morphology was comparable to untreated wild type worms (data not shown). C, control L4 larvae of TP50 strain (CB364dpy-18/Jr667unc-119) displayed normally aligned and non-fused lateral seam cells. The seam cells were shorter and wider with enlarged nuclei when compared with wild type. D, adult TP50 control worms show the fused seam cell producing a single linear syncytium, containing regularly spaced and aligned nuclei on the lateral side of the worm. The fused seam cell band was wider than that of wild type animals. E, following pdi-3 RNAi, L4 larvae of TP50 displayed misshapen and severely mislocalized seam cells, the position of which correspond to the severely Dpy regions of the animal. F, following pdi-3 RNAi, TP50 adult worms displayed an abnormally shaped syncytium, which is irregular and branched together with severely misaligned nuclei. These defective cells are located at the most severely Dpy regions of the animal and correspond to the position of the severely disrupted alae. All pictures were taken at the same magnification (×630).

appear that although closely related to human ERp60, PDI-3 and D. immitis ERp60-like protein may perform different biological functions to that of the vertebrate ERp60s.

In this study, antibody localization and reporter gene analysis revealed that the pdi-3 transcript was expressed in numerous tissues including the hypodermis and the gut of the nematode from embryonic through to mature adult stages. Interestingly, the PDI-3 ortholog from the parasitic nematode D. immitis (ERp60) displays a similar expression pattern, being expressed in the adult hypodermis and gut and also being detected in the developing embryos (20). The reporter gene study also uncovered an interesting regulatory feature regarding the relatively large first intron. When the first intron was included in the initial pdi-3 promoter reporter construct (pPDI-3PLacZ1), no β-galactosidase staining pattern was obtained in the transgenic lines studied. However, a second pdi-3 promoter reporter construct (pPDI-3PlacZ2), which lacked this first intron, generated β-galactosidase staining patterns in all of the lines studied. This observation suggested that this first intron may perform a down-regulatory control function in pdi-3 expression. A similar negative regulatory function has been proposed for the second intron of the prolyl 4-hydroxylase α-subunit-encoding gene dpy-18 (10). The pdi-3 transcript was temporally constitutively expressed throughout the post-embryonic life cycle with highest peaks of abundance in the L4 and adult stage. The temporal and spatial patterns support a potential cuticle assembly, perhaps cross-linking a role for this enzyme although additional roles cannot be excluded.

Recombinant PDI-3 was expressed, biochemically characterized, and shown to exhibit both disulfide isomerase and transglutaminase-like activity following in vitro assays, an unusual feature that is supported by the recently reported biochemical in vitro analysis of H06d01.1 (38). The PDI-3 ortholog from the parasitic nematode D. immitis (ERp60) was likewise demonstrated to display this dual isomerase and transglutaminase activity (17), providing three independent cases supporting this unexpected observation. In all three studies, the specific transglutaminase-like cross-linking activity was demonstrated against a dimethylcasein substrate using standard assays (27).

Transglutaminases are important post-translational modification enzymes that are able to covalently cross-link cellular proteins and thereby contribute to overall tissue stabilization (22). They play essential and varied functions in eukaryotic cells such as in apoptosis (21) and human epidermal development (22). Despite a complete genome sequence for C. elegans (39), no conserved TGase homologs have been identified in this organism. However, TGase-like activity has been detected in C. elegans extracts by biochemical assay using the same method applied in this study (40). Additionally, as for PDI-3, constitutive expression levels of this enzyme activity were noted in the C. elegans intestine (40). Together with PDI-3 (38) (Fig. 5, A and B), C. elegans PDI-1, PDI-2 (Fig. 5, A and B), D. immitis ERp60-like protein (17), and more surprisingly human PDI (17) all possess TGase-like activity. Therefore, this may be a common function of PDIs and may account for the absence of TGase genes in C. elegans despite the detectable TGase activity in the C. elegans extracts (40). The function of pdi-3 was investigated using an RNAi approach, which failed to generate an easily distinguishable phenotype in wild type C. elegans. The disrupted localization of the DPY-7 cuticle collagen however indicated that RNAi was having a mild effect at least on this component of the cuticle. Additional pdi-3 RNAi experiments in 12 cuticle-related mutant backgrounds, including cuticle collagen mutants and enzymes involved in cuticle collagen biosynthesis, were carried out. This involved the examination of several mutant alleles, and a total of twenty independent strains were examined. Synthetic phenotypes were only observed in two mutant genotypes, namely dpy-18 (alleles e364, bx26, and ok162) and sqt-3 (temperature-sensitive severe mutant allele e2117). All of the affected alleles represent strains that have thermally weakened cuticles and corresponding body-form defects. The sqt-3(e2117) Dpy phenotype is the result of glycine substitution in the COL-1 cuticle collagen, and it has been predicted to result in the decreased thermal stability and increased flexibility of the resulting collagen trimers (31). In the dpy-18 strains CB364, EM76, and JK2729, there is a marked decrease in 4-hydroxyproline residues because of the deletion of an important prolyl 4-hydroxylase enzyme subunit (9, 11, 12). It has been demonstrated that the catalytic hydroxylation of proline to 4-hydroxyproline represents an essential post-translational modification event, important for the thermal stability of collagen trimers (13). It can be concluded that PDI-3 has a function in ECM assembly that is not essential in wild type animals but becomes critical for maintenance of the proper post-embryonic body shape in strains with a significantly weaker cuticular ECM. It is also interesting to note that the temperature-sensitive embryonic lethal phenotype associated with sqt-3(e2117) and the combined prolyl 4-hydroxylase α-subunit (phy-1 and phy-2) depletions phenotypes are very similar and that both support the critical role played by the first larval cuticle in the maintenance of the normal vermiform body shape (9). CB1421(sqt-3) embryos raised at 25 °C elongate normally and then collapse to their pre-elongated form (32), a post-elongation embryonic lethal phenotype that is both temporally and phe-
notopy to similar to phy-2 RNAi in the CB364(dpy-18) mutant background (9).

The RNAi effect of pdi-3 in dpy-18 mutant backgrounds resulted in branching alae, protruding vulva, and abnormal male and hermaphrodite tails, all of which are structures produced by the lateral seam cell hypodermis (3, 41). Further characterization using the seam cell-specific gap marker strain (JRK671) crossed into CB364(dpy-18) worms in combination with MH27 antibody staining demonstrated a serious disruption to the morphology of the normal seam cells and the resulting syncytium. Following pdi-3 RNAi in the CB364(dpy-18) background, it appears that the seam cells were mislocalized and misshapen and that they fused in an abnormal fashion as a direct consequence of this mislocalization. Interestingly, the seam cell mislocalization corresponds exactly to the region where the worm is most dumpy and where the DPY-7 collagen was most severely mislocalized. Indeed, proper worm morphology relies on the generation of correctly localized cells and on the ultimate morphogenetic properties of these cells (42). The cuticle synthesized by these cells plays an essential role in maintaining post-embryonic body shape of the worm (32), thus the synthesis of an abnormal or weakened cuticle will ultimately affect the morphology of all post-embryonic stages.

The protruding vulva phenotype associated with dpy-18 mutant strains following pdi-3 RNAi is reminiscent of the squashed vulva class of mutants (squ) (43) such as squ-8. squ-8 codes for a protein with homology to glucuronyl transferase that may play a role in the synthesis of glycosoylates (44). Interestingly, the vulva is also derived from hypodermal cell lineage (41), and an additional phenotype associated with squ-8 was bulging at the tail or mid-body of the worm (43). This finding suggested that squ-8 may be involved in additional aspects of epithelial morphogenesis and that the vulval defects and morphology defects in squ-8 may be a direct consequence of a leaky weakened cuticle (43). Recently, it has been shown that glycosylation plays an important role in stabilization of a collagen from the hydrothermal vent worm Riftia pachyptila (45).

In addition, vertebrate Erp60 has been shown to form complexes with calreticulin and calnexin in the ER and to interact with newly synthesized glycoprotein; therefore, it may act as a chaperone to assist in protein folding (46). It could be hypothesized that pdi-3 plays a similar function in C. elegans, acting as a chaperone for newly synthesized glycoproteins that are part of the ECM.

The ortholog of pdi-3 may be widely distributed across the nematode phylum, and translations of many parasite EST clones have homology to PDI-3: parasitic nematodes remains unresolved, but it appears to be posed as potential chemotherapy targets (19).

In C. elegans, the precise function of pdi-3 in cuticular ECM assembly remains to be conclusively established. PDI-3 may be involved in ECM cross-linking via TGase activity, which may ultimately stabilize the C. elegans ECM. Alternatively, pdi-3 may act as chaperone and folding catalyst for newly synthesized glycoproteins that are important stabilizing components of the ECM. Alternatively, pdi-3 may play a role in both functions. Although pdi-3 does not appear essential in wild type animals under standard laboratory conditions with constant physico-chemical parameters and optimal food conditions, it may however prove to be vital in the natural soil environment. In wild type C. elegans, the actual morphology of the animal was not affected following pdi-3 RNAi, but the proper localization of cuticle collagen studied (DPY-7) was affected. The only synthetic RNAi phenotypes observed were in specific genetic backgrounds that possess an unstable cuticular ECM. Together, these observations reinforce the proposition that pdi-3 may be involved in ECM stabilization via cross-linking activity and/or via a protein chaperone role.

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