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THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 288, NO. 3, pp. 1750–1761, January 18, 2013

Prolyl 4-Hydroxlase Activity Is Essential for Development and Cuticle Formation in the Human Infective Parasitic Nematode *Brugia malayi**[S]

Received for publication, July 3, 2012, and in revised form, November 16, 2012 Published, JBC Papers in Press, December 7, 2012, DOI 10.1074/jbc.M112.397604

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Background: Collagen prolyl 4-hydroxylases (C-P4H) are involved in the formation of extracellular matrices.
Results: The full complement of C-P4H enzymes from the human infective parasite *Brugia malayi* have been bioinformatically, biochemically, and functionally characterized.
Conclusion: C-P4H enzymes are essential for development in *B. malayi*.
Significance: Unique features of these essential enzymes may be exploited in future control mechanisms.

Collagen prolyl 4-hydroxylases (C-P4H) are required for formation of extracellular matrices in higher eukaryotes. These enzymes convert proline residues within the repeat regions of collagen polypeptides to 4-hydroxyproline, a modification essential for the stability of the final triple helix. C-P4H are most often oligomeric complexes, with enzymatic activity contributed by the α subunits, and the β subunits formed by protein disulfide isomerase (PDI). Here, we characterize this enzyme class in the important human parasitic nematode *Brugia malayi*. All potential C-P4H subunits were identified by detailed bioinformatic analysis of sequence databases, function was investigated both by RNAi in the parasite and heterologous expression in *Caenorhabditis elegans*, whereas biochemical activity and complex formation were examined via co-expression in insect cells. Simultaneous RNAi of two *B. malayi* C-P4H α subunit-like genes resulted in a striking, highly penetrant body morphology phenotype in parasite larvae. This was replicated by single RNAi of a *B. malayi* C-P4H β subunit-like PDI. Surprisingly, however, the *B. malayi* proteins were not capable of rescuing a *C. elegans* α subunit mutant, whereas the human enzymes could. In contrast, the *B. malayi* PDI did functionally complement the lethal phenotype of a *C. elegans* β subunit mutant. Comparison of recombinant and parasite derived material indicates that enzymatic activity may be dependent on a non-reducible covalent link, present only in the parasite. We therefore demonstrate that C-P4H activity is essential for development of *B. malayi* and uncover a novel parasite-specific feature of these collagen biosynthetic enzymes that may be exploited in future parasite control.

The parasitic filarial nematode *Brugia malayi* is one of the causative agents of lymphatic filariasis, a condition affecting around 120 million people in 73 countries worldwide (1, 2). The *B. malayi* genome has been sequenced and is similar in size and gene number to the free-living nematode *Caenorhabditis elegans* (3). All nematodes display similar body plans and are encased in a collagenous exoskeleton, known as the cuticle, which covers the outermost layer of epithelial cells. We have previously demonstrated in *C. elegans* that collagen prolyl 4-hydroxylase (C-P4H)[3] (EC 1.14.11.2) and protein disulfide isomerase (PDI) (EC 5.3.4.1) activities are essential for nematode development due to their cuticle collagen modification function (4–6). We therefore examined whether the *B. malayi* forms of these enzymes were similarly required for development and as such would represent effective anti-parasitic drug targets.

The nematode cuticle is a collagen-rich extracellular matrix, which is required for maintenance of body morphology and interaction with the environment (7). The collagens that constitute the cuticle are formed via a multi-step process of intra- and extracellular post-translational enzymatic modifications (reviewed in Ref. 7). An essential step in this process is catalyzed by C-P4H and occurs within the endoplasmic reticulum where secreted proteins are folded. C-P4H converts peptide bound proline residues in the *X*-Pro-Gly repeat region of procollagen polypeptides to 4-hydroxyproline. Mature collagen triple helices that lack this modification are thermally unstable at physiological temperatures. In most species examined, C-P4H are oligomeric complexes with the C-P4H active sites present in

* This work was supported by grants from the Medical Research Council and the Biotechnology and Biological Sciences Research Council of Great Britain.
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[S] This article contains supplemental "Experimental Procedures," Tables S1 and S2, Figs. S1–S6, and additional references.
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[3] The abbreviations used are: C-P4H, collagen prolyl 4-hydroxylase(s); PDI, protein disulfide isomerase; hsiRNA, heterogeneous short-interfering RNA; co-IP, co-immunoprecipitation.

the α subunits and the enzyme PDI present in the β subunit (8). PDI is also found at high levels free from the C-P4H complex and functions to ensure correct disulfide bond formation and, therefore folding, of many proteins, including collagens, which transit the endoplasmic reticulum (6).

The form and function of collagen C-P4H have been reviewed in detail (8) and are summarized in supplemental Fig. S1. Humans possess three catalytically active α subunits (named P4HA1, P4HA2, and P4HA3), each of which form $\alpha_2\beta_2$ complexes containing matching α subunits. The β subunit, which is common to all forms of the human complexes, is PDI (P4HB). In *C. elegans*, there are two α subunits (named DPY-18 and PHY-2) and a β subunit (PDI-2) that also form three C-P4H complexes. However, unlike humans, these consist of a mixed α subunit tetramer, [DPY-18][PHY-2][PDI-2]$_2$, and two dimers, [DPY-18][PDI-2] and [PHY-2][PDI-2] (5). The diversity of complexes formed in nematodes is highlighted in *Caenorhabditis briggsae*, a close relative of *C. elegans*, where, in addition to a mixed α subunit tetramer and [DPY-18][PDI-2] dimer identical to those found in *C. elegans*, a [PHY-2]$_2$[PDI-2]$_2$ tetramer can also form (9).

Nematodes produce a new cuticle for each developmental stage, and in *C. elegans*, the C-P4H complex encoding genes are expressed almost exclusively in the cuticle collagen secreting cells of the hypodermis in waves of abundance that correspond to that of collagen (4). C-P4H activity is developmentally required in *C. elegans*; worms that have lost the α subunit DPY-18 are viable but show defects at the levels of worm body morphology, cuticle structure, and collagen localization (4). Complete combined loss of both α subunits, DPY-18 and PHY-2, or complete single loss of the β subunit, PDI-2, results in embryonic lethality (4, 6). In *C. briggsae*, C-P4H function is similarly essential (9). The phenotypes resulting from loss of C-P4H can be replicated using enzyme inhibitors (5).

The *in vivo* action of the *C. elegans* C-P4H β subunit PDI-2 has been examined in detail with incomplete loss of function producing sterile adults with severely abnormal body morphology, cuticle structure, and collagen localization (6). Interestingly, although it was not possible to analyze separately the C-P4H-dependent and -independent roles of PDI-2, intact PDI-2 thioredoxin active site residues are essential for correct development (6). In addition, loss of *C. elegans* PDI-2 could be completely rescued by human PDI (P4HB), suggesting it may be possible to heterologously express *B. malayi* PDIs in *C. elegans* to examine their function.

The likely importance of C-P4H activity during *B. malayi* development and cuticle collagen modification is suggested by several lines of evidence. Chemical inhibition of C-P4H produces cuticle associated defects and a reduction in 4-hydroxyproline levels in cultured *B. malayi* adults (10), whereas for cultured third stage larvae, the essential C-P4H co-factor ascorbate is necessary for correct molting and development (11). Furthermore, adult *B. malayi* cuticle collagens contain 7.9% hydroxyproline (40% of prolines are hydroxylated) (12), a level comparable with that found in *C. elegans* (12% hydroxyproline, 51% of prolines are hydroxylated) (13).

We have previously characterized a protein from *B. malayi* with homology to C-P4H α subunits, *Bma*-PHY-1, which showed a number of unusual properties (14). Recombinant α subunits expressed alone are in general insoluble, and therefore, complexes are usually produced by co-expression of α and β subunits in systems such as insect cells. In most cases, complexes can be formed by combining α and β subunits from different species. However, when *Bma*-PHY-1 was expressed in an insect cell expression system, it was soluble without a β subunit, did not form complexes with β subunits from other species, and instead formed an active homotetramer (14). Despite this *in vitro* activity, when *Bma*-PHY-1 was expressed in *C. elegans*, it was not capable of replacing the *in vivo* functions of the α subunit DPY-18 (14). We therefore speculated that either additional C-P4H α subunit-like genes were present in *B. malayi*, or, that only complexes containing native partner protein(s) would produce fully functional enzyme. In this study, we utilized the *B. malayi* genome sequence (3) to ensure identification of all potential C-P4H subunits and used a recently developed approach for RNAi in this parasite (15) to demonstrate that these genes are required for development and formation of the cuticle. In addition, we uncovered a novel parasite-specific non-reducible covalent modification required for association of this critical enzyme complex.

EXPERIMENTAL PROCEDURES

Procedures for RNA extraction, cDNA synthesis, genomic DNA isolation, plasmid construction, single worm PCR, and RT-PCR assessment of transgene expression in *C. elegans* are provided in the supplemental "Experimental Procedures." All primer names, sequences, and descriptions of their uses are given in supplemental Table S1. The abbreviations *Bma* and *Cel* are used throughout before gene and protein names to indicate *B. malayi* and *C. elegans* respectively.

Nematode Strains, Culture, and Microscopy—*C. elegans* strains used in this study were N2 (wild type), CB364 (*dpy-18*(*e364*) III) (4), and TP69 (*pdi-2*(*tm0689*)/*lon-2*(*e678*) X) (6). The *pdi-2*(*tm0689*) allele was maintained by selecting *pdi-2*(*tm0689*) heterozygotes, which are the only phenotypically wild type progeny segregating from strain TP69. *C. elegans* strains were cultured as described previously (16). *B. malayi* nematodes were kindly provided by Rick Maizels and Yvonne Harcus (University of Edinburgh). For microscopy, live *C. elegans* were transferred to slides with a 2% agarose, 0.065% sodium azide pad. *B. malayi* were prepared for microscopy by transferring microfilaria to slides with a coverslip and heating the slides to 60 °C on a hot plate for 10 s to immobilize the larvae. All images were captured using an Axioskop 2 microscope (Zeiss) and an AxioCam MRm camera (Zeiss).

RNAi of B. malayi by Soaking and C. elegans by Feeding—For RNAi of *B. malayi*, *Bma-phy-1*, *Bma-phy-2*, *Bma-pdi-2*, and *Cel-phy-3* (negative control) were targeted following previously described methods (15). Briefly, *in vitro* transcription templates were produced by linearizing the *Bma-phy-1*, *Bma-phy-2*, *Bma-pdi-2*, and *Cel-phy-3* RNAi plasmids each in two separate reactions (using the cloning restriction enzymes) and single-stranded RNAs produced using a MEGAscript kit (Ambion). Equal quantities of the complementary single stranded RNAs were then annealed and the long double-stranded RNA digested with ShortCut RNase III (New England Biolabs) to

produce heterogeneous short-interfering RNAs (hsiRNAs). Freshly extracted *B. malayi* adult females were cultured in 24-well plates containing two females per well in 1 ml of culture medium. Medium for culture of *B. malayi* was RPMI 1640 (with glutamine and HEPES) (Invitrogen), 1% glucose, 100 units/ml penicillin and 100 μg/ml streptomycin (Invitrogen), and 10% heat-inactivated fetal calf serum (Invitrogen). Following an overnight culture period in medium alone, the parasites were exposed to RNAi for a total of 48 h by adding fresh medium containing hsiRNAs, at a final concentration of 0.5–2 μM, at the start of the culture and again after 24 h. For RNAi of *C. elegans* lines expressing *Bma-pdi-2*, the *Bma-pdi-2* RNAi plasmid was transformed into bacterial strain HT115(DE3) (17) (HT115(DE3) available from the *Caenorhabditis* Genetics Center). RNAi by feeding was performed as described previously (18). As a negative control all processes were replicated using control RNAi bacteria containing the RNAi vector without an insert.

Database Searches for Additional B. malayi phy Genes—tBLASTn was performed on whole genome shotgun sequences using *Bma*-PHY-1 and *Bma*-PHY-2 (maximum values selected for "alignment" and "descriptions" options). The genomic sequences of *Bma-phy-1* and *-2* were then used as BLASTn queries against the same database. Those hits that showed complete nucleotide identity to each gene were considered to represent that gene (to identify any potential new *Bma-phy*, this analysis assumes some degree of sequence divergence). This enabled all whole genome shotgun sequences with amino acid homology to PHYs (tBLASTn results) to be assigned as being derived from either *Bma-phy-1* (complete nucleotide identity to *Bma-phy-1*) or *Bma-phy-2* (complete nucleotide identity to *Bma-phy-2*). The BLASTn results were then subtracted from the tBLASTn results to determine if any whole genome shotgun sequences were present which were not derived from the known genes.

Expression of B. malayi Proteins in Insect Cells—Recombinant vectors were co-transfected into *Spodoptera frugiperda* Sf9 cells with a modified *Autographa californica* nuclear polyhedrosis virus DNA (BaculoGold, BD Pharmingen) by calcium phosphate precipitation. Sf9 or High Five (Invitrogen) insect cells were cultured as monolayers in TNM-FH (modified Grace's insect cell medium, Sigma) supplemented with 10% fetal bovine serum (BioClear) or in suspension in Sf900IISFM serum-free medium (Invitrogen). The cells were seeded at a density of 5×10^6 cells/100-mm plate or 1×10^6 cells/ml and infected at a multiplicity of 5 for either single or multiple virus coding for *B. malayi* proteins. Cells were harvested 72 h after infection, washed with a solution of 0.15 M NaCl and 0.02 M phosphate, pH 7.4, homogenized in a 0.1 M NaCl, 0.1 M glycine, 10 μM dithiothreitol, 0.1% Triton X-100, and 0.01 M Tris buffer, pH 7.4, and centrifuged at 10,000 $\times$ *g* for 20 min. The pellets were further solubilized in 1% SDS. C-P4H activity was assayed by a method based on the hydroxylation-coupled decarboxylation of 2-oxo-[1-^{14}C]glutarate, and K_m and K_i values were determined as described previously (19).

Transgenic Expression in C. elegans—Transgenic *C. elegans* carrying extrachromosomal arrays were generated by microinjection following standard protocols (20) with at least three independent lines analyzed in all cases. Repetitive extrachromosomal arrays were produced by injecting circular plasmids. These were performed by co-injecting the test plasmid at 2–25 μg/ml, the transformation marker *dpy-7prom*::*gfp* plasmid (from Iain Johnstone, University of Glasgow) at 5 μg/ml, with pBluescript SKM (Stratagene) added to give a total plasmid concentration of 150 μg/ml. To produce complex extrachromosmal arrays (21), plasmids were linearized by restriction digest and co-injected with fragmented *C. elegans* genomic DNA. Genomic DNA was prepared for injection by digestion with PvuII followed by phenol/chloroform extraction and ethanol precipitation. The *dpy-7prom*::*gfp* transformation marker was digested with FspI, and plasmids for expression of *B. malayi* or human genes were digested with blunt cutting enzymes where possible or were subsequently treated with DNA polymerase I large (Klenow) fragment (New England Biolabs) (see supplemental Table S2). All linearized plasmids were purified using a QIAquick PCR purification kit. Injections to produce complex arrays were performed by co-injecting the linearized plasmid(s) each at 1 μg/ml, the transformation marker at 2 μg/ml, and fragmented *C. elegans* genomic DNA at 100 μg/ml. For unknown reasons, direct introduction of complex arrays by injection of the *dpy-18* mutant strain CB364 resulted in high toxicity, even in the absence of *B. malayi* PHY and PDI expression constructs. Therefore, in these cases, injections were performed in the *C. elegans* wild type N2 strain, and the transgenic extrachromosomal array subsequently crossed into the *dpy-18* mutant strain.

Nematode Extracts—B. malayi parasite extracts were made by resuspending adults in extraction buffer (0.1 M NaCl, 0.1 M glycine, 10 μM dithiothreitol, 0.1% Triton X-100, and 10 mM Tris, pH 8.0, supplemented with protease inhibitors 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 1 mM EGTA, 2 μM E64, and 0.1 μM pepstatin) and disrupting using a 3-ml glass hand-held homogenizer (Jencons). To examine the expression of transgenic proteins, *C. elegans* extracts were made by adding 50 transgenic worms to 30 μl of 1XM9 (16) with 12 μl of 4$\times$ SDS sample buffer and freezing the samples at −80 °C. For reducing gels, 5% (v/v) 2-mercaptoethanol was added, and the samples were boiled before loading.

Western Blotting—Electrophoresis and Western blotting of nematode and insect cell-derived material was carried out using Bio-Rad equipment as described by the manufacturer, with Tris-HCl gels (Bio-Rad) and Hybond-P PVDF membrane (GE Healthcare). *Bma*-PHY-1 was detected using an anti-peptide antibody described previously (14). An anti-peptide antibody was raised to the C-terminal sequence of *Bma*-PHY-2 (CLGAPEPKRHLNIRSEKARK, the *underlined* cysteine was added to couple the peptide to keyhole limpet hemocyanin) (Sigma). An anti-peptide antibody raised previously to *Cel*-PDI-2 (5) was found to cross-react with *Bma*-PDI-2. Secondary antibodies for PHY and PDI primaries were anti-rabbit IgG (whole molecule) peroxidase (goat) (Sigma) or anti-rabbit IgG (whole molecule) alkaline phosphatase (Sigma). Stripped blots were reprobed for β-actin using monoclonal anti-β-actin clone AC-15 (Sigma) and anti-mouse IgG HRP conjugate (Promega). Peroxidase conjugates were detected with Amersham Biosciences ECL Plus Western blotting detection system (GE Health-

TABLE 1
Amino acid similarity and identity of C-P4H α subunits

	Cel-PHY-2	*Bma*-PHY-1	*Bma*-PHY-2	*Hsa*-P4HA1	*Hsa*-P4HA2
Cel-DPY-18	53.7/65.6[a]	57.1/70.9	53.1/66.3	40.7/56.1	39.4/54.7
Cel-PHY-2		55.1/67.5	47.6/63.3	44.8/58.8	43.9/58.9
Bma-PHY-1			51.9/69.1	43.0/55.5	42.3/56.7
Bma-PHY-2				39.1/55.1	39.1/54.7
Hsa-C-P4HA1					63.4/77.0

[a] Identity/similarity.

care), and alkaline phosphatase conjugates with SigmaFast BCIP/NBT (Sigma).

Co-immunoprecipitation (co-IP)—The peptide used to raise the anti-Cel-PDI-2 antibody (5) was linked to a beaded agarose support, and the antibody was affinity purified using a SulfoLink immobilization kit (Thermo Scientific). Purified anti-*Cel*-PDI-2 antibody was dialyzed using a Slide-A-Lyzer dialysis cassette (Thermo Scientific) and the Pierce co-immunoprecipitation kit (Thermo Scientific) protocol was followed to immobilize the antibody to an agarose support and perform co-IP using adult *B. malayi* lysate. Lysate was generated by resuspending adult parasites in IP lysis buffer containing halt protease and phosphatase inhibitor mixture (Thermo Scientific) and disrupting using a 1-ml Radnoti Econo-Grind Micro Tissue Grinder. Control co-IP reactions were performed in parallel using isotype-matched, affinity-purified antibody reactive to *C. elegans* BLI-5.

RESULTS

Cloning of Bma-phy-2 and Bma-pdi-2—We had previously cloned and characterized a *B. malayi* protein, *Bma*-PHY-1, with C-P4H activity (14) (cDNA and genomic accession nos. AJ297845 and AJ421993). Prior to publication of the complete annotated *B. malayi* genome (3), we searched for further proteins with homology to C-P4H α subunits (known as PHY in nematodes). Sequences of an additional PHY-encoding gene, which was distinct from *Bma-phy-1*, were identified in the *B. malayi* genome preassembly using tBLASTn. The complete sequence was identified by finding overlapping sequences and identifying coding regions with homology to known PHYs. The full-length cDNA was verified by PCR amplification, cloning, and sequencing (accession no. AJ628347) with the 5′ end verified by RT-PCR utilizing the trans-spliced leader SL1 sequence (22) (data not shown).

An EST (accession no. AI784701) containing a partial sequence for a *B. malayi pdi* (named *Bma-pdi-2* for reasons described in later sections) was identified by tBLASTn at EBI using *Cel*-PDI-2 as a query. The sequences identified enabled PCR screening of lysates from a BAC library (kindly provided by Mark Blaxter, University of Edinburgh) and sequencing of a positive BAC provided the full-length *Bma-pdi-2* genomic sequence (accession no. AJ577085). A full-length cDNA was generated by PCR (accession no. AJ577086), and the 5′ end confirmed by SL1-RT-PCR (data not shown). In the annotated *B. malayi* genome (3), the genes representing *Bma-pdi-2* (Bm1_39250) and *Bma-phy-2* (Bm1_45460) both have inaccurately predicted splicing, and therefore, all work here refers to our experimentally confirmed sequences and the accession numbers for those that are listed above.

Bma-phy-2 encodes a 551-amino acid protein with a predicted signal peptide (23) occurring between residues 17 and 18 (VSA-DS) and a single predicted *N*-glycosylated asparagine. The mature protein shows 51.9% identity and 69.1% similarity with *Bma*-PHY-1 (Table 1), and is most similar to *Onchocerca volvulus* PHY-1 (10), with which it shows 69.3% identity and 77.0% similarity. As described for *Bma*-PHY-1 (14), all residues identified as being required for protein structure, complex formation, and enzyme activity (24–26) are conserved in *Bma*-PHY-2 (supplemental Fig. S2). *Bma-pdi-2* encodes a 503-amino acid protein with a predicted signal peptide occurring between residues 22 and 23 (AHD-AS) and an endoplasmic reticulum retention signal at the C terminus. *Bma*-PDI-2 shows higher homology to C-P4H β subunit PDIs (Table 2) than to other PDIs. The degree of conservation of residues defined previously as the active sites (27), active site role charged residues (28), hydrophobic ligand binding site (29), and residues involved in C-P4H complex formation (30), is shown in supplemental Fig. S3.

Database Searches Indicate Bma-phy-1 and Bma-phy-2 Are the Only B. malayi phy Genes—Analyzing the available completed genome sequences for other parasitic nematodes we noted that although the *Trichinella spiralis* genome (31) is likely to encode only two PHYs (results not shown), an expanded family of five PHY-encoding genes exists in the compact genome of the plant parasite *Meloidogyne hapla* (32),[4] and *Ascaris suum*, which, similar to *B. malayi*, is an animal parasite in nematode clade III, has three PHY-encoding genes (accession nos. ADY44523, ADY43128, and ADY44875) (33, 34). A careful analysis of the *B. malayi* genome was therefore undertaken to determine whether any additional PHY-encoding genes, other than *Bma-phy-1* and *-2*, were present in this organism. tBLASTn analysis of the assembled *B. malayi* scaffolds using PHY protein sequences did not reveal any additional *Bma-phy* genes, other than *Bma-phy-1* and *-2* and a *Bma-phy* gene fragment. *Bma-phy-1* and *-2* are arranged in tandem in the genome with the *Bma-phy* gene fragment situated between them (supplemental Fig. S4 and supplemental "Experimental Procedures"). tBLASTn and BLASTn was then carried out using the whole genome shotgun database so that sequences that may not have assembled into scaffolds could also be examined. The BLASTn results were used to identify sequences that showed complete nucleotide identity with the known genes (and were therefore assumed to represent these genes) and were subtracted from the tBLASTn results. This analysis did not reveal any sequences with homology to PHY proteins that

[4] A. D. Winter and A. P. Page, unpublished results.

TABLE 2
Amino acid similarity and identity of C-P4H β subunits

	Bma-PDI-2	*Hsa*-PDI (P4HB)
Cel-PDI-2	71.7/82.7[a]	54.3/68.9
Bma-PDI-2		56.7/67.7

[a] Identity/similarity.

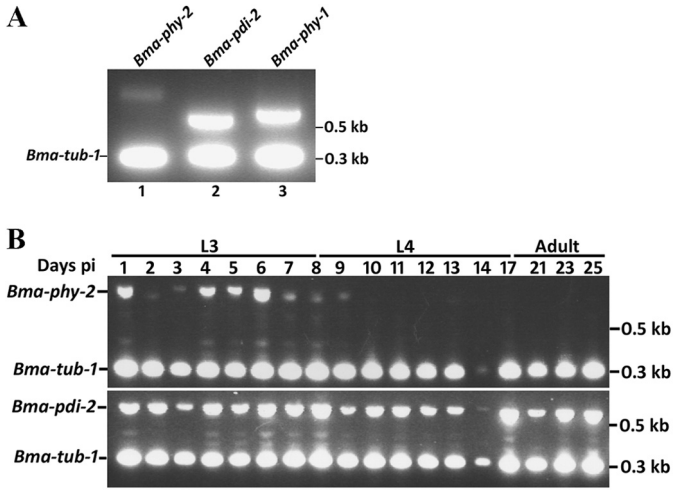

FIGURE 1. **Developmental expression profiles of *B. malayi* C-P4H genes assessed by RT-PCR.** For each gene, simultaneous amplifications were performed using gene-specific primers and primers to *B. malayi* tubulin (*Bma-tub-1*) (accession no. AJ551180) as an internal control. *A*, expression of *Bma-phy-2*, *Bma-pdi-2*, and *Bma-phy-1* in microfilaria (first stage larvae). *B*, expression of *Bma-phy-2* and *Bma-pdi-2* in post-infective stages. Parasite material was taken from the time points indicated post-infection (*days pi*) from infected jirds. *L3* and *L4* denote larval stages 3 and 4, and *Adult* indicates juvenile adults. Sizes of cDNA amplicons were as follows: *Bma-phy-2*, 808 bp; *Bma-pdi-2*, 597 bp; *Bma-phy-1*, 655 bp; *Bma-tub-1*, 308 bp. Sizes of genomic amplicons, *Bma-phy-2*, 3529 bp; *Bma-pdi-2*, 1301 bp; *Bma-phy-1*, 1909 bp; *Bma-tub-1*, 390 bp.

were not completely identical to the known *Bma-phy* genes at the nucleotide level. Similar analysis of the *B. malayi* BAC end sequences held at TIGR and the Sanger Institute, EST sequences at EBI, and NematodeNet, and analysis of genomic scaffolds from a resequenced *B. malayi* genome (kindly provided prepublication by Elodie Ghedin, University of Pittsburgh) also failed to uncover any sequences considered to represent new genes. In addition, sequencing of an ~2-kb genome assembly gap, situated between the *Bma-phy-1* and *-2* genes and downstream of the *Bma-phy* gene fragment region, revealed no further PHY-encoding genes (see supplemental "Experimental Procedures"). Therefore, as exhaustive searches of multiple databases revealed no additional genes encoding proteins with homology to C-P4H α subunits, *Bma-phy-1* and *Bma-phy-2* represent the only identifiable genes of this type in all databases and are very likely to encode the only PHYs in this organism.

Expression Profiling—The expression of *Bma-phy* and *Bma-pdi-2* genes was examined in a range of developmental stages by RT-PCR. In each case, the test gene was amplified simultaneously along with the constitutively expressed tubulin gene *Bma-tub-1*. Expression in the first larval stage (termed microfilaria) of *Bma-phy-2*, *Bma-pdi-2*, and *Bma-phy-1* is shown in Fig. 1*A*, with *Bma-phy-2* found at a lower level than *Bma-pdi-2* and *Bma-phy-1*. A panel of cDNAs used previously to investigate the expression of *Bma-phy-1* (14), representing stages from post-infective third stage larvae (L3) to juvenile adult, was then employed for *Bma-phy-2* and *Bma-pdi-2*. The *Bma-phy-2* message was predominantly found in the early and mid L3 stages and was barely detectable in the late fourth stage larvae (L4) and adult stages (Fig. 1*B*). In contrast, the *Bma-pdi-2* message was abundant in all stages examined, displaying a very similar profile to that shown previously for *Bma-phy-1* (14).

RNAi of Cultured B. malayi—To investigate the functional roles of our identified genes during *B. malayi* development, we employed a recently described RNAi approach (15). In contrast to previously described RNAi studies in animal parasitic nematodes (reviewed in Ref. 35), this approach uses a pool of hsiRNAs generated enzymatically from a long double-stranded RNA template, to give robust, reproducible RNAi. The effect of targeting *Bma-phy-1*, *Bma-phy-2*, and *Bma-pdi-2* by RNAi was determined by culturing adult females in the presence of hsiRNAs corresponding to each gene singly, as well as *Bma-phy-1* and *Bma-phy-2* in combination. For each RNAi, multiple pairs of adult females (at least four groups of two females over two experiments) were treated, and the viability and morphology of the first stage larvae (microfilaria) produced was analyzed. Applying either *Bma-phy-1* or *Bma-phy-2* RNAi singly at 1 μM produced no visible effects on either the females or the microfilaria produced (Fig. 2, *A* and *B*). *Bma-pdi-2* RNAi at 1 μM (data not shown) and especially 2 μM produced mutant microfilaria that were immotile and presented with a coiled lumpy appearance (Fig. 2*C*). The first larval cuticle surrounding the microfilaria is highly irregular, whereas the extracuticular microfilarial sheath, a remnant of the eggshell, remained intact. This phenotype was more penetrant after the second day of culture. A practically identical highly penetrant microfilarial mutant phenotype was found by simultaneous RNAi of *Bma-phy-1*/*Bma-phy-2* at a combined concentration of 1 μM (each gene at 0.5 μM) (Fig. 2, *D* and *E*) and was also observed at a combined concentration of 0.5 μM and at 2 μM (data not shown). Untreated controls with no hsiRNA added and controls treated with 2 μM hsiRNA from *Cel-phy-3* (Fig. 2*F*) (36), for which no homologous sequences were identifiable in the *B. malayi* genome, were included in all cases.

Expression of B. malayi Proteins in Insect Cells—Recombinant baculoviruses were produced containing *Bma-phy-2* and *Bma-pdi-2* singly. These were used, along with a recombinant virus for *Bma-phy-1* generated previously (14), to investigate the enzymatic activity and protein-protein interactions of these presumed C-P4H subunits. Insect cells were then infected either with a single recombinant virus or with combinations of viruses. Cells were harvested 72 h after infection and extracts analyzed by Western blotting of native PAGE gels with peptide-specific antibodies. C-P4H activity of extracted material was assessed using a method based on the hydroxylation-coupled decarboxylation of radiolabeled co-substrate and a defined artificial substrate (19). These methods have been used to determine recombinant C-P4H activities from a range of species (8). The insect cell extracts were compared with parasite-derived extracts (Fig. 3, *lane 6*) with the designation of the type of complex being based on comparisons with previously characterized forms of the enzyme (5, 14). Sole expression of *Bma*-PHY-1

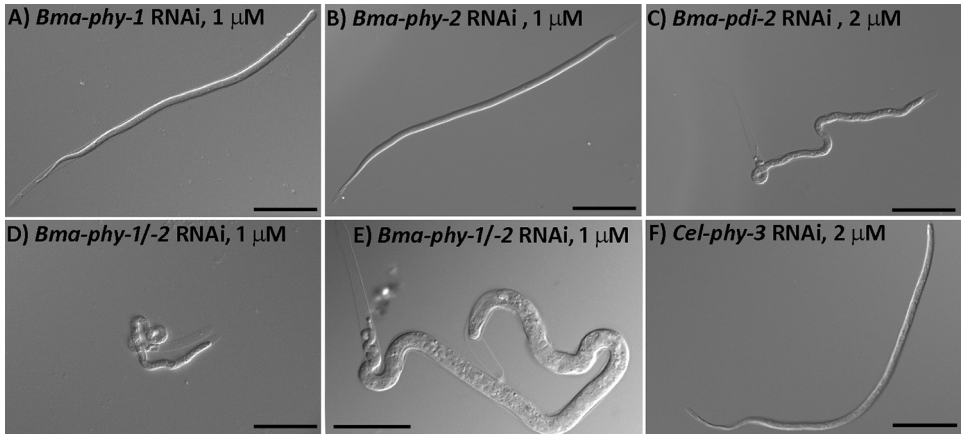

FIGURE 2. **RNAi effects in *B. malayi* microfilariae (first stage larvae).** Cultured adult female *B. malayi* were treated with hsiRNA derived from *Bma-phy-1* (*A*), *Bma-phy-2* (*B*), and *Bma-pdi-2* (*C*), and the effects were determined in the microfilariae produced. Single targeting of *Bma-pdi-2* shows severe morphological defects. *D* and *E* depict the severe morphological defects resulting from the simultaneous targeting of *Bma-phy-1* and *-2*. *F*, *Cel-phy-3* (negative control). All images are of microfilariae produced from females treated with hsiRNA at a concentration of 1–2 μM over a 48-h period. *Scale bars* represent 50 μm in *A–D* and *F* and 25 μm in *E*.

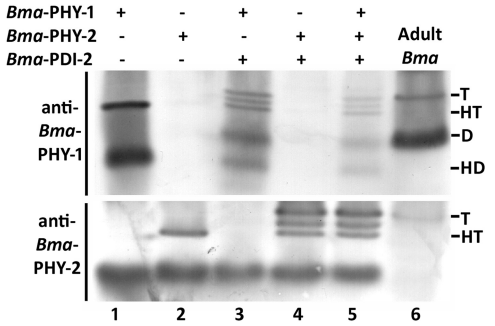

FIGURE 3. **Western blot (native PAGE) of Triton X-100 extracts of *B. malayi* proteins expressed in insect cells.** *Bma*-PHY-1 and *Bma*-PHY-2 were expressed singly (*lanes 1* and *2*), co-expressed with *Bma*-PDI-2 (*lanes 3* and *4*), and triple-expressed (*lane 5*). The complexes produced are compared with *B. malayi* adult parasite material (*lane 6*, *Adult Bma*). The size of homotetramer (*HT*), homodimer (*HD*), heterodimer (*D*), and higher molecular weight heterotetramer (*T*) bands are indicated.

produced a soluble homotetramer and homodimer as described previously (Fig. 3, *lane 1*) (14), whereas single expression of *Bma*-PHY-2 resulted in a soluble protein that formed a higher order structure, considered to be a homotetramer (Fig. 3, *lane 2*). The homotetramer was found to be enzymatically inactive (data not shown). To investigate whether *Bma*-PHY-1 and -2 required a PDI from *B. malayi* to produce a fully active C-P4H enzyme complex, each subunit was co-expressed with *Bma*-PDI-2. For *Bma*-PHY-1, co-expression with *Bma*-PDI-2 resulted in an additional dimer and a band running above the homotetramer (Fig. 3, *lane 3*), both of which show the same mobility as the two bands from the parasite extract (Fig. 3, *upper panel*, *lane 6*). For *Bma*-PHY-2, co-expression with *Bma*-PDI-2 produced a band running above the homotetramer that was equivalent to the band in parasite extract (Fig. 3, *lower panel*, *lane 6*). Co-expression of *Bma*-PDI-2 along with either *Bma*-PHY-1 or -2 had no effect on enzymatic activity (data not shown). Finally, to determine whether, similar to *C. elegans*, C-P4H tetramer formation required the presence of all three proteins, *Bma*-PHY-1, -2 and *Bma*-PDI-2 were all co-expressed. Surprisingly, the availability of all identified potential C-P4H subunits had no additional effect on complex formation (Fig. 3, *lane 5*) or enzymatic activity (data not shown). A low molecular weight nonspecific band was noted in Western blots using the anti-*Bma*-PHY-2 antibody in insect cell derived material but not in the parasite extracts (Fig. 3).

Bma-pdi-2 Can Replace the Essential Function of C. elegans pdi-2—In vertebrates and *C. elegans*, a single PDI is the C-P4H β subunit. We have previously shown that the human C-P4H β subunit PDI (P4HB) could functionally substitute for the *C. elegans* C-P4H β subunit PDI (*Cel*-PDI-2) (6). Although examination of the *B. malayi* genome suggested that *Bma*-PDI-2 was the only likely orthologue of *Cel*-PDI-2 (data not shown), we looked to confirm this *in vivo*. A *C. elegans pdi-2* mutant strain described previously (6) has severe body morphology defects and results in sterile adults. The ability of *Bma-pdi-2* to functionally substitute for loss of *Cel-pdi-2* was tested by expressing *Bma-pdi-2* in this mutant background. Two *Bma-pdi-2* expression plasmids, one genomic and one cDNA (with synthetic intron, SI), were made using vector pAW2 (*Cel-pdi-2* promoter and *Cel-dpy-18* 3′ UTR (6)). Striking rescue of the severe phenotype of the *Cel-pdi-2* mutant was achieved using complex extrachromosomal arrays carrying either of the *Bma-pdi-2* expression plasmids (Fig. 4, *A–D*). In *C. elegans*, extrachromosmal arrays are lost naturally in a percentage of the progeny, and viability of the *Bma-pdi-2* rescued transgenic lines was completely dependent on the presence of the extrachromosmal array, with animals that lost the array all dying during embryogenesis. SW-PCR was performed to ensure the *Bma-pdi-2* expression plasmids were present (data not shown), and the transgenic lines were genotyped to confirm they were *Cel-pdi-2*(*tm0689*) mutant homozygotes (Fig. 4*E*). Genotyping also ensured that no wild type *Cel-pdi-2* sequences had been introduced with the co-injected fragmented *C. elegans* genomic DNA used to generate the complex arrays. RT-PCR was performed to verify *Bma-pdi-2* expression (Fig. 4*F*). Transgenic lines carrying only the transformation marker and fragmented genomic DNA failed to rescue. Finally, the rescued transgenic lines were RNAi-treated with a construct targeting *Bma-pdi-2*. As shown in Table 3, viability was completely dependent on

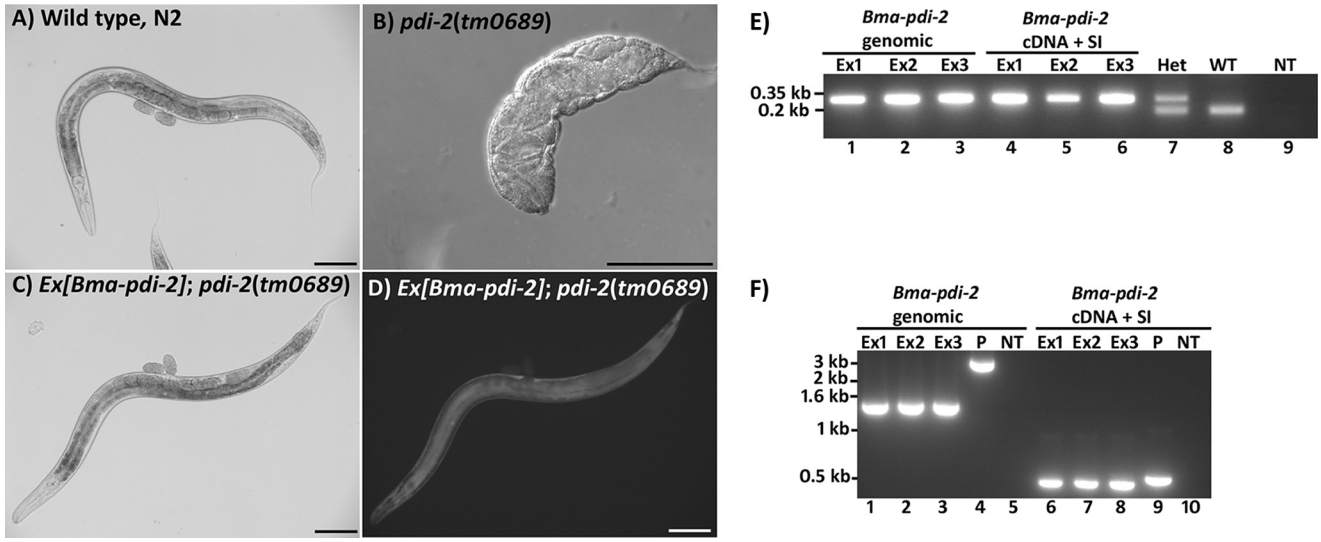

FIGURE 4. *A–D*, *Bma-pdi-2* introduced into a *C. elegans pdi-2* null mutant results in complete repair of the mutant phenotypes. *A*, wild type *C. elegans* N2 strain; *B*, *C. elegans pdi-2* homozygote mutant (from a heterozygous mother); *C* and *D*, *C. elegans pdi-2* homozygote mutant carrying *Bma-pdi*-2 on an extrachromosomal array with *D* showing GFP expression from the transformation marker *dpy-7prom::gfp* carried on the same extrachromosomal array. *Scale bars* represent 100 μm. *E*, genotyping by single-worm PCR. A set of three primers designed around the *Cel-pdi-2*(*tm0689*) deletion were use to genotype all transgenic lines to ensure mutant homozygosity. The upper 320-bp band identifies the deleted region, and the 185-bp band indicates the wild type. The three transgenic lines (*Ex1–Ex3*) generated for each construct, *Bma-pdi-2* genomic and *Bma-pdi-2* cDNA + synthetic intron (*SI*), all produce only the deletion sized amplicon. *Lane 7*, the larger deletion-derived amplicon and smaller wild type-derived amplicon from a *pdi-2(tm0689)* heterozygote (*Het*). *Lane 8* is wild type (WT), and lane 9 represents no template control (*NT*). *F*, RT-PCR showing expression of *Bma-pdi-2* in transgenic *C. elegans*. Expression is shown for three transgenic lines (*Ex1–Ex3*) for each *Bma-pdi-2* construct and *Bma-pdi-2* genomic and *Bma-pdi-2* cDNA + synthetic intron (*SI*). For the *Bma-pdi-2* genomic construct, primers amplifying the full-length *Bma-pdi-2* sequence were used. *Lanes 1–3* show expression of the 1512-bp spliced *Bma-pdi-2* cDNA in lines carrying the genomic construct. *Lane 4*, the 3579-bp amplicon generated using the *Bma-pdi-2* genomic plasmid (*P*) as template. *Lane 5*, no template (*NT*) control. For the *Bma-pdi-2* cDNA + synthetic intron (*SI*) construct, primers corresponding to *Bma-pdi-2* sequences situated either side of the 51-bp synthetic intron were used. *Lanes 6–8* show the 486-bp spliced amplicon derived from three transgenic *C. elegans* lines compared with the 537-bp plasmid-derived product (*P*, *lane 9*) and no template (*NT*) (*lane 10*). *Bma-pdi-2* from genomic construct; spliced, 1512 bp; unspliced, 3579 bp. *Bma-pdi-2* from construct with cDNA and a synthetic intron; spliced, 486 bp; unspliced, 537 bp.

TABLE 3
RNAi of *C. elegans pdi-2* mutants carrying *Bma-pdi-2* transgenes

	Bma-pdi-2 RNAi		Control	
Construct/line	**Hatched**	**Unhatched**[a]	**Hatched**[b]	**Unhatched**[c]
***Bma-pdi-2* genomic**				
Ex1	0	222	57	181
Ex2	0	215	78	96
Ex3	0	80	63	39
***Bma-pdi-2* cDNA + SI**				
Ex1	0	184	150	80
Ex2	0	181	62	119
Ex3	0	23	1	15
Total	0 (0%)	905 (100%)	411 (43.7%)	530 (56.3%)

[a] Unhatched consisted of GFP-positive and -negative embryos for all lines.
[b] All hatched larvae were GFP-positive for all lines.
[c] Some GFP-positive dead embryos were present, possibly due to expression levels being either borderline too high or too low.

transgenic *Bma-pdi-2*, confirming that rescue was due entirely to the introduced *B. malayi* gene.

Rescue of a C. elegans C-P4H α Subunit Mutant with Human but Not B. malayi Proteins—We again used *C. elegans* as an expression system to examine the possibility that formation of active *B. malayi* C-P4H complexes might require a factor, such as a specific post-translational modification or auxillary protein(s) required for folding, which was present in *C. elegans* but not in insect cells. A previously characterized homozygous viable *C. elegans* C-P4H α subunit mutant strain, *Cel-dpy-18*(*e364*), shows a marked reduction in C-P4H activity and has a malformed cuticle resulting in a readily identifiable body morphology defect (4, 5). We therefore expressed heterologous protein(s) in this mutant and observed whether they were able to repair the mutant body morphology, thereby providing a simple assay for enzyme activity and the ability of these proteins to functionally substitute for *C. elegans dpy-18*. Previously, this approach was successful when using *C. briggsae* sequences (9) but not with *Bma-phy-1* introduced singly (14). *Bma-phy-2* was expressed from a vector, pAW1 (14), which contains the promoter and 3′ UTR from *Cel-dpy-18*. To express multiple genes simultaneously, a similar construct for *Bma-phy-1* (generated previously (14)) and a *Bma-pdi-2* expression construct (described above) were also used. *Bma-phy-2* was introduced singly and in combination with *Bma-phy-1* and *Bma-pdi-2* into the *C. elegans dpy-18* mutant strain on repetitive extrachromosomal arrays. Neither *Bma-phy-2* alone, in multiple pairings of the three genes, nor all three genes in combination was able to rescue the body morphology defect of *Cel-dpy-18*(*e364*) (results not shown). However, RT-PCR of transgenic lines carrying the three *B. malayi* genes highlighted the difficulty of achieving robust expression of multiple transgenes from repetitive arrays (results not shown). We therefore next attempted rescue of the *Cel-dpy-18* mutant strain using complex extrachromosomal arrays. As explained under "Experimental Procedures," complex arrays to be tested in the *Cel-dpy-18* strain were first generated in the *C. elegans* wild type N2 strain and then crossed into the mutant. Three constructs for expression of *B. malayi* genes in *C. elegans* were used (*Bma-phy-1* cDNA + SI, *Bma-phy-2* cDNA + SI, and *Bma-pdi-2* genomic). The res-

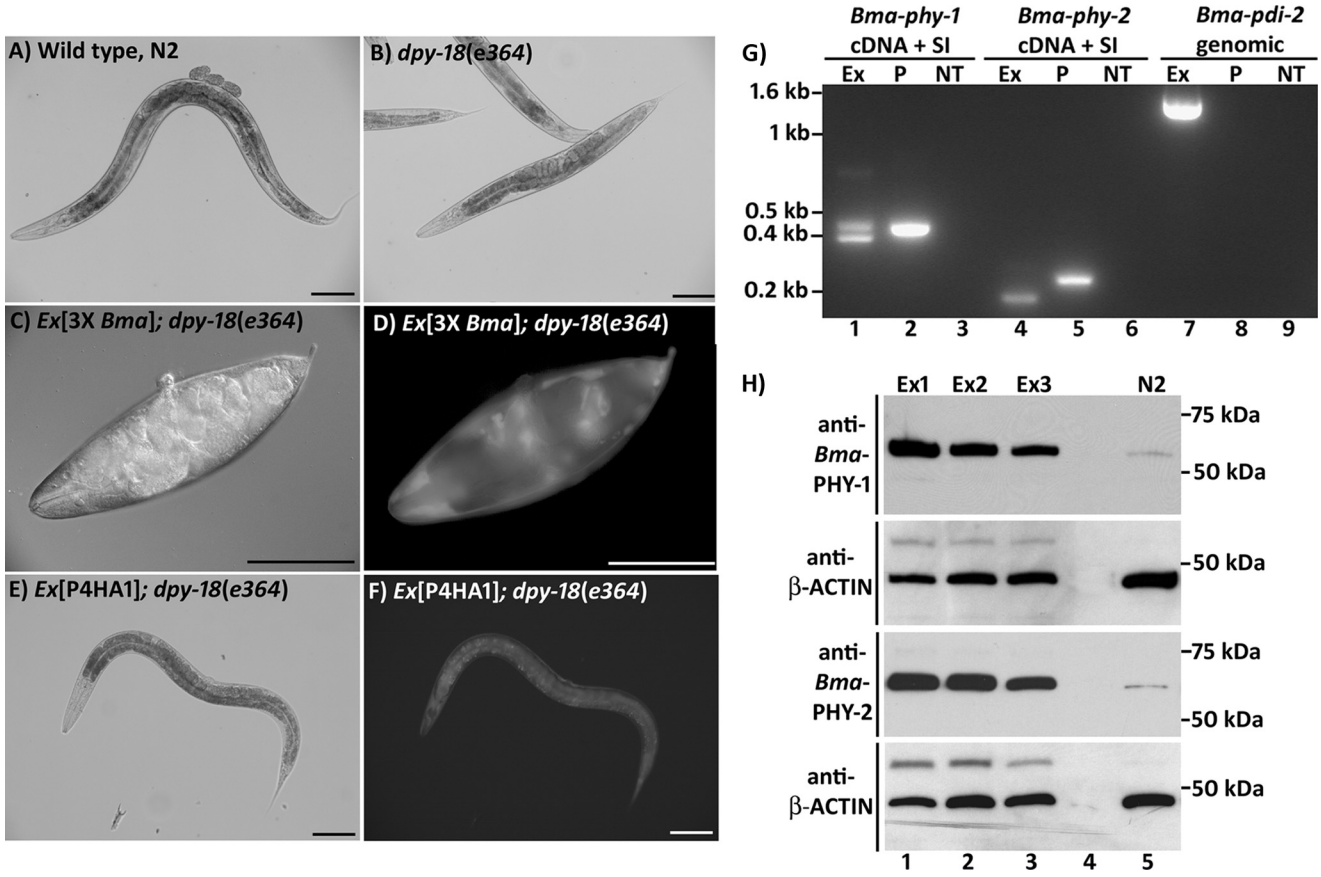

FIGURE 5. *A–F*, introduction of transgenes into a *C. elegans* C-P4H mutant strain, *dpy-18*. *A*, wild type *C. elegans* N2 strain; *B*, *C. elegans dpy-18(e364)* homozygote mutant; *C* and *D*, introduction of an extrachromosomal array carrying three *B. malayi* genes (*Bma-phy-1*, *Bma-phy-2*, and *Bma-pdi-2*) results in an exacerbation of the body morphology defects of the *dpy-18* mutant strain, with *D* showing GFP expression from the transformation marker *dpy-7prom::gfp* carried on the same extrachromosomal array. *E* and *F*, introduction of an extrachromosomal array carrying the human C-P4H α subunit gene P4HA1 partially repairs the body morphology defect of the *dpy-18* strain. *Scale bars* represent 100 μm. *G*, RT-PCR demonstrating expression of all transgenes from the extrachromosomal array carrying *Bma-phy-1*, *Bma-phy-2* and *Bma-pdi-2*. The results from one transgenic line are shown with other lines showing comparable expression levels. For the cDNA + synthetic intron (*SI*) construct, primers corresponding to the *B. malayi* gene sequences situated on either side of the 51-bp synthetic intron were used, whereas expression from the *Bma-pdi-2* genomic construct was detected using primers amplifying the full-length *Bma-pdi-2* sequence. *Lanes 2* and *5* show the unspliced amplicon size derived using the plasmid (*P*) carrying the cDNA + synthetic intron, in *lane 7* the 3.5-kb amplicon from the *Bma-pdi-2* genomic plasmid (*P*) is not amplified under the conditions used. *Lanes 3*, *6*, and *9* are the no template (*NT*) controls for each primer set. *Bma-phy-1* from construct with cDNA and a synthetic intron; spliced, 369 bp; unspliced, 420 bp. *Bma-phy-2* from construct with cDNA and a synthetic intron; spliced, 178 bp; unspliced, 229 bp. *Bma-pdi-2* from genomic construct; spliced, 1512 bp; unspliced, 3579 bp. *H*, Western blots (reducing SDS-PAGE) of transgenic *C. elegans* with complex extrachromosomal arrays carrying *Bma-phy-1*, *Bma-phy-2*, and *Bma-pdi-2*. Expression of *Bma*-PHY-1 and *Bma*-PHY-2 protein was demonstrated in three transgenic lines (*Ex1–Ex3*). Blots were stripped and reprobed for β-actin as a loading control. *Lane 4* is blank, and *lane 5* is wild type N2; the faint band produced with both *Bma*-PHY-1 and -2 antibodies is likely to be a cross-reaction with the *C. elegans* proteins. The sizes of the mature (minus signal peptide) proteins are as follows: *Bma*-PHY-1, 60 kDa; *Bma*-PHY-2, 62 kDa.

cue results above for *Bma-pdi-2* demonstrate the production of functional protein in transgenic *C. elegans* using this construct. Surprisingly, introduction of an array carrying the three *B. malayi* genes into the *Cel-dpy-18* mutant strain resulted in an exacerbation of the existing body morphology defect (Fig. 5, *A–D*) and could not be maintained in the homozygote mutant genetic background. Control experiments where complex arrays bearing only the transformation marker and fragmented genomic DNA were crossed into *Cel-dpy-18* strain did not result in an enhanced mutant phenotype. The presence of the three *B. malayi* constructs on the array was verified by SW-PCR (data not shown), RT-PCR indicated that all three transgenes were expressed (Fig. 5*G*), and production of *Bma*-PHY-1 and *Bma*-PHY-2 protein was demonstrated by Western blotting (Fig. 5*H*). Due to the toxicity of these arrays in the *Cel-dpy-18* mutant, RT-PCR and Western blotting had to be performed with the array in the wild type genetic background.

The possibility that failure to rescue the *C. elegans* C-P4H α subunit mutant using *B. malayi* proteins might be a result of evolutionary distance was next investigated by similarly analyzing the human C-P4H α subunits P4HA1 and P4HA2. Introduction of either of these genes into the *Cel-dpy-18* mutant using complex arrays resulted in partial rescue with a clear return to wild type body shape being evident (shown for P4HA1 in Fig. 5, *E* and *F*). The human proteins are therefore able to compensate to a large degree for loss of *Cel*-DPY-18.

Comparison of Parasite-derived and Insect Cell-expressed Protein Identified a Non-reducible Covalent Complex Association—Due to the difficulties encountered producing active *B. malayi* C-P4H both in insect cells and *C. elegans* systems, we next re-examined the insect cell expressed *B. malayi* proteins and compared these with parasite-derived material by reducing SDS-PAGE followed by Western blotting (Fig. 6). In addition to the monomer-sized band, a band running at the size predicted

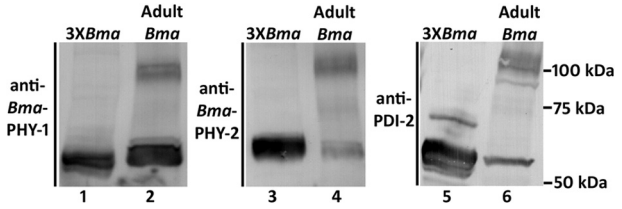

FIGURE 6. **Western blots (reducing SDS-PAGE) of insect cell extracts compared with *B. malayi* adult worms.** *Lanes 1*, *3*, and *5* are extracts from insect cells co-expressing *Bma*-PHY-1, *Bma*-PHY-2, and *Bma*-PDI-2 (*3XBma*). *Lanes 2*, *4*, and *6* are *B. malayi* extracts (*Adult Bma*). The antibodies used are indicated at the *side* of each blot. All three antibodies detect a band of the same size, running at approximately the size expected of a dimer (migrating at approximately 100 kDa), in the *B. malayi* worm extracts only. The sizes of the mature (minus signal peptide) proteins are as follows: *Bma*-PHY-1, 60 kDa; *Bma*-PHY-2, 62 kDa; *Bma*-PDI-2, 54 kDa.

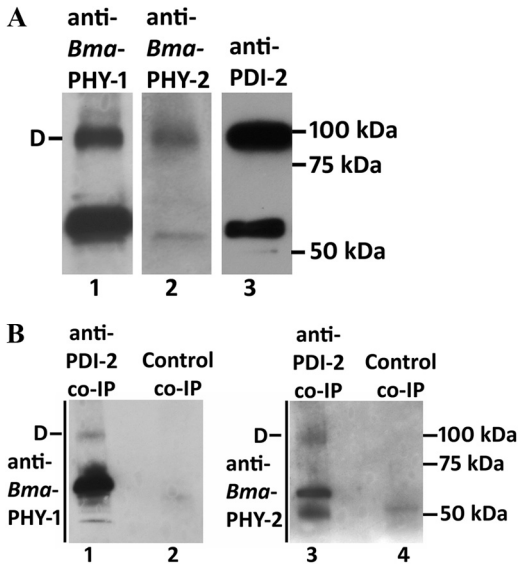

FIGURE 7. *A*, Western blots (reducing SDS-PAGE) of *B. malayi* adult material extracted in cell lysis buffer for co-IP, detected using the sensitive ECL plus system. The position of the dimer (*D*) is indicated. *B*, co-IP using an anti-PDI-2 antibody on native *B. malayi* extracts. Eluted complexes were separated by reducing SDS-PAGE and detected by the sensitive ECL Plus Western blotting system with anti-*Bma*-PHY-1 antibody (*lanes 1* and *2*), anti-*Bma*-PHY-2 antibody (*lanes 3* and *4*). The position of the dimer (*D*) in *lanes 1* and *3* is indicated. The results of a control co-IP performed using anti-*Cel*-BLI-5 antibody are shown in *lanes 2* and *4*.

for a dimer was found in the parasite material, whereas *B. malayi* proteins produced in insect cell displayed only the monomer size for all three proteins. The same sized band was observed using three different antibodies reactive to the three *B. malayi* proteins (Fig. 6) and was replicated in three independent extracts of parasite material (results not shown). We also note that this band was absent from extracts of *C. elegans* expressing the *B. malayi* proteins (result not shown). These findings suggested that a non-reducible post-translational modification was present in the parasite material. We suspected that such a linkage would be most likely to occur between *Bma*-PDI-2 and each *Bma*-PHY to produce linked dimers. This was further investigated by co-IP using affinity-purified anti-*Cel*-PDI-2 antibody to isolate proteins that interact with *Bma*-PDI-2 from adult parasite extract. Testing the parasite extract via sensitive Western blotting methods prior to co-IP confirmed the presence of dimer-sized bands and shows they are present at high abundance (Fig. 7*A*). Co-IP was carried out on these extracts and analyzed by reducing SDS-PAGE and sensitive Western blotting using antibodies against *Bma*-PHY-1 and -2. This showed both *Bma*-PHYs were co-immunoprecipiated using the anti-PDI-2 antibody, whereas control co-IPs using isotype-matched antibody, reactive to *C. elegans* BLI-5, did not result in co-IP of either *Bma*-PHY (Fig. 7*B*). As well as monomer forms, *Bma*-PHY-1 and *Bma*-PHY-2 are both detected as dimer-sized bands after reducing SDS-PAGE, confirming that *Bma*-PHY-1 and -2 are each individually linked to *Bma*-PDI-2 via a non-reducible covalent linkage.

DISCUSSION

The C-P4H enzymes play critical roles in matrix formation and development in metazoans (8). Our previous studies in *C. elegans* had shown that C-P4H function was vital for development and collagen biogenesis in this species (4–6), whereas an analogous function remained to be established in any parasitic nematode species. We therefore investigated the role of C-P4H in *B. malayi* and in this study demonstrate that these enzymes are developmentally essential in this important human parasite.

The size of the *phy* gene family in *B. malayi* was examined in detail revealing that *Bma*-PHY-1 (14) and *Bma*-PHY-2 are the only proteins with homology to C-P4H α subunits in this organism. The temporal expression profile of *Bma-phy-2* is more restricted than that of either *Bma-pdi-2* or the previously described *Bma-phy-1* (14). *Bma-phy-1* and *Bma-pdi-2* are both abundantly expressed in all stages examined, whereas *Bma-phy-2* is expressed only in the first larval stage (microfilaria) and the early and mid-L3 stages. The lower abundance of *Bma-phy-2* in microfilaria may reflect a more restricted cell/tissue expression pattern.

C-P4H Is Essential for Larval Development in B. malayi—RNAi approaches to examine gene function in parasitic nematodes of animals have, to date, met with very limited success (35). However, in this study, we employed a recently adapted RNAi approach (15) to examine the *B. malayi phy* and *pdi* genes in cultured parasites. Pronounced mutant body morphology phenotypes of stunted, lumpy immotile larvae, were found in first stage larvae when *Bma-phy-1* and *Bma-phy-2* were disrupted simultaneously, but not singly, or when *Bma-pdi-2* was targeted singly. The severity of the phenotype from simultaneous RNAi of *Bma-phy-1* and *-2* also supports the contention that other *Bma*-PHYs are unlikely to be present in this organism. These results echo those of *C. elegans* where single RNAi of either *Cel-dpy-18* or *Cel-phy-2* are viable but combined RNAi results in mutant larvae with body morphology defects and malformed cuticles (4). Interestingly, in *C. elegans*, single disruption of *Cel-pdi-2* results in phenotypes equivalent to the combined disruption of *Cel-dpy-18* with *Cel-phy-2* (4, 6). Likewise, in *B. malayi, Bma-pdi-2* RNAi produces a similar phenotype to that of combined *Bma-phy-1/Bma-phy-2* RNAi. In *C. elegans*, complete removal of both *Cel-dpy-18* and *Cel-phy-2* by genetic mutation is completely embryonic lethal (4, 6), suggesting that this could also be the case in *B. malayi*.

That *Bma-pdi-2* RNAi resulted in a phenotype suggests that the *Bma*-PHY-1 complex we described previously (14) is either

not sufficiently active *in vivo* for normal development or, as it does not possess a recognizable endoplasmic reticulum retention signal, becomes mislocalized. Our RNAi results in *B. malayi* represent one of a limited number of successful RNAi experiments reported in animal parasitic nematode species. In addition, the striking, highly penetrant morphological mutant phenotypes found have, to our knowledge, not been described previously in this species.

Functional Analysis of B. malayi Proteins in C. elegans—In parasitic nematodes such as *B. malayi*, experimental options using the parasite itself are limited, with no genetic approaches available and transgenesis only possible to a very limited extent (37). The function of the *B. malayi* proteins was therefore further investigated utilizing *C. elegans* mutants and transgenically introducing proteins to interrogate their functional roles. Surprisingly, despite confirmation of expression of multiple transgenic *B. malayi* genes in *C. elegans*, we were unable to rescue the phenotype associated with the *C. elegans* C-P4H α subunit mutant strain, *dpy-18(e364)*. This result is all the more intriguing as we were able to rescue the same mutant using the human enzymes. However, at the amino acid level, *Cel*-PHY-1 and -2 are more similar to *Bma*-PHY-1 and -2 than they are to the human C-P4H α subunits P4HA1 and P4HA2 (Table 1). It therefore seemed unlikely that evolutionary distance would explain the inability of the *B. malayi* proteins to rescue the *C. elegans* mutant. In addition, we found that the cuticle collagen gene families of *B. malayi* were very similar to those of *C. elegans* (see supplemental "Experimental Procedures"), making it unlikely that differences in the substrate might account for our results. In contrast, the natural collagen substrates of human P4HA1 and P4HA2 differ from *C. elegans* cuticle collagens in a number of respects; yet, as reasonable repair was found by expressing the human enzymes in *C. elegans*, they must be capable of correctly modifying worm collagen substrates. Importantly, we also find that the failure to rescue the *C. elegans dpy-18* mutant strain is not a reflection of the general lack of complementation using *B. malayi* genes because we conclusively demonstrated that *B. malayi pdi-2* can rescue the more extreme phenotypes associated with loss of *C. elegans pdi-2*. The highlighted differences in C-P4H complex formation between the host and parasite may represent novel drug targets in otherwise highly conserved enzymes.

Bma-PDI-2 Is a C-P4H β Subunit—Oligomeric C-P4H in every species examined contain only one type of PDI that is present in all forms of the complex. That we had correctly identified the C-P4H forming PDI in *B. malayi* and that this represented the only PDI with this function was suggested by sequence analysis, which showed that only *Bma*-PDI-2 had high homology to C-P4H forming PDIs (data not shown). That *Bma*-PDI-2 was the sole protein involved in C-P4H complex formation was also shown by the effect of single *Bma-pdi-2* RNAi being equivalent to combined loss of *Bma-phy-1* and *-2*, suggesting the PDI was present in complexes with both *Bma*-PHYs. This hypothesis is also supported by the fact that the PDI-2 antibody immunoprecitated complexes containing *Bma*-PHY-1 and *Bma*-PHY-2. In addition, transgenic rescue of a *C. elegans pdi-2* mutant with *Bma*-PDI-2 demonstrated this was the orthologue of the C-P4H forming PDI in *C. elegans*. All these lines of evidence indicate that *Bma*-PDI-2 is the C-P4H β subunit in *B. malayi*.

Dominant Negative Phenotype from Expression of B. malayi Proteins in dpy-18 Mutant—Interestingly, expression of the three *B. malayi* genes in the *C. elegans dpy-18* mutant strain caused a pronounced enhancement of the aberrant body morphology phenotype. However, in the wild type genetic background, the same transgenic arrays did not disrupt worm body morphology. In the wild type background, all C-P4H complexes are able to form. Where the three *B. malayi* genes were expressed in the wild type background, assuming *Cel*-PDI-2 is not preferentially incorporated into complexes, some of these will contain *Bma*-PDI-2 and others *Cel*-PDI-2. In the wild type background, where all forms of the C-P4H complexes can be produced, if any reduction to C-P4H activity resulted from *Bma*-PDI-2-containing complexes, it must not have been enough to affect worm morphology. That *Bma*-PDI-2-containing C-P4H complexes generate sufficient activity is supported by expression of *Bma*-PDI-2 in the *Cel*-PDI-2 mutant; here, full rescue was found when all C-P4H complexes would have contained *Bma*-PDI-2. However, in the *dpy-18* mutant strain, the only C-P4H complex present is the (*Cel*-PHY-2)(*Cel*-PDI-2) dimer (5). This dimer does not form naturally and is found only after loss of DPY-18, where its low C-P4H activity must be enough to permit survival (5). In the *dpy-18* mutant strain expressing the three *B. malayi* genes, *Bma*-PDI-2 most likely competes with *Cel*-PDI-2 for association with *Cel*-PHY-2 but forms a complex that is less active or inactive. As *Bma*-PHY-1 and *Bma*-PHY-2 containing complexes provide no or minimal C-P4H activity, due to lack of the required linkage, the net result is a reduction in the already low level of C-P4H activity, causing it to drop below the threshold required for viability.

Subunit Linkage via a Non-reducible Covalent Bond—The subunit associations and enzymatic activity of C-P4H complexes are usually assessed in an insect cell co-expression system, an approach that has been invaluable in defining these enzymes in organisms as diverse as *Drosophila melanogaster*, *C. elegans*, mouse, and human (8). The ability of the PHY and PDI proteins from *B. malayi* to form active C-P4H complexes was therefore also examined using this recombinant system. *Bma*-PHY-2 was soluble when expressed alone in the absence of a PDI and formed what is likely to be a homotetramer, a similar finding to that described previously for *Bma*-PHY-1 (14). However, unlike *Bma*-PHY-1, the *Bma*-PHY-2 homotetramer was not enzymatically active. In addition, *Bma*-PHY-2, again similar to *Bma*-PHY-1, failed to form an active complex with C-P4H β subunit PDIs from other species.[4] Surprisingly, however, co-expression of all combinations of *Bma*-PHY-1, *Bma*-PHY-2, and *Bma*-PDI-2, including all three together, also resulted in no enzyme activity, despite the formation of complexes analogous to those found in parasite-derived material. Potential explanations for our findings in insect cells, such as additional *Bma*-PHYs, with *Bma*-PDI-2 not being the correct and only C-P4H β subunit, or *B. malayi* enzymes evolving to process unusual collagen substrates, were ruled out by our systematic examination described above.

Previously described nematode C-P4H subunits combine in unique fashions (7), and our results in *B. malayi* indicate a fur-

ther level of complexity in this species. Our co-IP analysis showed that *in vivo Bma*-PHY-1 and -2 are each found in two types of complex with *Bma*-PDI-2. In the first type, each *Bma*-PHY protein is linked to *Bma*-PDI-2 via a non-reducible covalent linkage, and in the second, each *Bma*-PHY is associated with *Bma*-PDI-2 but not linked by such a bond (see supplemental Fig. S6). We suspect that *Bma*-PDI-2 epitope may be partially masked in the covalently linked complex, resulting in this binding less efficiently during co-IP than the non-linked form. We therefore predict based on our combined results that in *B. malayi* C-P4H activity is generated by the covalent linked form of the complex. This novel modification has not been found in C-P4H identified from any other species to date. The enzyme generating this modification is currently unknown but could potentially be identified by RNAi screening of candidate enzymes to identify a similar phenotype to that produced by knockdown of *B. malayi* PHY-1/-2 and PDI-2.

Acknowledgments—Some nematode strains used in this work were provided by the Caenorhabditis Genetics Center, which is funded by the National Institutes of Health National Center for Research Resources. We thank the following people for reagents and materials: Rick Maizels and Yvonne Harcus (University of Edinburgh) for B. malayi nematodes and staged cDNA, Steven Williams (NIAID/National Institutes of Health FR3, Smith College) for EST clones, Mark Blaxter (University of Edinburgh) for BAC library lysates and clones, and Elodie Ghedin (University of Pittsburgh) for access to the resequenced B. malayi genome data.

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