A Hypodermally Expressed Prolyl 4-Hydroxylase from the Filarial Nematode

Brugia malayi is Soluble and Active in the Absence of Protein Disulfide Isomerase

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Running title: Prolyl 4-Hydroxylase in the Nematode Brugia malayi
SUMMARY

The collagen prolyl 4-hydroxylase (P4H) class of enzymes catalyze the hydroxylation of prolines in the X-Pro-Gly repeats of collagen chains. This modification is central to the synthesis of all collagens. Most P4Hs are $\alpha_2\beta_2$ tetramers with the catalytic activity residing in the $\alpha$ subunits. The $\beta$ subunits are identical to the enzyme protein disulfide isomerase (PDI). The nematode cuticle is a collagenous extracellular matrix required for maintenance of the worm body shape. Examination of the model nematode Caenorhabditis elegans has demonstrated that its unique P4Hs are essential for viability and body morphology. The filarial parasite Brugia malayi is a causative agent of lymphatic filariasis in humans. We report here on the cloning and characterization of a B. malayi P4H with unusual properties. The recombinant B. malayi $\alpha$ subunit, PHY-1, is a soluble and active P4H by itself, and it does not become associated with PDI. The active enzyme form is a homotetramer with catalytic and inhibition properties similar to those of the C. elegans P4Hs. High levels of B. malayi phy-1 transcript expression were observed in all developmental stages examined, and its expression localized to the cuticle synthesizing hypodermal tissue in the heterologous host C. elegans. Although active by itself, the B. malayi PHY-1 was not able to replace enzyme function in a C. elegans P4H mutant.
INTRODUCTION

Biosynthesis of vertebrate collagens requires processing by up to eight specific intra- and extracellular posttranslational enzymes (1). The collagen prolyl 4-hydroxylase (P4H) class of enzymes (EC 1.14.11.2) catalyze the hydroxylation of prolines in the X-Pro-Gly repeats of collagen chains. This endoplasmic reticulum (ER) resident enzyme is central to collagen synthesis, as collagen triple helices are thermally unstable in the absence of 4-hydroxyproline residues (2,3). P4H also acts as a chaperone in the assembly of collagen ensuring that only correctly folded collagens are released for secretion (4). In vertebrates and Drosophila melanogaster the enzyme is an $\alpha_2\beta_2$ tetramer (2,3,5,6), with hydroxylation activity residing in the catalytic $\alpha$ subunits. Two $\alpha$ subunit isoforms, $\alpha$(I) and $\alpha$(II), have been characterized in vertebrates (7,8). They become assembled into $[\alpha(I)]_2\beta_2$ and $[\alpha(II)]_2\beta_2$ tetramers, with insect cell coexpression data arguing strongly against the formation of mixed $\alpha$(I)$\alpha$(II)$\beta_2$ tetramers (8). The $\beta$ subunits of P4Hs are identical to the enzyme and chaperone protein disulfide isomerase (PDI) (EC 5.3.4.1) (9) and are required to maintain the $\alpha$ subunits in a catalytically active nonaggregated conformation (10,11). The P4H is also maintained within its correct subcellular compartment by virtue of an ER retention signal at the C-terminus of PDI (11). When expressed alone in a recombinant expression system the $\alpha$ subunits are insoluble and inactive, whereas coexpression with PDI results in the formation of an active, soluble P4H (7,12-14). The PDI s from different organisms can often substitute for the authentic partner, such as the human PDI that can function as a $\beta$ subunit in the mouse and Drosophila P4H tetramers and in a P4H dimer with Caenorhabditis elegans PHY-1 (5,7,13).

In the model nematode C. elegans the cuticular collagen modifying function of P4H is essential for body morphology and viability (15,16). In nematodes the exoskeleton (known as the cuticle) is an extracellular matrix (ECM) composed of small collagen-like molecules (17). The nematode cuticle is synthesized by the underlying hypodermal tissue and performs multiple functions including maintenance of worm body shape. Mutations in collagens forming the cuticle, and in the enzymes involved in collagen biosynthesis, can result in lethality and severe alterations to body shape as illustrated by the C. elegans sqt-3 (18) and bli-4 (19) mutant phenotypes.

The P4Hs in C. elegans that are involved in the synthesis of cuticle collagens are formed from the $\alpha$ subunits PHY-1 and PHY-2, and the $\beta$ subunit PDI-2 (14,15). The expression, function and assembly of...
these subunits have been examined in detail showing that unique P4H forms exist in *C. elegans*. The most abundant form is a tetramer, this however differs from others described, in being a mixed PHY-1/PHY-2/(PDI-2) tetramer (14). PHY-1 and PHY-2 can also each individually associate with PDI-2 to form dimers (14). Such P4H forms have not been described for any other species to date. Genetic disruption of *phy-1* and *phy-2* simultaneously, or *pdi-2* singly, results in embryonic lethality where embryos develop normally until the first cuticle is required to maintain the elongated worm shape (15,16). The weakened cuticle is then unable to maintain this form after which embryos collapse to a disorganized state and eventually die. The body shape defect and reduced 4-hydroxyproline levels in the cuticle collagens of the viable *phy-1* null genetic mutant, *dpy-18*, underline the importance of collagen modification by P4H for nematode body morphology (15,16).

Applying the knowledge of P4H function in *C. elegans* we examined a P4H in the filarial parasitic nematode *Brugia malayi*. *B. malayi* along with *B. timori* and *Wuchereria bancrofti* are the causative agents of lymphatic filariasis in humans, with over 120 million people infected and over 1 billion people at risk of infection worldwide (20). Lymphatic filariasis is a debilitating disease, with approximately a third of those infected being incapacitated and/or disfigured by the infection (21). Commercially available inhibitors of P4H have been shown to be toxic to *B. malayi* adults, producing associated cuticular defects (22). These observations and the requirement in *C. elegans* for P4H activity, highlight this enzyme class as a potential drug target in the control of human and veterinary parasitic nematode infections.

In this paper we describe the identification of a P4H *phy-1* gene from *B. malayi*, characterize the molecular and enzymatic properties of the recombinant *B. malayi* P4H produced in an insect cell expression system, and examine the expression profile and putative function of the *B. malayi phy-1* gene by heterologous expression in the model nematode *C. elegans*. Unusually, the *B. malayi* PHY-1 is a soluble and active P4H when expressed alone in a recombinant system in the absence of PDI, and it does not associate with PDIs from other organisms, including *C. elegans*. The developmental temporal expression pattern of the *B. malayi phy-1* gene was analyzed by RT-PCR using stage specific mRNA samples. Reporter gene experiments showed that the *B. malayi phy-1* promoter directs tissue-specific spatial expression to the hypodermal cells of *C. elegans*. 
MATERIALS AND METHODS

Nematode Strains and Culture Conditions - *C. elegans* strains were cultured as described elsewhere (23). The wild type Bristol N2, CB364[dpy-18(e364)] and DR96[unc-76(e911)] *C. elegans* strains were provided by the Caenorhabditis Genetics Centre. The *B. malayi* nematodes were provided by Rick Maizels (University Edinburgh).

Isolation of cDNA and Genomic Clones - ESTs SW3D9CA480SK, MBAFCX8G05T3 and MBAFCZ7H09T3 (Fig. 1A) were received from the Filarial Genome Project (FGP), subcloned and sequenced. The primers X8G5F1, 5′-CAGTCGCTCAACACCGG-3′, and BMNPHYR, 5′-CCAATAGTATTTAAGCAC-3′, were designed from the EST sequences and used to obtain a 312-bp PCR product (Fig. 1A) from *B. malayi* adult-stage cDNA that was prepared as described previously (15). The purified PCR product was labeled with α-32P dCTP and used to screen a *B. malayi* adult male cDNA library SAW94NLbAm (from Steven Williams, Filarial Genome Project, Northampton, MA). Eleven positive clones all representing the same cDNA, named *B. malayi* phy-1, were identified from a total of 2 x 10⁴ recombinants. Amplification of the 5′ end of the *B. malayi* phy-1 cDNA was performed using the Gibco BRL Life Technologies 5′ RACE System. Two 418-bp products from independent 5′ RACE PCR reactions (Fig. 1A) were sequenced, to generate a consensus 5′ sequence. The primers BMPHY-1RESF(BamHI), and BMPHY-1RESR(NolI), (for primer sequences see later) were used to generate the *B. malayi* phy-1 genomic sequence from the translation start codon ATG to the stop codon TAA using *B. malayi* genomic DNA as a template. Three identical full-length clones were then fully sequenced to generate a consensus genomic sequence. Computer prediction for analysis of conceptual translation of *B. malayi* phy-1, protein alignments, signal peptide analysis and predicted posttranslational modifications were performed on the ExPASy proteomics tools database (http://www.expasy.ch).

Generation of a Recombinant Baculovirus coding for *B. malayi* PHY-1, and Expression and Analysis of Recombinant Proteins in Insect Cells – The full-length coding sequence of *B. malayi* phy-1 cDNA was cloned from *B. malayi* adult stage cDNA by PCR using *Pfu* polymerase (Stratagene) with the primers BMPHY-1BVF(NolI), 5′-gaacagccgccATGATAGCTACCGTGCTGTTCT-3′, and BMPHY-1BVR(XbaI), 5′-gtctagaTTAAGCACCCTAGATCGCCCAC-3′ (artificial restriction sites in lower case and underlined). The PCR product was cloned into a NolI-XbaI-digested transfer vector pVL1392 (Pharmingen).
and sequenced. The recombinant vector was cotransfected into *Spodoptera frugiperda* Sf9 cells with a modified *Autographa californica* nuclear polyhedrosis virus DNA (BaculoGold, Pharmingen) by calcium-phosphate precipitation.

Sf9 or High Five (Invitrogen) insect cells were cultured as monolayers in TNM-FH medium (Sigma) supplemented with 10% fetal bovine serum (BioClear) or in suspension in Sf900ISFM serum-free medium (Invitrogen). The cells were seeded at a density of 5 x 10^6 cells/100-mm plate or 1 x 10^6 cells/ml and infected at a multiplicity of five with the virus coding for the *B. malayi* PHY-1 alone or together with viruses coding for *C. elegans* PDI-1, PDI-2, or human PDI. In control experiments, the cells were coinfectected with the viruses coding for *C. elegans* PHY-1, PHY-2 and PDI-2, with PHY-1 and human PDI or with the various PDI viruses alone. The cells were harvested 72 h after infection, washed with a solution of 0.15 M NaCl and 0.02 M phosphate, pH 7.4, homogenized in a 0.1 M NaCl, 0.1 M glycine, 10 µM dithiothreitol (DTT), 0.1% Triton X-100, and 0.01 M Tris buffer, pH 7.4, and centrifuged at 10,000 x g for 20 min. The pellets were further solubilized in 1% SDS. Aliquots of the samples were analyzed by 8% SDS-PAGE under reducing conditions and by nonenaturing PAGE followed by Western blotting with polyclonal antibodies against *B. malayi* PHY-1 (see below), *C. elegans* PDI-1 or PDI-2 (14) or a monoclonal antibody against human PDI (5B5, DAKO). P4H activity was assayed by a method based on the hydroxylation-coupled decarboxylation of 2-oxo-[1-14C]glutarate, and K_m and K_i values were determined as described previously (24). The molecular weight of the recombinant *B. malayi* P4H was analyzed by applying the Triton X-100-soluble fraction of insect cells expressing *B. malayi* PHY-1 to a calibrated HiPrep Sephacryl S-200 HR gel filtration column (Amersham Biosciences), equilibrated and eluted with 0.1 M NaCl, 0.1 M glycine, 10 µM DTT, and 0.01 M Tris buffer, pH 7.4, and P4H activity in the eluted fractions was assayed. Purified recombinant human type I P4H (12) was used as a control in the gel filtration experiments.

**Protein Analysis of B. malayi Extracts** - Extracts from *B. malayi* were made by disrupting 100 adult females with a hand-held glass homogenizer in the following buffer; 0.1 M NaCl, 0.1 M glycine, 10 µM DTT, 0.1% Triton -X100, and 10 mM Tris buffer, pH 8.0, supplemented with the protease inhibitors; 1 mM PMSF, 1 mM EDTA, 1mM EGTA, 2 µM E64 and 0.1 µM pepstatin. The soluble extracts were analyzed by reducing SDS-PAGE and nondenaturing PAGE in 4-12% NuPAGE Bis-Tris polyacrylamide
gels and 4-12% Tris-glycine gels (Invitrogen), respectively, followed by Western blotting. N-glycosidaseF (PNGaseF, New England Biolabs) treatment was performed according to the manufacturers recommendations.

Polyclonal antiserum was raised in two rabbits against a synthetic peptide corresponding to the C-terminal region of \textit{B. malayi} PHY-1. The peptide CRRPCGLSRSVEEQFVGDLSA was conjugated to keyhole limpet heamocyanin (Sigma Genosys) via an added cysteine residue (underlined) and used for immunization.

\textit{dpy-18} Rescue Experiments with \textit{B. malayi} phy-1 - A 2.8-kb PstI-BamHI \textit{C. elegans} phy-1 promoter fragment from the pPD95-03-phy-1 construct (15) was cloned into pBlueScript SKM (Stratagene). The \textit{C. elegans} phy-1 3' UTR sequence was generated by PCR from \textit{C. elegans} N2 genomic DNA using the primers CEPHY-1 3'UTRF(Sacl), 5'-gcggagctcCTCTAAGCATTGGTTTTCATTG-3', and CEPHY-13'UTRR(Sacl), 5'-gcggagctcACTAGGGAATTGTCGGCTGC-3' with Vent polymerase (NEB), and cloned into the pBlueScript-Cephy-1promoter construct to generate the plasmid pAW1 (Fig. 8B).

The coding sequence of \textit{B. malayi} phy-1 cDNA and the genomic sequence of \textit{B. malayi} phy-1 from the translation initiation codon to the stop codon were generated by PCR using the primers BMPHY-1RESF(BamHI), 5'-gagcggccgcGATGATAGCTACCGTGGGTTC-3', and BMPHY-1RESR(NotI), 5'-gagcggccgcTTAAGCACTTAGATCGCCCAC-3', with Pfu and Pfu Turbo (Stratagene) polymerases, respectively. The PCR products were cloned into BamHI-NotI-digested vector pAW1 to generate \textit{B. malayi} phy-1 cDNA and genomic rescue constructs (Fig. 8B). A synthetic intron (5'-GTAAGTTTAAACTATTCGTTACTAACTTTAAACATTTAAATTTTCAG -3') was inserted into the \textit{B. malayi} phy-1 cDNA rescue construct by ligating a double stranded oligo into a Stul blunt-ended restriction site.

The \textit{B. malayi} phy-1 cDNA (± a synthetic intron) and genomic rescue constructs were microinjected into the syncytial gonad of \textit{C. elegans} phy-1 null, \textit{dpy-18} (c364), nematodes at concentrations of 10 µg/ml and 100 µg/ml. A marker plasmid with a \textit{dpy}-7 cuticle collagen promoter in the green fluorescent protein (GFP) fusion vector pPD95-67 (from Iain Johnstone, University of Glasgow) was co-injected at 5 µg/ml, and the injection mixes were made up to a final concentration of 150 µg/ml with
Transformants were selected by GFP fluorescence, and more than five semi-stable transmitting lines were examined for each concentration.

**Developmental Timecourse RT-PCR** - PCR was performed on cDNA samples generated from daily extracts of *B. malayi* infected jirds, up to day 14 post infection, after which extracts from 2-4 day intervals were taken (25). Two sets of primers were used for each PCR, BMPHY1.1S1F, 5'-GCTTCTGGTGTTCAACCG-3', and BMPHY1.1S2R, 5'-GGTATGATGCTGTGTGTTCAAG-3', corresponding to the *B. malayi* phy-1, and BMTUBA, 5'-AATATGTGCCACGAGCAGTC-3', and BMTUBB, 5'-CGGATACTCCTCAGAATTT-3', corresponding to the control *B. malayi* tubulin gene.

**Cloning of the B. malayi phy-1 Promoter** - To identify the putative promoter region, a genomic *B. malayi* BAC library was screened with a 1.7-kb biotin labelled probe generated by PCR, using the primers T7PL, 5'-CTCACTATAGGGCGAATTGG-3' (biotin labelled, New England Biolabs), and BMPHY1.1S3R(B), 5'-GCGTGGATGATTGGATC-3', and a plasmid containing a T7 site and the 5' genomic coding sequence from *B. malayi* phy-1 as a template. The gridded BAC filters were hybridized using NEBlot Phototope and Phototope-star (New England Biolabs) detection kits. BLASTX analysis was performed on the ExPASy proteomics tools database.

**Construction of a B. malayi phy-1 Promoter-Reporter Plasmid** - A 2.2-kb putative promoter fragment of the *B. malayi* phy-1 gene was amplified from *B. malayi* genomic DNA using *Pfu* polymerase and the primers BMPHY-1PF(SphI), 5'-ggcgcatgcGAATGAGACAATTGCACAAG-3', and BMPHY-1PR(BamHI), 5'-ggcggatccGCTATCATCACTGGCTCTGGA-3'. This fragment, extending from –2189 to +8 relative to the translation start site, was cloned into SphI-BamHI-digested *C. elegans* reporter gene vector pPD96-04. This was microinjected into the syncytial gonad of the *C. elegans* strain DR96(unc-76) together with the unc-76 rescue plasmid (p7616B); both at 100 µg/ml. Six semi-stable transgenic lines were identified and examined for reporter gene expression by viewing GFP expression in live worms and by sensitive staining of fixed worms for β-galactosidase activity (26). Live nematodes were transferred to 2% agarose/0.065% sodium azide pads, and images were taken with an Axioskop 2 microscope using a Hamamatsu digital camera and Improvisation Openlab processing software.
RESULTS

Cloning of *B. malayi* phy-1 - Three ESTs from the *B. malayi* database were identified that encode amino acid sequences homologous to *C. elegans* PHY-1 and PHY-2. MBAFCX8G05T3 (AA509222) and MBAFCZ7H09T3 (AA406985) were both derived from a *B. malayi* adult female cDNA library, and SW3D9CA480SK (AA585698) from a *B. malayi* L3 cDNA library. Sequencing of the ESTs established that they were all derived from a single gene, termed *B. malayi* phy-1 (Fig. 1A). A 312-bp PCR probe was generated from *B. malayi* adult stage cDNA based on the EST sequences, and used to screen a *B. malayi* adult male cDNA library (Fig. 1A). Eleven positive clones were identified, full-length sequencing was performed on two 1.6-kb clones, and both were found to represent the *B. malayi* phy-1 cDNA. The nine other identified clones contained inserts with sizes ranging from 0.6 kb to 1.6 kb, and all corresponded to the *B. malayi* phy-1 gene. The 1669-bp sequence generated from the library screening contained a 1515-bp open reading frame and 154-bp of 3' UTR sequence (Fig. 1A). The 3' untranslated region did not contain a consensus polyadenylation signal (AATAAA). A divergent poly(A) signal sequence (GATAAA) was however located 11-bp upstream of the poly(A) tail, representing a variant poly(A) signal sequence also found in approximately 5% of *C. elegans* genes examined (27).

Comparison of amino acid sequence encoded by the 1515-bp *B. malayi* phy-1 cDNA sequence with known P4H α subunits signified that the 5' coding sequence was incomplete. Additionally, according to the signal sequence prediction program SignalP, the N terminus did not contain a characteristic signal peptide. The 5' UTR, the transsplice leader sequence and the coding sequence for 37 additional N-terminal amino acids was obtained using the 5' RACE system for rapid amplification of cDNA ends (Fig. 1A). This data was assembled with the 1669-bp *B. malayi* phy-1 sequence obtained from the cDNA libraries to give the full-length cDNA sequence, which was confirmed by the sequencing of a full-length *Pfu*-generated PCR product (Fig. 1A). The complete *B. malayi* phy-1 cDNA sequence contains a consensus 22-bp SL1 trans-splice leader sequence, an 8-bp 5' UTR, a single open-reading frame of 1626-bp that encodes a 541 amino acid polypeptide (AJ297845), and a 154-bp 3' UTR (Fig. 1A).

The 4596-bp consensus genomic sequence of the *B. malayi* phy-1 was determined from three individual full-length PCR products, and contains 12 exons and 11 introns (Fig. 1B) (AJ421993). The intron sizes range from 119-bp to 479-bp and have an average size of 270-bp.
Comparison of the B. malayi PHY-1 Amino Acid Sequence with Those of Other P4H α Subunits -

A signal peptide cleavage site between Ala17 and Asp18 was predicted by the SignalP program, and thus the processed B. malayi PHY-1 consists of 524 amino acids. Highest amino acid sequence homology was found between the processed B. malayi PHY-1 and C. elegans PHY-1 sequences (13), the identity being 59% and similarity 76%, while the identity and similarity between the B. malayi PHY-1 and C. elegans PHY-2 (15,16) are 53% and 71%, respectively (Fig. 2). The amino acid sequence homology between the B. malayi and C. elegans PHY polypeptides is slightly higher than that between the B. malayi PHY-1 and the PHY-1 from a closely related filarial nematode Onchocerca volvulus (22), the B. malayi and O. volvulus PHY-1 polypeptides being 49% identical and 70% similar. The amino acid sequence identities between the B. malayi PHY-1 and the human α(I) and α(II) subunits are 45% and 44%, and similarities are 62% and 63%, respectively. The cysteine residues essential for intrachain disulfide bonding (28,29) and the active site histidine, aspartic acid and lysine residues (29,30) are all conserved in B. malayi PHY-1 (Fig. 2). The extended C-terminal regions present in the C. elegans and O. volvulus PHY-1 polypeptides are not found in B. malayi PHY-1 (Fig. 2).

B. malayi PHY-1 is a Soluble and Active P4H when Expressed in the Absence of PDI in a Baculovirus System - The B. malayi phy-1 cDNA was cloned into the baculovirus expression vector pVL1392, a recombinant baculovirus was generated and used to infect insect cells. The cells were harvested 72 h after infection, homogenized in a 0.1% Triton X-100 containing buffer, and centrifuged. The remaining pellet was solubilized in 1% SDS, and the samples were analyzed by reducing SDS-PAGE followed by Western blotting (Fig. 3A). The C. elegans PHY polypeptides (13,14) and vertebrate P4H α subunits (7,12) require association with PDI to form soluble and active P4Hs. When expressed alone in a recombinant system, these polypeptides form inactive aggregates and 1% SDS is required for their efficient solubilization (7,12-14). In contrast to these P4H α subunits, Western blotting showed that the majority of the recombinant B. malayi PHY-1 was soluble in the Triton X-100 containing buffer (Fig. 3A, lane 1), and only a minor amount formed insoluble aggregates that required 1% SDS for solubilization (Fig. 3A, lane 2). The Triton X-100 extracts from insect cells expressing the B. malayi PHY-1 were analyzed for P4H activity with an assay based on the hydroxylation-coupled decarboxylation of 2-oxo-[1-14C]glutarate (24), and a significant amount of P4H activity was observed (Table I). The B. malayi PHY-1 thus differs from the C.
elegans PHY polypeptides and the vertebrate P4H α subunits, which require association with PDI to form soluble and active P4Hs (7,12-14).

To study whether *B. malayi* PHY-1 has the potential to associate with PDI, the recombinant protein was expressed in insect cells either alone, with the *C. elegans* β subunit PDI-2 (31), with the *C. elegans* PDI-isof orm PDI-1 (31), or with the human PDI (12). In control experiments the insect cells were infected with viruses coding for the various PDIs alone, or coinfect ed with viruses coding for the *C. elegans* PHY-1/PHY-2/(PDI-2) tetramer or the hybrid *C. elegans* PHY-1/human PDI dimer. Triton X-100-soluble extracts of the cell lysates were analyzed by nondenaturing PAGE followed by Coomassie Blue staining (Fig. 3B). In the control experiments, bands corresponding to the *C. elegans* PHY-1/PHY-2/(PDI-2) tetramer (Fig. 3B, lane 5) and the *C. elegans* PHY-1/human PDI dimer (Fig. 3B, lane 6) were detected. In contrast, neither a tetramer nor a dimer were detected by Coomassie Blue staining in the extracts from cells expressing the *B. malayi* PHY-1 alone or in combination with different PDIs (Fig. 3B, lanes 1-4).

However, three *B. malayi* PHY-1 immunoreactive bands were detected by nondenaturing Western analysis in extracts from cells expressing *B. malayi* PHY-1 alone (Fig. 4A, lane 1). Three bands with approximately the same mobilities were also detected in the extracts from cells coexpressing the *B. malayi* PHY-1 and the different PDIs (Fig. 4A, lanes 2-4). The mobility of the *B. malayi* PHY-1 upper band was similar to those of the *C. elegans* PHY-1/PHY-2/(PDI-2) tetramer (Fig. 4A, lane 8) and the human P4H tetramer (data not shown), and the mobility of the middle band was likewise similar to that of the PHY-1/PDI-2 dimer (Fig. 4A, lane 8). No *B. malayi* PHY-1 immunoreactive bands were detected in the extracts from cells expressing the different PDIs alone (Fig. 4A, lanes 5-7). Coexpression of the recombinant *B. malayi* PHY-1 with the different PDIs did not significantly increase the amount of P4H activity obtained (Table I), and Western blotting of the nondenaturing PAGE with antibodies against the different PDIs showed that the *B. malayi* PHY-1 does not associate with either the human PDI (Fig. 4B), *C. elegans* PDI-1 (Fig. 4C) or *C. elegans* PDI-2 (Fig. 4D). This is in contrast to the vertebrate, *C. elegans* and *Drosophila* α subunits that can each associate with orthologous P4H β subunits (5,7,13).

Gel filtration experiments in a calibrated HiPrep Sephacryl S-200HR column of the Triton X-100 extracts from cells expressing the recombinant *B. malayi* PHY-1 alone showed that P4H activity was eluted in fractions that corresponded to a molecular weight of approximately 350,000 (Fig. 5A). Control
experiments with purified recombinant human type I P4H tetramer showed that it eluted in exactly the
same position as the recombinant *B. malayi* P4H (Fig. 5A). Previous gel filtration studies have also shown
that the elution position of a purified chick embryo P4H tetramer corresponds to a molecular weight of
350,000 (32). The calculated molecular weights of a *B. malayi* PHY-1 tetramer and the human type I P4H
tetramer are 241,468 and 228,808, respectively. The fractions containing *B. malayi* P4H activity were
pooled and analyzed by reducing SDS-PAGE and non-denaturing PAGE followed by Western blotting (Fig.
5B and C). Two *B. malayi* PHY-1 immunoreactive bands corresponding to the nonglycosylated and
glycosylated forms (see Fig. 7) were detected in SDS-PAGE (Fig. 5B), and two bands with mobilities
similar to the human or *C. elegans* P4H tetramers and a *C. elegans* P4H dimer, respectively, were detected
in the non-denaturing PAGE (Fig. 5C). No detectable *B. malayi* P4H activity was eluted from the gel
filtration column in a position that corresponds to that of the *C. elegans* P4H dimer (Fig. 5A) (13), and
therefore our results indicate that the *B. malayi* PHY-1 self-associates into active P4H tetramers (Figs. 4A
and 5C), but during the non-denaturing PAGE partial dissociation of the *B. malayi* P4H tetramers into
dimers (Figs. 4A and 5C) and monomers (Fig. 4A) occurs.

The $K_m$ values for the cosubstrates 2-oxoglutarate, Fe$^{2+}$ and ascorbate of the *B. malayi* PHY-1
(Table II) were very similar to those of the *C. elegans* PHY-1/PHY-2/(PDI-2)$_2$ (14) and other P4Hs (13,30).
The $K_m$ value for the substrate (Pro-Pro-Gly)$_{10}$ of the *B. malayi* PHY-1 (Table II) was slightly lower than
those reported for other P4Hs (13,14,30). The *B. malayi* PHY-1 was not efficiently inhibited by poly(L-
proline) (data not shown), and it thus resembles the *C. elegans* P4Hs (13,14) and the vertebrate type II
P4Hs (7,8). The $K_i$ values of the *B. malayi* PHY-1 for the 2-oxoglutarate analogues pyridine-2,4-
dicarboxylate and pyridine-2,5-dicarboxylate (Table II) were approximately 2-fold lower than those
reported for *C. elegans* (13,14) and human P4Hs (3,30).

**Analysis of Tissue-Extracted *B. malayi* PHY-1 and Native Glycosylation** – Three immunoreactive
recombinant *B. malayi* PHY-1 bands were observed when Triton X-100 soluble extracts from insect cells
were analyzed by non-denaturing PAGE and Western blotting (Fig. 4A, lane 1). Native extracts were
prepared from the nematodes and were likewise analyzed to determine if similar PHY-1 immunoreactive
bands are found in *B. malayi in vivo*. Two major immunoreactive bands were observed in freshly-prepared
parasite extracts (Fig. 6A, lane 1), that have a similar native gel migration pattern to the characterized *C.
The Proscan programme (ExPASy) predicted that *B. malayi* PHY-1 has two N-linked glycosylation sites at positions 49-52 (NRSL) and 140-143 (NASG) (amino acid positions given relate to the mature processed protein) (Fig. 2). The extracts from *B. malayi* nematodes and from insect cells expressing recombinant *B. malayi* PHY-1 were treated with N-glycosidase F and analyzed by reducing SDS-PAGE followed by Western blotting (Fig. 6B) to compare the glycosylation of native and recombinant *B. malayi* PHY-1 polypeptides. Following glycosidase treatment of insect cell and worm extracts (Fig. 6B, lanes 1 and 3), a single band of approximately 60 kDa was observed in both samples, the size of the immunoreactive band being consistent with the predicted size of *B. malayi* PHY-1. In addition to the 60 kDa band, an additional band migrating slightly higher was detected in the untreated insect cell extract (Fig. 6B, lane 2). The 60 kDa band was not detected in the untreated worm extract (Fig 6B, lane 4), but instead two bands with higher mobilities were observed, representing *B. malayi* PHY-1 polypeptides in which one or both glycosylation sites have been modified (Fig. 6B, lane 4). Thus, approximately half of the recombinant *B. malayi* PHY-1 polypeptides expressed in insect cells remain unglycosylated, while in the remaining polypeptides, only one of the glycosylation sites is modified. In *B. malayi* in vivo no unglycosylated forms are present, and a minority of PHY-1 polypeptides are glycosylated at both sites.

**Examination of the Functional Conservation Between the *B. malayi* and *C. elegans* PHY-1 Polypeptides** - Phenotypic rescue of the *C. elegans* phy-1 null mutant [*dpy-18(e364)*] was attempted in order to assess interspecies conservation of the *phy-1* gene function. Attempts were made to rescue the *C. elegans* mutant strain CB364[*dpy18(e364)*] using the *B. malayi* phy-1 coding sequence expressed under the control of the *C. elegans* phy-1 promoter (Fig. 7). The *B. malayi* PHY-1 should therefore be expressed in the relevant tissues with appropriate developmental timing and at comparable levels to that of the *C. elegans* PHY-1. A vector was constructed which contained the previously defined *C. elegans* phy-1 promoter (15), and a splice site containing 3’ UTR with the polyadenylation signal and poly-A transfer sequences (13) (vector pAW1) (Fig. 7B). The *B. malayi* phy-1 coding sequence, with and without introns, was inserted between the two *C. elegans* sequences (Fig. 7B), transformed into the CB364 strain, and the
ability to rescue the medium dumpy phenotype in the transformed animals carrying *B. malayi* PHY-1-encoding transgene was analyzed.

Multiple semi-stable lines of nematodes transformed with 10 µg/ml and 100 µg/ml of the rescue construct containing the *B. malayi* phy-1 cDNA sequence were examined and found not to rescue the *dpy-18* phenotype (data not shown). The lack of introns may result in extremely low levels or absence of expression of the heterologous protein (33,34), and therefore an artificial intron was synthesized based on standard *C. elegans* introns and inserted into the *B. malayi* phy-1 cDNA coding sequence (Fig. 7B). Microinjection of this cDNA construct containing a synthetic intron at 10 µg/ml and 100 µg/ml concentrations likewise failed to rescue the dumpy phenotype (data not shown). The ability of the genomic *B. malayi* phy-1 sequence to rescue the *dpy-18* phenotype was also studied. High concentrations (100 µg/ml) of this construct were however toxic, as all the transformed nematodes died during embryogenesis, and no transformed lines could be generated. Injections at lower concentrations (10 µg/ml) yielded transformed lines but examination of multiple lines again revealed a failure to rescue the dumpy phenotype (data not shown).

*Temporal Expression Analysis of B. malayi phy-1 mRNA* - Data from ESTs and library screenings indicated that the *B. malayi* phy-1 mRNA was expressed in adult females, males and L3 larvae. A more detailed analysis of the temporal expression pattern of *B. malayi* phy-1 in developmental stages from L3 to adults was performed by RT-PCR using mRNA samples prepared from extracts isolated from infected jirds; daily extracts taken up to day 14 post infection, after which extracts were taken every 2-4 days (25). The 655-bp PCR product from *B. malayi* phy-1 cDNA can be distinguished from that produced by any genomic contamination as the primers applied in this study were designed to span introns. A second set of primers simultaneously applied in the same PCR reactions, were used to amplify a fragment of the tubulin gene, as an internal control. The *B. malayi* phy-1 transcript was detectable in all L3, L4 and adult samples analyzed (Fig. 8). Expression was likewise found in an L1 (microfilaria) cDNA sample (data not shown), and taken together with the EST and library screening data, the RT-PCR results from stage-specific samples show that the *B. malayi* phy-1 is expressed in L1 (microfilaria), at all time points examined throughout L3 and L4 development, and in both adult sexes. Visual examination of this expression profile reveals peaks of abundance corresponding to the L3-L4 and L4 to adult molts (Fig. 8 lanes 8 and 17).
Analysis of the Function of the B. malayi phy-1 Promoter Region - A 2.2-kb region of the putative B. malayi phy-1 promoter sequence (AJ421994) was identified by screening a genomic BAC Brugia library. This 2.2-kb region does not contain any other coding sequences as determined by BLASTX analysis. A 3' trans-splicing acceptor site (27) is located at position –15 to –8 with respect to the translation start codon. The 2.2-kb promoter fragment followed by the codons for the first three amino acids of B. malayi PHY-1 was generated by PCR, and fused in frame to lacZ/GFP in the multi-intron reporter gene vector pPD96-04. The reporter gene construct was transformed into C. elegans at 100 µg/ml and multiple lines were examined, with expression found consistently in hypodermal cells of all lines examined (Fig. 9A-D). GFP expression was particularly prominent in the hypodermal cell nuclei hyp5, 6 and 7 of the head (Fig. 9A), and in the pair of hyp7 cell nuclei in the tail of adult worms (Fig. 9B). Using sensitive staining techniques for the detection of β-galactosidase activity, expression was also detected in larval stages, again strongly in the head and tail regions (Fig. 9C and D). The lacZ expression was less pronounced in the hypodermal cells of the body (Fig. 9C), with expression being detected in the hypodermal cell nuclei hyp4, 5, 6 and 7 of the head. With prolonged staining (overnight at room temperature) lacZ expression was also observed in the vulval cells of the adult, which are of hypodermal origin, in the adult body wall muscle cells, and in embryonic stages (data not shown).

DISCUSSION

In this study we have identified and characterized a P4H with unusual properties from the filarial parasite B. malayi. The recombinant B. malayi PHY-1 assembles into an active soluble P4H tetramer when expressed in insect cells in the absence of PDI. High-levels of B. malayi phy-1 transcript expression was detected in all parasite stages examined. Analysis of B. malayi phy-1 promoter-reporter gene fusions showed that the construct is expressed in hypodermal cells in a heterologous C. elegans system, thus indicating a conservation of control elements between these species. Although B. malayi PHY-1 appears to function as a P4H independently, without a PDI subunit, the B. malayi phy-1 was not able to replace P4H function in a C. elegans phy-1 mutant.

The essential nature of the cuticle collagen modifying function of P4H has previously been demonstrated in the nematode C. elegans where animals lacking or deficient in this function are non viable.
or malformed (15,16,35). Chemical inhibitors of P4H have also been shown to have similar cuticle-specific effects in both *C. elegans* (14, 16) and *B. malayi* (22). As P4H is involved in the synthesis of the nematode cuticle, it is therefore an excellent target for parasite control by chemical inhibition.

The general structure and composition of the cuticle or exoskeleton is well conserved throughout the nematode phyla (17). The cuticle is synthesized five times during development and is critical for many functions such as movement and protection from the environment. In *C. elegans* the structure of the cuticle has been studied in detail, demonstrating that despite overall similarity, cuticles from each developmental stage are chemically and structurally distinct (36). Following the completion of embryonic elongation the cuticle of the unhatched first larval stage maintains the elongated form of the worm (18). A weakened or malformed cuticle may cause altered body morphology in viable worms or in some cases, as in the combined disruption of the P4H α subunit encoding genes *phy-1* and *phy-2* in *C. elegans*, the resulting cuticle is too weak for survival (15,16). The cuticle is composed predominantly of collagen, and over 150 cuticular collagen genes are present in the *C. elegans* genome (37). Similar large gene families are likewise present in parasitic nematodes (17), and a representative cuticular collagen, COL-2 (38), has been cloned from *B. malayi*. The size of *B. malayi* COL-2 (33 kDa) is similar to the small *C. elegans* collagens (26-35 kDa), and shows overall amino acid sequence similarity to the *C. elegans* cuticle collagens. Comparison at the amino acid level, reveals 46% identity to the *C. elegans* group 2 (37) collagen T07H6.3. Precise positioning of the cysteine residues, that are required for registering and stabilizing the collagen triple helix, are likewise conserved between the *B. malayi* COL-2 and the *C. elegans* group 2 collagens. We propose that *B. malayi* PHY-1 may function to modify cuticular collagens such as *B. malayi* COL-2, a process, which in *C. elegans* is an essential feature of development.

*B. malayi* PHY-1 is unusual among the metazoan P4Hs in that it is a soluble and active P4H in the absence of a PDI subunit. The function of PDI subunits in the vertebrate and *D. melanogaster* P4Hs, and in the *C. elegans* P4Hs that are involved in the synthesis of cuticle collagens, is to keep the α, or PHY, subunits in a catalytically active, nonaggregated conformation and to maintain the enzyme within the ER (10,11). Active soluble monomeric P4Hs have been cloned and characterized from the *Paramecium bursaria* Chlorella virus-1 (39), *Arabidopsis thaliana* (40), and partially purified from algae (41). It is also possible that the *C. elegans* PHY-3, which is involved in the hydroxylation of collagens in early embryos,
acts as a monomeric P4H (42). Furthermore, the recently identified P4Hs that hydroxylate the hypoxia inducible transcription factor HIFα are monomeric cytoplasmic enzymes (43,44). Interestingly, PHY-1 from the related parasite *O. volvulus* may require a PDI subunit to form an active P4H, but neither subunit contains a recognizable ER retention signal (22,45). These unusual parasite P4Hs and the unique *C. elegans* P4H forms suggest that nematodes have evolved specialized forms of these enzymes, possibly in order to process the large number of collagens required to form the cuticular ECM.

When recombinant *B. malayi* PHY-1 was coexpressed in insect cells with PDI from other organisms, no detectable association of the polypeptides was observed. In contrast, the *C. elegans* PHY-1, and the *Drosophila* and vertebrate α subunits have been shown to assemble into an active P4H with PDIs from other species (5, 7, 13). The assembly of the recombinant *C. elegans* PHY-2 with PDI-2 is very inefficient, and only when it is coexpressed with *C. elegans* PHY-1 and PDI-2 it assembles into a fully active mixed tetramer (14). Thus, although the recombinant *B. malayi* PHY-1 can function as an active P4H by itself, it may assemble with additional, as yet unidentified assembly partners.

The *B. malayi* *phy-1* expression constructs under the control of the *C. elegans* *phy-1* promoter were not able to rescue the *C. elegans* *phy-1* null (*dpy-18(e364)*) mutant phenotype. A similar approach has however been successfully applied in the rescue of the same mutant strain with a wild type copy of the endogenous gene (15) (Fig. 7A). Inability of the *B. malayi* PHY-1 to functionally compensate for the lack of *C. elegans* PHY-1 could be attributable to a number of causes such as low expression levels, differences in codon usage, mis-splicing of introns or low activity of the transgenic protein. Alternatively, although the recombinant *B. malayi* PHY-1 was shown to be an active P4H by itself, it may have higher activity *in vivo* when assembled with an as yet unidentified partner. In support of this contention, the *C. elegans* PDI-2 does not associate with the *B. malayi* PHY-1 in a recombinant insect cell expression system (Fig. 4D). Thus, if additional subunits are needed for *B. malayi* PHY-1 to rescue *dpy-18*, these could not be provided by *C. elegans*. This possibility will be addressed following the completion of sequencing the *B. malayi* genome, and all possible partners can be assessed for associations with *B. malayi* PHY-1. Heterologous rescue of *C. elegans* mutants by *B. malayi* genes has not been described to date, but *C. elegans* rescue has been obtained with genes from the more closely related parasitic nematode *Haemonchus contortus*. Using a similar system to that employed in our study the embryonic lethal phenotype resulting from the lack of a
cathepsin L protease in *C. elegans* was rescued by transformation with a homologous gene from *H. contortus* (46). This demonstrates the conservation of essential developmental function of these two genes in different species. Amino acid sequence identity of mature cathepsin L proteases between *C. elegans* and *H. contortus* is 87%, which is significantly higher than the 59% identity found between PHY-1 polypeptides from *C. elegans* and *B. malayi*. The lower identity reflects the more distant evolutionary relationship between the latter pair of nematodes.

The *B. malayi phy-1* promoter is capable of directing tissue-specific expression in *C. elegans* to the cuticle synthesizing hypodermis, a location that is consistent with the proposed function of its gene product. Appropriate tissue-specific expression of reporter genes in *C. elegans* has been described for promoters from a number of genes from related parasitic nematodes (47-49), although to date, none have been described from *B. malayi*. This study therefore confirms that *C. elegans* represents an excellent heterologous system for the analysis of gene expression in the filarial nematodes. Although the native localization of PHY-1 in *B. malayi* is not known, its function as a P4H, taken together with data from reporter gene studies of the homologous genes from *C. elegans* (and the reporter-gene studies reported here), would predict a hypodermal localization for *B. malayi phy-1* expression, and is consistent with a role in cuticle collagen modification. The secondary staining of muscle cells and vulval cells is also identical to that found for the *C. elegans* homologues *phy-1* and *phy-2*, which are both expressed in vulval cells with additional muscle cell staining found for *phy-2* (15). Like the *C. elegans* homologues, the *B. malayi phy-1* transcript was strongly expressed throughout larval development, with greatest peaks of abundance consistent with a role in the molting cycle and cuticle synthesis.

The essential nature of P4H in the development and body morphology in *C. elegans* has been demonstrated both by genetic and RNAi analyses and by chemical inhibition studies. The extrapolation of the knowledge gained in *C. elegans* and other species, along with the emerging use of *C. elegans* as a surrogate system for expression of foreign proteins, provides powerful approaches for further characterization of parasite P4Hs and assessment of their potential as targets for chemical control.

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FOOTNOTES

1The abbreviations used are: P4H, prolyl 4-hydroxylase; PHY, prolyl 4-hydroxylase α subunit; PDI, protein disulfide isomerase; UTR, untranslated region; GFP, green fluorescent protein; lacZ, β-galactosidase encoding gene; ECM, extracellular matrix; ER, endoplasmic reticulum; hyp, hypodermal; EST, expressed sequence tag; BAC, bacterial artificial chromosome.

2The cDNA and genomic sequences described in this paper have been deposited in the Genbank database under the accession numbers AJ297845, AJ421993 and AJ421994.
FIGURE LEGENDS

FIG. 1. Cloning and gene structure of *B. malayi* phy-1. *A*, Cloning of the full-length cDNA sequence of *B. malayi* phy-1. The SL1 trans-spliced leader, 5' UTR and 3' UTR are depicted as open boxes, and the coding sequence by a filled box. ATG and TAA indicate the positions of the translational start and stop codons, respectively. The relative positions of the ESTs, the probe used for library screens, and the largest phage clone isolated are indicated. The full-length coding cDNA sequence derived from phage clones and 5' RACE data were confirmed by sequencing a full-length PCR product. *B*, Gene structure of *B. malayi* phy-1. The 5' UTR and intronic regions are represented by lines and exons depicted as filled boxes. The 3' UTR is shown as an open box and the poly-adenylation site is indicated. The sizes of exons and introns are given in base pairs above the exon boxes and below the intron lines, respectively. The exons are numbered with roman numerals.

FIG. 2. Amino acid alignments of *B. malayi* PHY-1 with *C. elegans*, human and *O. volvulus* P4H α subunits. Alignment of *B. malayi* (Bm), *C. elegans* (Ce), *O. volvulus* (Ov) PHY polypeptides and human α subunits using ClustalW and Boxshade. Gaps (-) were introduced for maximal alignment and signal peptides were removed, therefore numbering refers to the mature processed proteins. The N-glycosylation sites predicted by PROSITE for *B. malayi* PHY-1 are indicated with (+++). The cysteine residues (C) required for intrachain disulfide bonding (28,29), and the catalytically critical aspartate (D), histidine (H) and lysine (K) residues (29,30) identified by site-directed mutagenesis studies on the human P4H α(I) subunit are indicated with (*) and are all conserved in the *B. malayi* PHY-1. Accession numbers: *B. malayi* PHY-1 (AJ297845), *C. elegans* PHY-1 (Z81134), *C. elegans* PHY-2 (Z69637), human α(I) subunit (M24486), human α(II) subunit (U90441), *O. volvulus* PHY-1 (AF369787).

FIG. 3. Analysis of the expression of recombinant *B. malayi* PHY-1 in insect cells (*A*) and coexpression of *B. malayi* PHY-1 with various PDIs (*B*). *A*, Insect cells were infected with a recombinant baculovirus coding for the *B. malayi* PHY-1 polypeptide, harvested 72 h after infection, homogenized in a Triton X-100-containing buffer, and centrifuged. The remaining pellet was solubilized in 1% SDS. The
fractions were analyzed by 8% SDS-PAGE under reducing conditions followed by Western blotting using an antibody against *B. malayi* PHY-1. *B*, Insect cells were infected with the virus coding for the *B. malayi* PHY-1 alone (lane 1) or together with viruses coding for *C. elegans* PDI-1 (lane 2), PDI-2 (lane 3) or human PDI (lane 4). In control experiments, insect cells were coinfected with viruses coding for *C. elegans* PHY-1, PHY-2 and PDI-2 (lane 5), and *C. elegans* PHY-1 and human PDI (lane 6). Cells were harvested and homogenized as above, and the Triton X-100-soluble fractions were analyzed by nondenaturing PAGE followed by Coomassie Blue staining. The *C. elegans* PHY-1 PHY-2/(PDI-2)2 tetramer (T) and the *C. elegans* PHY-1/human PDI dimer (D) are indicated by arrows. The band indicated by an asterisk is also found in extracts from cells infected with a wild-type baculovirus (not shown), and the faint bands below it represents the non-associated PDI subunits.

**FIG. 4. Analysis of the assembly of *B. malayi* PHY-1 with various PDIs by nondenaturing PAGE and Western blotting.** Recombinant *B. malayi* PHY-1 was expressed in insect cells alone (lanes 1) or together with human PDI (lanes 2), *C. elegans* PDI-1 (lanes 3) or PDI-2 (lanes 4). In control experiments, the human PDI (A and B, lanes 5), *C. elegans* PDI-1 (A, lane 6 and C, lane 5) and PDI-2 (A, lane 7 and D, lane 5) were expressed alone, or the cells were coinfected with viruses coding for the *C. elegans* PHY-1, PHY-2 and PDI-2 (A, lane 8). The cells were harvested and homogenized as described in the legend to Fig. 3. Triton X-100-soluble fractions were analyzed by nondenaturing 8% PAGE followed by Western blotting using antibodies against *B. malayi* PHY-1 (A, lanes 1-7), *C. elegans* PHY-1 (A, lane 8), human PDI (B), *C. elegans* PDI-1 (C) and *C. elegans* PDI-2 (D). The positions of the *C. elegans* PHY-1 PHY-2/(PDI-2)2 tetramer (T) and the PHY-1/PDI-2 dimer (D) are indicated by arrows.

**FIG. 5. Gel filtration analysis of a Triton X-100-soluble fraction from insect cells expressing recombinant *B. malayi* PHY-1.** A, A Triton X-100-soluble fraction from insect cells expressing recombinant *B. malayi* PHY-1 was applied to a HiPrep Sephacryl S-200 HR column and the eluted fractions were assayed for P4H activity (—♦—). In a control experiment, purified recombinant human type I P4H was applied to the column and absorbance at 280 nm (----∆----) in the eluted fractions was measured. The arrow indicates the elution position of the *C. elegans* PHY-1/human PDI dimer. *B* and *C*, The gel
filtration fractions containing \textit{B. malayi} P4H activity were pooled, concentrated, and analyzed by 8\% SDS-PAGE under reducing conditions (B) and non-denaturing 8\% PAGE (C) followed by Western blotting using an antibody against \textit{B. malayi} PHY-1. The positions of the \textit{B. malayi} PHY-1 polypeptide and the \textit{B. malayi} PHY-1 tetramers (T) and dimers (D) are indicated by arrows.

**FIG. 6. Analysis of tissue-extracted \textit{B. malayi} PHY-1 and determination of native glycosylation forms.**

\textit{A}, \textit{B. malayi} protein extracts were made from the parasites and analyzed by non-denaturing gradient (4-12\%) PAGE followed by Western blotting using a \textit{B. malayi} PHY-1 antibody (lane 1). \textit{C. elegans} protein extracts were separated on the same gel, blotted and probed with an anti-\textit{C. elegans} PHY-1 antibody (lane 2). Arrows represent the \textit{C. elegans} PHY-1-PHY-2/PDI-2 tetramer (T) and PHY-1/PDI-2 dimer (D), with \textit{B. malayi} PHY-1 immunoreactive bands showing comparable sizes to the characterized \textit{C. elegans} tetramer and dimer. \textit{B}, Extracts from insect cells expressing the recombinant \textit{B. malayi} PHY-1 and the \textit{B. malayi} extracts were incubated either in the absence (lanes 2 and 4) or presence (lanes 1 and 3) of PNGase F N-glycanase. The samples were analyzed by denaturing (4-12\% gradient) SDS-PAGE followed by Western blotting with an anti-\textit{B. malayi} PHY-1 antibody. The arrow indicates the 60 kDa deglycosylated \textit{B. malayi} PHY-1.

**FIG. 7 Representation of \textit{phy-1} null \textit{dpy-18(e364)} \textit{C. elegans} rescue constructs.** \textit{A}, Depiction of the \textit{C. elegans} \textit{phy-1} gene construct (promoter, genomic sequence and 3' UTR) successfully used to rescue \textit{dpy-18(e364)} mutants (15) (indicated with, +). \textit{B}, The \textit{B. malayi} PHY-1 rescue constructs contained the \textit{C. elegans} \textit{phy-1} promoter and 3' UTR sequences and either the full-length \textit{B. malayi} \textit{phy-1} coding cDNA, the full-length \textit{B. malayi} \textit{phy-1} coding cDNA with a single \textit{C. elegans} synthetic intron or the \textit{B. malayi} \textit{phy-1} genomic sequence from the translation initiation codon to the stop codon. Sizes of the fragments are not drawn to scale and the full number of introns in the genomic fragments are not depicted. The constructs were injected into the \textit{C. elegans} \textit{phy-1} null strain CB364(\textit{dpy-18}) to examine interspecies rescue. None of the \textit{B. malayi} constructs were able to rescue the dumpy phenotype of \textit{C. elegans} \textit{phy-1} null mutants (indicated with, -)
FIG. 8. **Developmental expression analysis of** *B. malayi phy-1*. Expression of the *B. malayi phy-1* transcript was compared to the tubulin transcript through L3 to L4 and L4 to adult molts. PCR reactions were performed on cDNA samples using primer pairs from both genes simultaneously. *B. malayi* extracts were prepared from daily samples of infected jirds through L3 and L4, then at longer intervals after day 14. Numbers below the figure refer to days post infection.

FIG. 9. **Expression of reporter genes from** *B. malayi phy-1* **promoter in a heterologous expression system.** Transgenic expression patterns in *C. elegans* of a reporter gene construct in which the *B. malayi* promoter directs tissue-specific expression of *lacZ* and GFP. Panel *A* is a merge of DIC and UV images showing expression of GFP in the hypodermal cells hyp5, 6 and 7 in the head. Panel *B* is a merge showing expression of GFP in the hyp7 cells of the tail. Panels *C-D* show *lacZ* staining, with panel *C* showing the expression in the hypodermal cells hyp4, 5, 6 and 7 of the head and body; panel *D* depicts reporter gene expression in the hyp7 cells of the tail (compare to panel *B*).
Table I

*P4H activity in Triton X-100 extracts of insect cells expressing various PDIs, B. malayi PHY-1 alone or with various PDIs or C. elegans PHY-1/PHY-2/(PDI-2)*

Values are given in dpm/50 µl of Triton X-100 extractable cell protein, mean ± SD for at least four experiments.

| Polypeptides expressed | P4H activity (dpm/50 µl) |
|------------------------|--------------------------|
| *C. elegans* PDI-1, PDI-2 or human PDI | 500 ± 70 |
| *B. malayi* PHY-1 | 3920 ± 1070 |
| *B. malayi* PHY-1 and *C. elegans* PDI-1 | 3640 ± 710 |
| *B. malayi* PHY-1 and *C. elegans* PDI-2 | 4210 ± 770 |
| *B. malayi* PHY-1 and human PDI | 4710 ± 1210 |
| *C. elegans* PHY-1/PHY-2/(PDI-2)*2 | 8650 ± 1970 |
TABLE II

*Km* values of the *B. malayi* PHY-1 and the *C. elegans* PHY-1/PHY-2/(PDI-2)₂ tetramer for *cosubstrates and the peptide substrate and *Kᵢ* values for two competitive inhibitors with respect to 2-oxoglutarate

| Cosubstrate, substrate, or inhibitor | Kᵢ or Kᵢ | Constant | Bm PHY-1 | Ce PHY-1/PHY-2/(PDI-2)₂ |
|------------------------------------|----------|----------|----------|------------------------|
| 2-Oxoglutarate                     | Kᵢ       | 80ᵃ      |          | 60 80ᵃ                 |
| Fe²⁺                               | Kᵢ       | 2ᵃ       |          | 0.5 2ᵃ                 |
| Ascorbate                          | Kᵢ       | 350ᵃ     |          | 400 350ᵃ               |
| (Pro-Pro-Gly)₁₀                    | Kᵢ       | 20ᵃ      |          | 7 20ᵃ                  |
| Pyridine-2,4-dicarboxylate         | Kᵢ       | 1ᵃ       |          | 0.5 1ᵃ                 |
| Pyridine-2,5-dicarboxylate         | Kᵢ       | 1ᵃ       |          | 0.35 1ᵃ                |

ᵃRef. 14.
Fig. 1

A

5' UTR  ATG  Full length cDNA 1626 bp  3' UTR

---

SW3D9CA480SK
MBAFCX8G05T3
MBAFCZ7H09T3
PCR probe
Phage clone

---

5' RACE  Full length PCR

B

5' UTR  ATG

---

SL1

117  211  118  216  185  171  71  100  54  66  69  198  154

3'

Poly-A

---

Base

+1  +1000  +2000  +3000  +4000  +4596
Fig. 3
Fig. 4

A. Anti-BmPHY-1 and anti-CePHY-1 (lane 8)

B. Anti-HuPDI

C. Anti-CePDI-1

D. Anti-CePDI-2
Fig. 5

A

B. malayi PHY-1, DPM

B

216

132

78

46

Human type I P4H, A280

C

T

D

BmPHY-1
**A. C. elegans dpy-18 rescue construct**

- **Ce phy-1 promoter, genomic sequence and 3’UTR**

**B. B. malayi dpy-18 rescue constructs**

- **Bm phy-1 cDNA**
- **Ce phy-1 promoter**
- **Ce phy-1 3’UTR**
- **Bm phy-1 genomic sequence**  
  + **Ce synthetic intron**
A hypodermally expressed prolyl 4-hydroxylase from the filarial nematode Brugia malayi is soluble and active in the absence of protein disulfide isomerase

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