International Journal for Parasitology 40 (2010) 533–542

Contents lists available at ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

Collagen processing and cuticle formation is catalysed by the astacin metalloprotease DPY-31 in free-living and parasitic nematodes ☆

Gillian Stepek, Gillian McCormack, Antony P. Page *

Division of Infection and Immunity, Institute of Comparative Medicine, Faculty of Veterinary Medicine, University of Glasgow, Bearsden Road, Glasgow G61 1QH, UK

ARTICLE INFO

Article history:
Received 18 August 2009
Received in revised form 5 October 2009
Accepted 8 October 2009

Keywords:
Caenorhabditis elegans
Haemonchus contortus
Brugia malayi
Astacin metalloprotease
BMP
Development
Cuticle

ABSTRACT

The exoskeleton or cuticle performs many key roles in the development and survival of all nematodes. This structure is predominantly collagenous in nature and requires numerous enzymes to properly fold, modify, process and cross-link these essential structural proteins. The cuticle structure and its collagen components are conserved throughout the nematode phylum but differ from the collagenous matrices found in vertebrates. This structure, its formation and the enzymology of nematode cuticle collagen biogenesis have been elucidated in the free-living nematode *Caenorhabditis elegans*. The *dpy-31* gene in *C. elegans* encodes a procollagen C-terminal processing enzyme of the astacin metalloprotease or bone morphogenetic protein class that, when mutated, results in a temperature-sensitive lethal phenotype associated with cuticle defects. In this study, orthologues of this essential gene have been identified in the phylogenetically diverse parasitic nematodes *Haemonchus contortus* and *Brugia malayi*. The DPY-31 protein is expressed in the gut and secretory system of *C. elegans*, a location also confirmed when a *B. malayi* transcriptional *dpy-31* promoter–reporter gene fusion was expressed in *C. elegans*. Functional conservation between the nematode enzymes was supported by the fact that heterologous expression of the *H. contortus dpy-31* orthologue in a *C. elegans dpy-31* mutant resulted in the full rescue of the mutant body form. This interspecies conservation was further established when the recombinant nematode enzymes were found to have a similar range of inhibitable protease activities. In addition, the recombinant DPY-31 enzymes from both *H. contortus* and *B. malayi* were shown to efficiently process the *C. elegans* cuticle collagen SQT-3 at the correct C-terminal procollagen processing site.

1. Introduction

All nematodes are encased in a protective exoskeleton known as the cuticle. This complex extracellular matrix (ECM) is synthesised repeatedly, through a process called moulting, to form larval- and adult-specific cuticles that permit growth and form a protective barrier. The major components of this structure are the highly cross-linked small collagen-like proteins, which are modified by a variety of biosynthetic enzymes (Page and Winter, 2003; Page and Johnstone, 2007; Thein et al., 2009). The N-terminus of these proteins contains 80–150 amino acids of non-repetitive sequence, preceding the signature Gly-X-Y repetitive domain followed by a conserved subtilisin-like pro-domain cleavage site (Page and Johnstone, 2007). A non-repetitive region is also present at the C-terminus, following the Gly-X-Y repeat domain and contains an astacin-like, bone morphogenetic protein (BMP) processing domain (Novelli et al., 2006). The C-terminal non-repetitive region and its flanking cysteine residues are highly conserved between *Haemonchus contortus* and *Caenorhabditis elegans* collagens and hence equivalent molecules in the two nematodes probably share a similar function (Shamansky et al., 1989). Cuticle collagen genes represent large families in all nematodes examined and have a high degree of similarity in gene size and structure, further suggesting a common function and a similar mechanism for their biogenesis throughout the nematode phylum (Page and Johnstone, 2007).

Proteases are essential for the viability of parasitic nematodes, performing crucial functions such as cuticle moulting, host tissue penetration and digestion. There are several classes of proteases found in nematodes: cysteine, serine, aspartic and metalloproteases. Important developmental roles performed by the metalloprotease class of enzymes include hatching, cuticle collagen processing and cuticle moulting in *C. elegans* (Hishida et al., 1996; Davis et al., 2004; Novelli et al., 2004), activation of the free-living to parasitic stage of *Ancylostoma caninum* (Hawdon et al., 1995), feeding or host tissue penetration of *Trichuris suis* (Hill et al., 1993), digestion in *A. caninum* (Jones and Hotez, 2002), tissue penetration by *Strongyloides stercoralis* (Gallego et al., 2005) and ecdysis of *H. contortus* (Gamble et al., 1989, 1996). There are several families of metalloproteases

☆ Note: Nucleotide sequence data reported in this paper are available in the GenBank™ database under the accession numbers FJ812517 (*H. contortus nas-35*) and FJ812518 (*B. malayi nas-35*).

* Corresponding author. Tel.: +44 141 330 1997; fax: +44 141 330 5603.
E-mail address: a.page@vet.gla.ac.uk (A.P. Page).

0020-7519/$36.00 © 2009 Australian Society for Parasitology Inc. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.ijpara.2009.10.007

in nematodes, with the astacin metalloproteases belonging to the M12A family (Möhrlen et al., 2003). Astacin metalloproteases are structurally distinct zinc metallo-endopeptidases, include the bone morphogenetic proteins and are characterised by two conserved motifs in the catalytically-active astacin domain: the catalytic active-site (HExxHxxGFxHExxRxDRD), for binding the essential Zn^{2+}, and the methionine-turn (SxMHY), which maintains the enzyme conformation. The active-site zinc is penta-coordinated and is bound by the three histidine residues and the glutamic acid residue in the active-site and the tyrosine residue in the methionine-turn (Stöcker et al., 1993).

There are 39 nematode astacin (NAS) metalloproteases in the free-living nematode, *C. elegans*, divided into six sub-groups (I–VI) depending on the C-terminal domain structure, which is believed to determine the function of the enzyme (Möhrlen et al., 2003). The enzymes in sub-group V (NAS-33 to NAS-38) have a unique nematode-specific domain arrangement, in that they have a signal peptide, a pro-domain, the N-terminal catalytic astacin domain, and the C-terminal epidermal growth factor (EGF), C1r/C1s, embryonic sea urchin protein Uegf, Bmp-1 (CUB) and thrombospondin type-1 repeat (TSP-1) domains. These C-terminal domains are important for the regulation of the proteolytic activity and are commonly found in proteins that play critical developmental roles (Möhrlen et al., 2003). The sub-group V enzymes perform important developmental functions in *C. elegans*. Nematodes with mutations in the *nas-34* gene display a delayed *hatch*ing phenotype, Hch-1 (Hishida et al., 1996). Mutations in the *nas-35* gene result in a severe *dumpy* appearance, hence the name Dpy-31, and this enzyme has been proven to be an essential procollagen C-peptidase involved in proper cuticle formation (Novelli et al., 2004). Nematodes with mutations in *nas-36* and *nas-37*, but particularly the *nas-37* gene, show defects in the ecdysis step of moulting and subsequent shedding of the cuticle (Davis et al., 2004; Suzuki et al., 2004). An unidentified protease from the exsheathing fluid of *H. contortus* infective larvae has previously been shown to stimulate ecdysis by promoting the escape of the L3 stage from the L2 sheath through the formation of an anterior refractile ring and removable cap structure (Gamble et al., 1989, 1996). An identical ring structure was formed on isolated *H. contortus* larval cuticles in the presence of recombinant *C. elegans* NAS-37, suggesting that similar specific astacin substrates are shared between these diverse nematode species (Davis et al., 2004). It is reasonable to predict that the remaining nematode-specific, sub-group V astacin metalloproteases, that have essential roles in the hatching and collagen processing in *C. elegans*, may likewise be functionally conserved in parasitic nematodes and may therefore represent potential vaccine and drug targets.

In this study, we investigate the role that the procollagen C-peptidase DPY-31 (NAS-35) plays in cuticle formation and identify and characterise orthologues of this enzyme from the parasitic nematodes *H. contortus* and *Brugia malayi*. In *C. elegans*, DPY-31 plays an essential role in SQT-3 cuticle collagen processing and normal cuticle formation (Novelli et al., 2004, 2006). Mutations in the gene encoding this enzyme affect post-embryonic viability and have profound effects on the cuticle structure and nematode morphogenesis.

2. Materials and methods

2.1. Nematode strains

The wild-type Bristol N2 and *dpy-31(e2770)* strains of *C. elegans* were provided by the *Caenorhabditis* Genetics Centre, University of Minnesota, USA and Professor Jonathan Hodgkin, University of Oxford, UK, respectively. *H. contortus* adults were provided by Dr. Frank Jackson (Moredun Research Institute, UK) and *B. malayi* adults by Professor Rick Maizels (University of Edinburgh, UK).

2.2. Preparation of genomic DNA, RNA and cDNA from C. elegans, H. contortus and B. malayi worms

Genomic DNA was isolated from adult nematodes using a standard protocol involving homogenisation in Proteinase K, followed by repeated phenol:chloroform extraction. Briefly, worms were homogenised in 6 vols. of lysis buffer containing 100 μg/ml Proteinase K and incubated at 65 °C for 4 h. Worm debris was removed by centrifugation and the DNA was purified by repeated phenol:chloroform and chloroform extractions, ethanol precipitated and resuspended in Tris–EDTA (TE) buffer, pH 8.0. The DNA was treated with a final concentration of 100 μg/ml RNAse A for 1 h at 37 °C, phenol:chloroform extracted, chloroform extracted, ethanol precipitated and resuspended in TE Buffer, pH 8.0. Total RNA was isolated from adult nematodes following Trizol (Invitrogen) extraction, and cDNA was prepared using the AffinityScript Multiple Temperature cDNA synthesis kit (Stratagene), following the manufacturer's recommendations, with 1 μg RNA per reaction with oligo-dT primer.

2.3. Isolation of the cDNA and genomic DNA of H. contortus dpy-31

The *C. elegans* DPY-31 protein sequence was used to search the *H. contortus* database (http://www.sanger.ac.uk/Projects/H_contortus/) using the BLAST algorithm, initially using the options labelled "assembled contigs (27/01/06)" and "sequence reads (01/08/05)". The sequence reads haem-479f01.p1k and haem-479f01.q1k, and contigs 049443 and 037116, had the highest homology score and were put into a Scaffold program designed by Dr. Robin Beech, McGill University, Canada, which assembles a group of physically linked sequences using the *Haemonchus* genome project databases. Primers were designed from the reads haem-479f01.p1k and haem-479f01.q1k, and used to PCR, clone and sequence the genomic DNA of *dpy-31* from *H. contortus*, using *PfuTurbo* or *PfuUltra* polymerases. Primers were then designed to sequence the full 2715 bp PCR product. From the Scaffold results, one bacterial artificial chromosome (BAC) clone, HaemApoBac 18h16, was identified as containing part of the *dpy-31* gene. Further reads were identified from the initial Scaffold procedure upstream of the 2715 bp clone and primers were designed for sequencing the genomic PCR products between the new read pairs. A contig was formed between the three cloned PCR products and the BAC clone, HaemApoBac 18h16, using ContigExpress and potential intron–exon splice sites were predicted using the GeneWise tool on the ExPASy proteomics site (http://us.expasy.org/). This predicted partial coding sequence was translated using the Translate tool on the ExPASy proteomics site and then aligned with the *C. elegans* protein sequence using ClustalX and BoxShade (http://www.ch.embnet.org/software/BOX_form.html). Amplification of the 5′ and 3′ ends of the *H. contortus dpy-31* cDNA was performed using the Invitrogen 5′- and 3′-rapid amplification of cDNA ends (RACE) systems with the following primers: Hc nas-35 5′-RT, Hc nas-35 5′ R1, Hc nas-35 5′ R2, Hc nas-35 3′ F1 and Hc nas-35 3′ F2. The complete coding sequence was formed from the 5′- and 3′-RACE products and the original partial sequence in ContigExpress. The primers Hc nas-35 F and Hc nas-35 R were used to PCR the genomic sequence of *H. contortus dpy-31*, and the 5′ and 3′ unknown genomic regions were sequenced using the primers Hc nas-35gF2, Hc nas-35gR2 and Hc nas-35gR3. Gene Structure Draw was used to produce a scaled schematic depicting the positions of the introns and exons in the gene. The relationship between the contigs and PCR fragments is depicted in the