A conserved metalloprotease mediates ecdysis in *Caenorhabditis elegans*

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Summary

Molting is required for progression between larval stages in the life cycle of nematodes. We have identified four mutant alleles of a *Caenorhabditis elegans* metalloprotease gene, *nas-37*, that cause incomplete ecdysis. At each molt the cuticle fails to open sufficiently at the anterior end and the partially shed cuticle is dragged behind the animal. The gene is expressed in hypodermal cells 4 hours before ecdysis during all larval stages. The NAS-37 protein accumulates in the anterior cuticle and is shed in the cuticle after ecdysis. This pattern of protein accumulation places NAS-37 in the right place and at the right time to degrade the cuticle to facilitate ecdysis. The *nas-37* gene has orthologs in other nematode species, including parasitic nematodes, and they undergo a similar shedding process. For example, *Haemonchus contortus* molts by digesting a ring of cuticle at the tip of the nose. Incubating *Haemonchus* larvae in extracted exsheathing fluids causes a refractile ring of digested cuticle to form at the tip of the nose. When *Haemonchus* cuticles are incubated with purified NAS-37, a similar refractile ring forms. NAS-37 degradation of the *Haemonchus* cuticle suggests that the metalloproteases and the cuticle substrates involved in exsheathment of parasitic nematodes are conserved in free-living nematodes.

Key words: Astacin, *Caenorhabditis elegans*, Cuticle, Metalloprotease, Molting

Introduction

Nematodes, like all invertebrate metazoans, have an exoskeleton, called the cuticle, which serves a structural and protective role for the organism. The importance of the cuticle to nematode biology is revealed in the *Caenorhabditis elegans* genome, which contains over 150 genes encoding collagen and cuticulin proteins involved in cuticle construction (Johnstone, 2000). Unlike arthropods, nematodes have an exoskeleton that is able to grow as the animal grows, but the ability of the cuticle to accommodate growth is not limitless, and nematodes must molt four times during progression from hatchling to adult.

Molting poses a difficult problem from an engineering point of view. Because the cuticle is the main barrier between the worm and its environment, the old cuticle must remain intact while the new cuticle is synthesized. The new cuticle is assembled between the old cuticle and the underlying hypodermis. Thus, connections of the old cuticle to the hypoderm must be severed before the new cuticle is deposited. This severing is called apolysis. Following apolysis, hypodermal cells secrete soluble collagens into the space between the hypoderm and the cuticle. These proteins self-assemble into multiple layers, with the collagen fibers oriented in different directions. When the new cuticle is complete, the old cuticle is shed in a process called ecdysis. These three steps – apolysis, cuticle synthesis and ecdysis – must each be properly executed before initiation of the next step if the animal is to survive and grow.

The third larval stages of certain parasitic nematodes loosely retain their second-stage cuticle as a protective sheath. The process of exsheathment in parasitic nematodes is an evolutionary elaboration on the process of ecdysis and is of particular interest because it is a key regulator of infection. In these nematodes, molting of the sheath is specifically triggered by contact with a host. During this exsheathment process, *Haemonchus contortus* larvae release a zinc metalloprotease that specifically degrades an anterior ring of the sheath, allowing the L3-stage larva to shed the second molt cuticle (Gamble et al., 1989b). This protease has been purified from exsheathment fluids and is sufficient to degrade isolated second molt cuticles in vitro (Gamble et al., 1989b). The molecular identity of this protease could eventually demonstrate how exsheathment is regulated, as well as provide a potential target of anthelmintics for the prevention of nematode infections.

To identify the proteases involved in ecdysis we isolated mutants of the nematode *C. elegans* in which the cuticle cannot be shed. We identified four mutations in a single gene. Because all four mutations are in one gene, this locus is probably the only one that can be mutated to this phenotype. We cloned the gene and demonstrated that it encodes an Astacin-class metalloprotease called NAS-37. The gene is broadly expressed in the hypodermis 4 hours before ecdysis at each larval stage and is expressed at points of cuticle attachment at ecdysis. The NAS-37 protein begins to accumulate in the anterior cuticle 4 hours before ecdysis. The protein appears to be acting on the old cuticle, as it is entirely associated with the molted cuticle following ecdysis; however, we also observe some defects in collagen deposition in addition to ecdysis defects. An ortholog

of this gene is found in parasitic nematodes. We find that purified NAS-37 protein is able to reproduce the refractile ring formation induced in isolated *H. contortus* second-molt cuticles by exsheathing fluids (Gamble et al., 1989b). This result suggests a conservation between the cuticle substrates involved in exsheathment of parasitic nematodes and cuticle substrates in molting of free-living nematodes. Further, NAS-37 is a metalloprotease with proteolytic activity specific for these substrates.

Materials and methods

Isolating *nas-37* alleles

We isolated *ox196*, *ox197* and *ox199*, based on their ecdysis defect in various screens for unrelated phenotypes. *ox190* was isolated fortuitously in a screen for mutations that affect axonal outgrowth. Although the ecdysis defect of *ox190* was not separable from the outgrowth defect, the two appear to be due to independent mutations, because the other three *nas-37* alleles do not have an outgrowth defect and the outgrowth defect is complemented by *ox199*.

The ecdysis defect of *ox199* was measured by placing mutant L4 hermaphrodites on a plate at 23°C and counting the fraction of adults with unshed cuticles 10 hours later.

SNP mapping and cloning *nas-37*

ox190 is linked to *oxIs12* (Mcintire et al., 1997), and therefore located on the right arm of the X chromosome. Further mapping was done using *ox199* crossed to the Hawaiian isolate CB4856, which has a large number of single nucleotide polymorphisms relative to the N2 Bristol strain (Swan et al., 2002; Wicks et al., 2001). Ecdysis-defective animals were isolated from this cross and singled onto individual plates. These were allowed to self-fertilize, and the progeny were assayed by PCR for the polymorphism status at the sites indicated in Fig. 2. A total of 317 recombinant F2s were analyzed to narrow the potential location of *ox199* to the interval between the polymorphisms located on cosmid F47A4 and cosmid F19C6.

Alleles were sequenced at the University of Utah sequencing facility using an ABI3730 96-capillary sequencer. Sequencing templates were mixtures of at least three independent PCR reactions. The nature of the point mutations in two of the *nas-37* alleles is unusual. *ox196* is an A-to-G transition caused by ethyl methane sulfonate (EMS) and *ox190* is a T-to-A transversion caused by N-ethylnitrosourea (ENU). *ox197* (EMS) and *ox199* (ENU) are G-to-A transitions that are commonly seen as the products of alkylation of guanine residues by ether EMS or ENU. The *ox190* transversion mutation is consistent with the observation that ENU is capable of a broader spectrum of DNA changes than is EMS, which generally causes G-to-A transitions (De Stasio and Dorman, 2001).

cDNA structure and GFP fusions

The 5′ end of the *nas-37* cDNA was determined by RT-PCR using the primer 5′-CGTTCTGCTCAACGTGTCTAATAGC for first-strand synthesis from whole RNA of mixed-stage *C. elegans*. This template was then used for a 35-cycle PCR using the primer 5′-GAGCTTGTCGCCTTGATCTTGG and the splice-leader 1 (SL1) specific primer 5′-GGTTTAATTACCCAAGTTTGAG. This product was TA-cloned and sequenced. The remainder of the *nas-37* cDNA was synthesized using PCR with T7 and T3 specific primers using the phage cDNA clone yk355c4 as template (provided to us by Yuji Kohara). Products were TA cloned and sequenced. The full-length, error-free cDNA was generated by ligating regions from three different 3′ clones and the 5′ clone.

The construct pWD90 is a fusion of the *nas-37* promoter driving GFP. The promoter fragment was made using PCR with the primers: 5′-TGGCTCTGGCAGTCGAAAGC and 5′-CGGATCCATTCTG-CAAAATAGAACATCAAGAATCGG and with N2 worm genomic DNA as template. This product was TA-cloned and then cloned as a *Pst*I–*Bam*HI fragment into the GFP expression plasmid pPD95.75 (A. Fire). This construct contains 3806 bp of genomic DNA between the predicted genes C17G1.7 and C17G1.6 (*nas-37*), and contains the predicted ATG start codon of C17G1.6 fused directly to the open reading frame of GFP. pWD90 was injected at 40 ng/μl with 20 ng/μl *lin-15(+)* as a co-injection marker and 100 ng/μl 1 kb plus DNA ladder (Invitrogen) as filler.

A destabilized GFP version of this construct, called pWD95, was made by fusing the PEST sequence from mouse ornithine decarboxylase (mODC) to the C-terminus of GFP. GFP destabilized in this way has been found to have a half-life of 2 hours in mammalian cells (Li et al., 1998). A translationally silent *Mfe*I site was introduced in the middle of the GFP:mODC (pd2EGFP, Clontech) to make this EGFP construct compatible with standard GFP in worm vectors. This 3′ fragment was then cloned as an *Mfe*I-*Eco*RI fragment into the GFP expression construct pPD95.75 to make pWD93. This GFP:mODC expression construct was then placed under the *nas-37* promoter by cloning an *Xho*I-*Apa*I fragment into the transcriptional fusion construct pWD90. pWD95 was injected at 90 ng/μl with 10 ng/μl *lin-15(+)* as a co-injection marker. This high concentration of *Pnas-37::GFP::mODC* was required to see robust expression of GFP::mODC. pWD95 was integrated using 4000 cGy of X rays, and the integrated transgene was used for determining the timecourse of *nas-37* expression. Timecourse experiments were done at 19°C using thinly seeded 2% agarose plates. Single L2 or L3 animals brightly expressing GFP were placed on plates and imaged every hour until they had molted twice (~17 hours).

pWD103 is a NAS-37::GFP translational fusion under the *nas-37* promoter. This was made by PCR of N2 genomic DNA using Phusion DNA polymerase (MJ Research) and the primers: 5′-GGCTACC-GGTCCGTTTTTGTAGCAAACTCCTCTTCTAGG and 5′-ACCC-TCTTTGTCTATCCTCCTCTG. This PCR product includes most of the *nas-37* promoter and all the predicted coding region; it introduces an *Age*I restriction site just before the *nas-37* stop codon. This PCR product was cloned into pWD90 as a *Sac*II-*Age*I restriction fragment. Because NAS-37 is a secreted product, pWD103T was created by replacing the S65C version of GFP in pWD103 with GFP(S65T) from pPD113.35. As expected, we found that this construct produces a slightly brighter fluorescence signal in worms than does pWD103. pWD103T was injected into the *ox199* mutant background with *Punc-122::GFP* as a co-injection marker. This translational fusion fully rescues the *ox199* mutant phenotype.

Cuticle collagen patterns in *nas-37* mutants

The COL-19::GFP integrated strain TP12 (Thein et al., 2003) was crossed with *ox199* to visualize the effect of loss of NAS-37 on the expression of an adult cuticle collagen. Live worms were mounted on agar pads and viewed under epifluorescence using a Zeiss axioscope 2. Embryonic and larval cuticle collagens were assessed by fixing mixed-stage cultures of *ox199* worms and staining with the DPY-7 monoclonal antibody (McMahon et al., 2003). The antibody was applied at 1/50 followed by Alexa Fluor 488 anti-mouse IgG conjugate. All fluorescence images were digitally captured and processed using Openlab (Improvision) and Adobe Photoshop software.

Haemonchus contortus cuticle refractile ring assays

We constructed a NAS-37 expression plasmid, pWD100.4, that included the region from the ATG up to and including the last amino acid of NAS-37. This region was PCR amplified from a full-length cDNA clone and inserted in-frame into pET42a (Novagen). This construct is designed to express a GST::6HIS::NAS-37::6HIS fusion protein under the control of the lactose operon.

pWD100.4 was transformed into BL21(DE3) cells, grown to log phase and induced with 1 mmol/l isopropyl-β-D-thiogalactopyranoside (IPTG) overnight (16 hours) at 25°C. Sonicates

were prepared using standard methods and cleared lysates purified on Qiagen Ni-NTA spin columns. The larvae were prepared and the refractile ring assays were performed essentially as described by Gamble et al. (Gamble et al., 1989b). Briefly, L3(2M) larvae were heat-killed by immersion in boiling water, cooled on ice, then transferred to a microscope slide and chopped open with a razor blade. Chopped worms were re-suspended in Earle's balanced salt solution at 4°C. Assays were set up in 96-well microtiter plates with prepared larvae transferred in 100 µl aliquots of 100 mmol/l Tris (pH 8). Purified protein was added at 1-2 µg to the wells; controls included no protein, excess crude bacterial sonicate, or 100 µg bovine serum albumin. Plates were incubated at 37°C for 1 hour; then samples were transferred to slides and viewed under a differential interference contrast microscope.

Results

nas-37 mutations cause ecdysis defects

We identified four mutant alleles of a gene that gave a striking molting phenotype (Fig. 1). All four alleles were indistinguishable from each other. At each molt the animals failed to open the anterior end of the old cuticle sufficiently to crawl out. Instead, the partially shed cuticle formed a tight constriction around the animal. The time required for the worm to shed the cuticle fully was variable. Approximately 40% (7/18) of young adult animals retained partially shed cuticles within 10 hours of the molt. Eventually, most animals were able to free themselves from the constricted cuticle; however, sometimes the cuticle broke off behind the constriction, leaving a tight band of cuticle girdling the worm and giving it a 'wasp waist' appearance. This phenotype was observed at all four molts and corresponded to the expected phenotype for a defect in a protease involved in ecdysis.

NAS-37 is an Astacin metalloprotease

The mutant locus was mapped using single nucleotide polymorphisms between Bristol and Hawaiian *C. elegans* isolates (Fig. 2A) (Wicks et al., 2001). Ultimately, *ox199* was mapped to a 187 kb interval between polymorphisms on cosmids F47A4 and F19C6 (Fig. 2B). This interval contained *nas-37* (C17G1.6), which encodes an Astacin extracellular-matrix metalloprotease. This gene had been shown to have a developmental or molting defect using high-throughput RNAi screens (Kamath et al., 2003; Maeda et al., 2001; Simmer et al., 2003). Because this gene was an excellent candidate to be involved in ecdysis, we sequenced *nas-37* in our four mutant strains. All four alleles had mutations in the *nas-37* open reading frame. Three alleles, *ox190*, *ox197* and *ox199*, caused premature stop codons and therefore probably represented the null phenotype of *nas-37*. We found that both *nas-37(RNAi)* and the deletion allele *nas-37(tm410)* gave the same phenotype as our mutant alleles (data not shown). Although NAS-37 has homology with proteases it is possible that it does not act as a protease in vivo. For example, mutations in the ACN-1 protease-related protein cause ecdysis defects in *C. elegans*, but ACN-1 lacks key zinc-binding residues in the metalloprotease domain and is thus unlikely to be an active protease (Brooks et al., 2003). However, the *ox196* allele of *nas-37* caused a histidine-to-arginine mutation in an invariant residue involved in coordinating the zinc ion in the metalloprotease catalytic site (Bode et al., 1992) (Fig. 2E). This mutation is just as penetrant as the three nonsense alleles, and thus NAS-37 is probably acting as a bona fide protease.

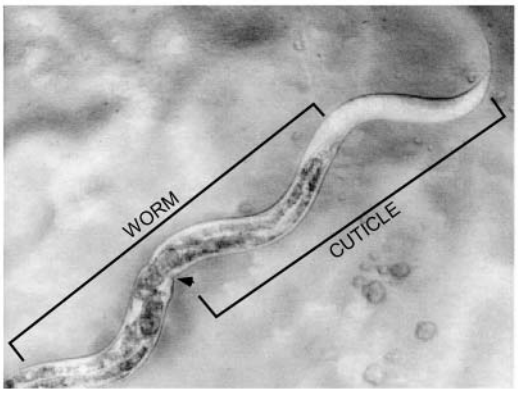

Fig. 1. *nas-37* mutations cause defects in cuticle shedding after each molt. An unshed cuticle (bracket) is dragging behind a *nas-37(ox199)* adult (bracket). The anterior end of the L4 cuticle forms a constriction just posterior to the vulva (arrowhead).

To determine the primary sequence of the protein encoded by *nas-37*, we constructed and sequenced a full-length cDNA (Fig. 2C and see Materials and methods). We found that *nas-37* is an SL1 trans-spliced gene. Sequencing of three independent cDNAs demonstrated that exon 12 is shorter than the predicted version in the public database, WormBase (www.wormbase.org). This region of the predicted protein is between the CUB and thrombospondin domains (see below) and is not conserved in paralogous proteins in the genome. Further, this region of exon 12 is poorly conserved in the corresponding region of the *C. briggsae* (CBG01954) and *C. remanei* (Washington University Genome sequencing center, unassembled whole genome shotgun sequence) orthologous genes. Although the amino acid sequence is only moderately conserved between the *C. elegans* and *C. briggsae* versions of this exon, a longer exon 12 can also be predicted in the *C. briggsae* and *C. remanei* sequences, suggesting that this exon may be longer in some transcripts. Other than this discrepancy, we found that the cDNA matched the WormBase prediction for *nas-37*. Database searches revealed that orthologs of *nas-37* are found in EST databases derived from several parasitic nematodes, including *Brugia malayi* (see below), *Strongyloides ratti* (GenBank BI741990) and *Meloidogyne chitwoodi* (GenBank CB831257). Among these, the most complete gene sequence is found in the partially completed genome sequence of *B. malayi*. Two fragments possibly representing a single gene (TIGR *Brugia malayi* WGS contigs 1132528 and 1133899) were orthologous to *C. elegans nas-37*. The splice sites of *nas-37* from *B. malayi* and *C. elegans* were conserved for four out of five introns (Fig. 2C), whereas only one out of five of *nas-37* splice sites were conserved in the closely related *C. elegans* genes *hch-1* or *toh-2*. Thus, the NAS-37 protease is conserved in parasitic nematodes and may be playing a role in ecdysis in these species as well.

The NAS-37 protein contains a secretion signal sequence and four recognizable domains (Fig. 2D). The N-terminus contains an Astacin-class zinc metalloprotease domain. This catalytic domain is found in a wide variety of proteases in both invertebrates and vertebrates and can carry out a wide variety of functions: as a digestive enzyme, a peptide-processing enzyme, a signaling maturase or a hatching enzyme. The

completed *C. elegans* genome has 39 Astacin family genes (Mohrlen et al., 2003). These all have similar protease domains; substrate specificity is probably conferred by the C-terminal domains of the proteases. The genes can be subdivided into six subgroups based on the domain composition of the C-terminus of the protein (Mohrlen et al., 2003). For example, subgroup I members contain only the protease domain, and the expression pattern of a member of this subgroup suggests that it may be serving as a general digestive enzyme in the gut (Mohrlen et al., 2003). NAS-37 belongs to subgroup V. These proteins all contain three potential protein–protein interaction domains at their C-termini: an EGF-like domain, a CUB domain and a thrombospondin domain.

nas-37 is expressed in hypodermal cells before ecdysis

Although the old cuticle remains attached in *nas-37* mutants, it is released from most of the body surface, and only remains attached by a tightly constricted ring of cuticle at its anterior end. This limited defect might be due to a specific role of NAS-37 in digesting the anterior end of the old cuticle to allow the worm to escape the cuticle. If NAS-37 has this role in ecdysis, we would expect the gene to be expressed at the anterior attachment points at the time of molting. To determine which cells express *nas-37*, we fused the promoter of this gene to the green fluorescent protein (GFP) open reading frame and generated transgenic animals. We included 3807 bp of the genomic DNA 5′ of the *nas-37* start codon, which encompasses the entire intergenic region between the 3′ end of the upstream gene C17G1.7 and the *nas-37* start codon. As expected, bright fluorescence was observed in the Hyp5 hypodermal cell at the anterior end of the larvae (Fig. 3D) as well as at other attachment points of the cuticle at the anterior end of larvae, in the arcade cells in the mouth (Fig. 3D), the anterior pharynx (not shown) and the amphid socket cells (Fig. 3C), and in the rectal epithelial cells at the posterior end of the larvae. These cell types are in contact with the cuticle and could be the sources of enzyme secretion to aid ecdysis.

Bright fluorescence was also observed in the hypodermal cells of each larval stage (Fig. 3A-C). Expression was not seen in the seam cells of L1 through L3 larvae (Fig. 3A,B); however, the seam cells of the L4 larvae strongly expressed *nas-37::GFP* (Fig. 3C). The L4 stage is when the seam cells terminally differentiate and fuse with the hypodermal syncytium. It is at this time that the seam cells secrete a set of three longitudinal ridges called alae; however, the alae in *nas-37* looked normal. The expression in the hypodermis and seam cells disappeared in adult worms after a few hours. However, expression continued in the vulval epithelial cells (Fig. 3E) and the rectal epithelial cells (Fig. 3F) in the adult. It is not clear what role *nas-37* plays in these cells in adults; it is possible that the protease continues to loosen the cuticle at these sites to accommodate growth in the adult, however there are no apparent defects in *nas-37* mutants after the L4 to adult molt.

To test whether peak NAS-37 expression coincided with ecdysis in each larval stage, we increased the temporal resolution of the GFP reporter by fusing a PEST protein degradation signal onto the C-terminus of our GFP construct (Li et al., 1998). This sequence increases protein turnover and should rapidly eliminate GFP fluorescence after expression has stopped. As expected, bright fluorescence was observed in the Hyp5 cell at the anterior tip of the animal and in the rectal epithelial cells at ecdysis (Fig. 4). After the molt, *nas-37* expression was very low in all cells, although expression continued in Hyp5 and the rectal epithelial cells. GFP expression driven by the *nas-37* promoter (*Pnas-37::GPF*) began to rise about 6 hours before ecdysis, with peak *nas-37*

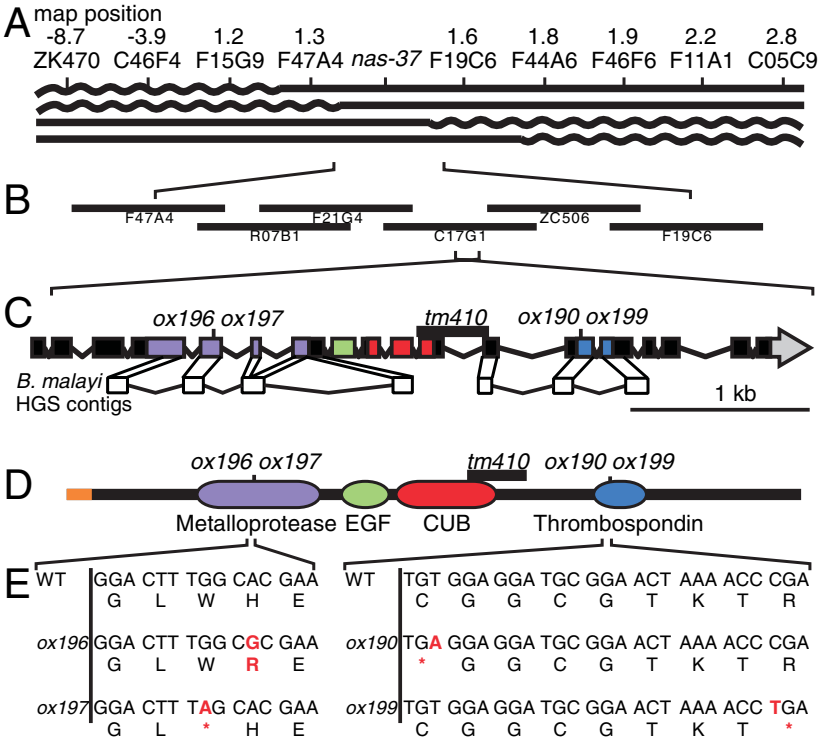

Fig. 2. Genomic structure of *nas-37*. (A) A schematic representation of the four most informative recombinant chromosomes identified using single nucleotide polymorphism mapping. Polymorphisms with Hawaiian (wavy lines) and Bristol (straight lines) DNA show that *nas-37* is between F47A4 and F19C6. (B) This region corresponds to an overlapping set of six cosmids on the X chromosome. (C) The *nas-37* gene has 18 exons. The positions of the metalloprotease, EGF-like, CUB and thrombospondin domains are indicated by colored exon regions. The 3′ untranslated region is indicated in gray (cDNA GenBank accession C54259). The transcript is SL1 trans-spliced. *ox196* and *ox197* mutations are in exon 5, *ox190* and *ox199* are in exon 13, *tm410* deletes exon 11 and part of 12. Two regions of *B. malayi* whole genome shotgun sequence with strong similarity to *nas-37* are shown below the *nas-37* structure. Intron–exon boundaries were predicted using *C. elegans* consensus splice site signals (Blumenthal and Steward, 1997) and homology to *nas-37*. (D) A schematic of the NAS-37 protein, showing the locations of each identified domain (protein GenBank accession #CAB01675). The secretion signal peptide is represented in orange. (E) The sequences of the *ox196*, *ox197*, *ox190* and *ox199* mutations. *ox196* is a missense mutation, while the other three are nonsense mutations. It is noteworthy that the natures of the point mutations in two of the *nas-37* alleles are unusual (see Materials and methods).

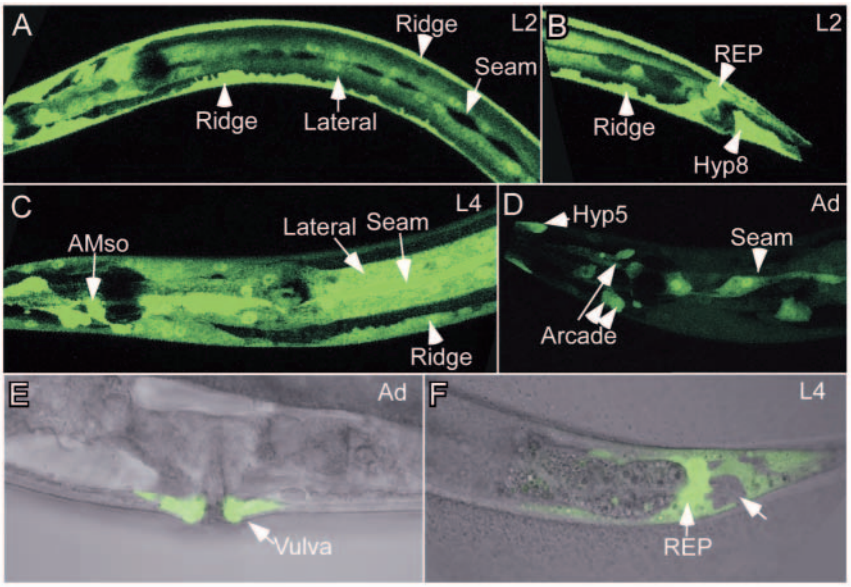

Fig. 3. *nas-37* is transcribed in the hypoderm. A *nas-37* promoter GFP transcriptional fusion (*Pnas-37::GFP*) 3807 bp of upstream promoter was fused to the GFP open reading frame at the start codon of *nas-37*. In these confocal micrographs, anterior is left and dorsal is up. (A) An L2 larva exhibits strong expression of the reporter construct in the hypodermis. Intense staining on the dorsal and ventral surfaces represents the hypodermal ridges; expression is not observed in the nervous system or in the intestine. Seam cells do not express *nas-37*. (B) The posterior region of the same animal shows strong expression in the rectal epithelial (REP) cells and the hypodermis. (C) An anterior view of an L4 larva shows hypodermal expression and strong expression in the lateral seam cells. (D) An anterior view of an early adult worm shows dim expression in the arcade cells, lateral seam cells and Hyp5. (E) *Pnas-37::GFP* strongly expresses in the vulval epithelial cells throughout the adult stage, and (F) in the rectal epithelial cells, which do not secrete a cuticle, but is not expressed in the adjacent hypodermal cells, which secrete the cuticle of the rectum (arrow).

expression in hypodermal cells (and seam cells exclusively in the L4 stage) about 4 hours before each molt (Fig. 4). Although the time of expression from the *nas-37* promoter does not indicate when the NAS-37 protein is acting, expression in the hypoderm 4 hours before molting is coincident with apolysis and with collagen gene expression.

NAS-37 is found in the cuticle

To determine where NAS-37 protein is localized, we constructed a NAS-37::GFP translational fusion under control of the same *nas-37* promoter region used in the transcriptional fusion. This construct rescues *nas-37(ox199)* to wild type. The NAS-37::GFP fusion protein was found in the cuticle, with high concentration in the anterior of the worm overlying Hyp5 (Fig. 5A,C). It was also concentrated in the alae (not shown), vulval cuticle (Fig. 5B) and cuticle of the rectum (Fig. 5D). In addition, the protein was abundantly expressed in the excretory duct cell and would therefore be released from the single anterior secretory pore (Fig. 5A). The anterior concentration of the protein is consistent with the ecdysis defect seen in the mutant, as this is the portion of the cuticle that fails to fully open during molting. We found that the protein began to appear in the anterior cuticle 4 hours before each molt (data not shown), consistent with our transcriptional GFP fusion results. Despite high levels of transcription of *nas-37* in hypodermal cells, high levels of protein accumulation were not observed in these cells. GFP fluorescence was also observed in shed cuticles (Fig. 5E), demonstrating that the fusion protein is indeed secreted and deposited into the old cuticle. Thus, NAS-37 is deposited at the right time and place for a protease that facilitates ecdysis. It is not clear whether the protease is active immediately after secretion, or whether it is activated specifically during ecdysis, potentially by other proteases.

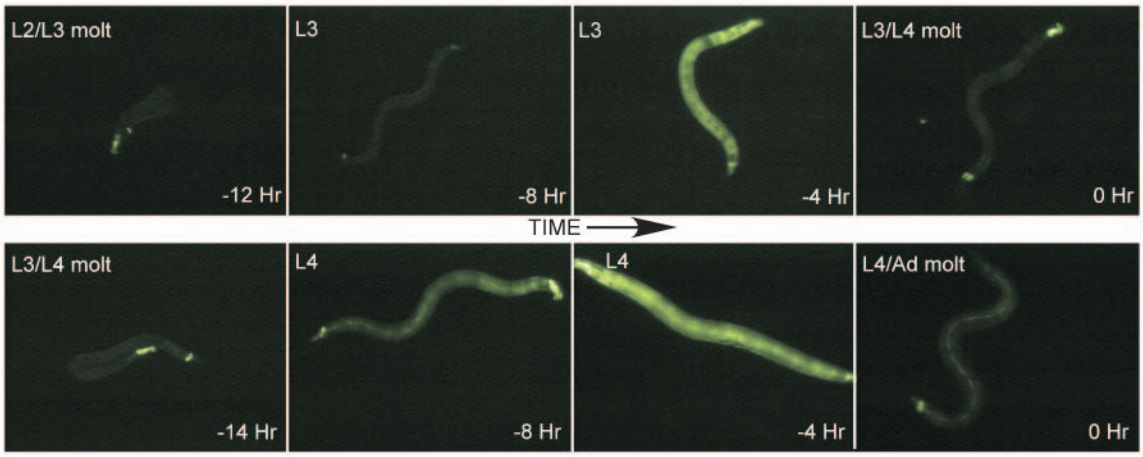

Fig. 4. *nas-37* expression peaks 4 hours before the molt. A destabilized GFP fused to the *nas-37* promoter shows the timing of *nas-37* expression relative to the molt. Single animals expressing *Pnas-37::GFP::PEST* were imaged repeatedly during the indicated larval stage, with times indicated relative to ecdysis. Expression is low during most of the larval stage, but begins to increase about 6 hours before ecdysis (not shown) and peaks about 4 hours before ecdysis. The GFP level then declines to a low level at the molt. Animals were maintained on agar plates at 19°C throughout the experiment.

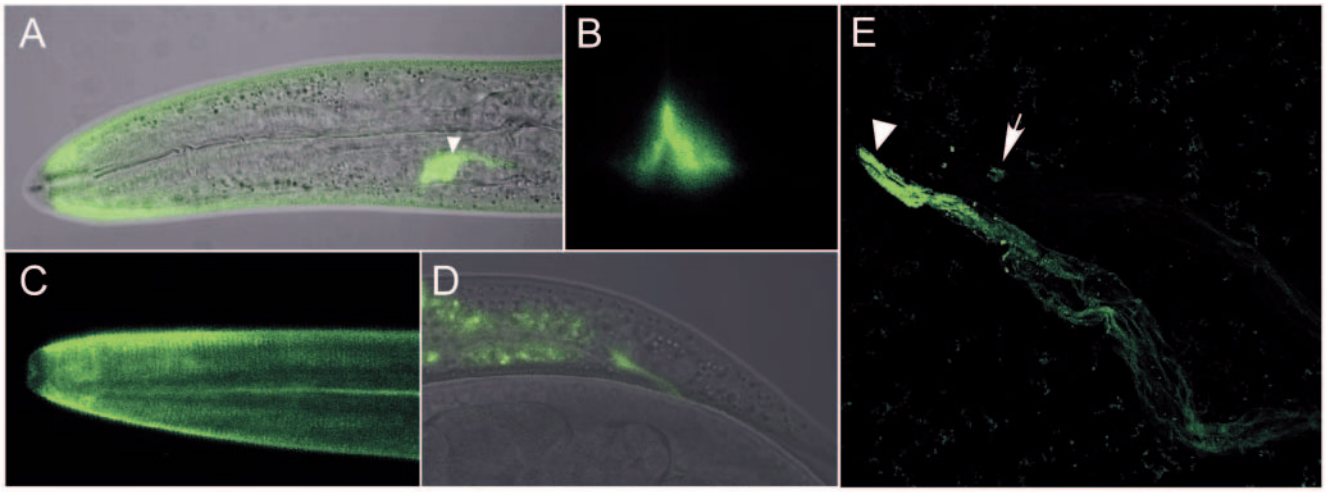

Fig. 5. NAS-37::GFP protein is secreted. A C-terminal GFP fusion to NAS-37 expressed under the *nas-37* promoter shows localization of NAS-37 protein. An overlay of a confocal projection of GFP fluorescence with the corresponding DIC image of an L4 worm (A) shows that NAS-37 is found in the cuticle and accumulates at the anterior end of the worm. It is also strongly expressed in the excretory duct cell (arrowhead). A confocal micrograph of the adult vulva (B) shows that NAS-37 accumulates in the vulval cuticle. A confocal projection of more lateral cuticle of an L4 animal (C) shows that NAS-37 is concentrated in the annular rings of the anterior cuticle, as well as in the lateral cuticle. A view of the posterior region of an L4 animal (D) shows that NAS-37 accumulates in the cuticle of the rectum. Fluorescence in the gut is due to autofluorescent particles and is not NAS-37::GFP. A confocal micrograph of a molted cuticle (E) shows that the NAS-37 protein accumulates in the anterior (arrowhead) and posterior (arrow) ends of the shed cuticle.

Cuticle collagen patterns are disrupted in *nas-37* mutants

Because NAS-37 is expressed coincident with collagen expression, it is possible that NAS-37 is required for processing newly synthesized collagen. To determine whether *nas-37* mutations affect the synthesis of cuticle, we examined the cuticle structure of *nas-37* mutants using Nomarski differential interference contrast, an adult-specific GFP-tagged COL-19 collagen strain (Thein et al., 2003), and antibodies to the DPY-7 collagen (McMahon et al., 2003). Using Nomarski microscopy, we observed that the inner layer of cuticle contained a meshwork of thick refractile filaments above the entire lateral hypodermis in *nas-37* mutant adults (Fig. 6A,B). This thick network is sometimes seen in collagen mutants (Thein et al., 2003) and may represent improperly processed collagen

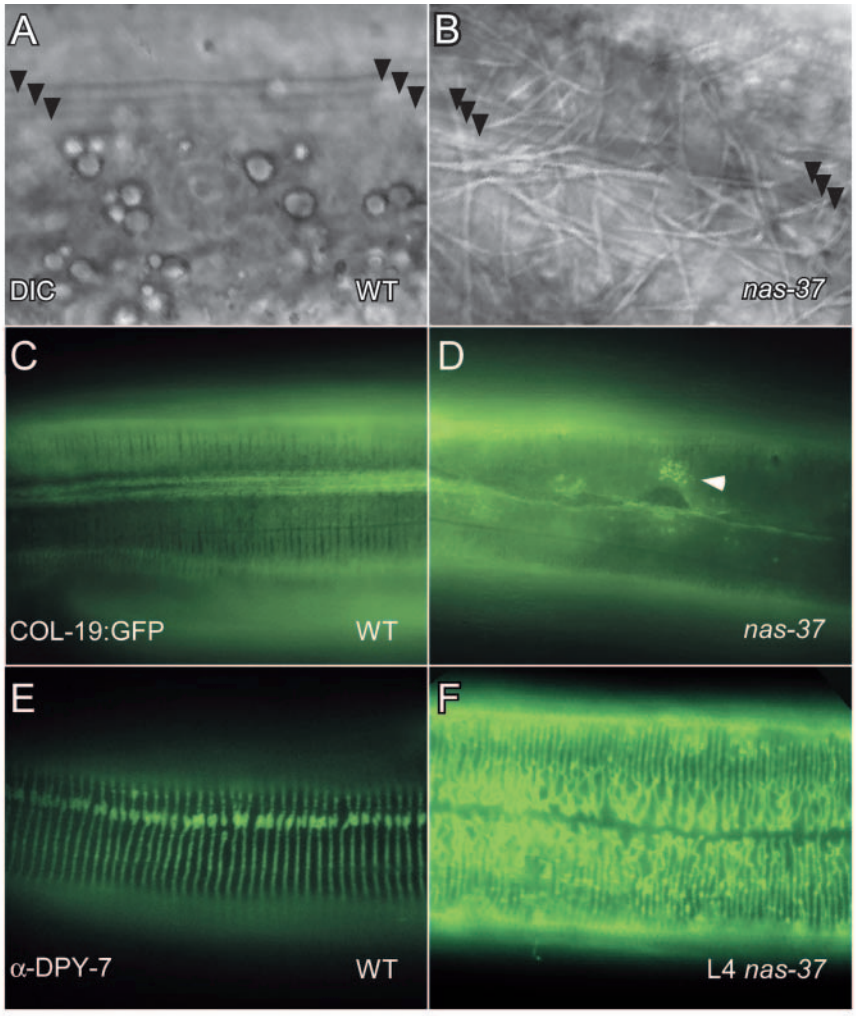

Fig. 6. Cuticle defects in *nas-37* mutants. A DIC view of wild-type (A) and *nas-37* (B) adults shows normal alae (slightly out of the plane of focus, left to right, arrowheads), but a network of fibers in deeper layers of the cuticle in *nas-37*. This network is not present in the wild type. The COL-19::GFP pattern of wild-type (C) and *nas-37* (D) shows that the pattern is disrupted in the adult stages of *nas-37* mutants (arrowhead). Anti-DPY-7 collagen staining in the wild type (E) and *nas-37* (F) shows that the annuli are disrupted and do not laterally overlap in L4 stages of *nas-37* mutants, compared with the overlapping pattern in wild-type L4s.

fibers. This network was not seen in larval stages of *nas-37* mutants (data not shown). GFP-tagged COL-19 collagen accumulated in small patches that were associated with gaps along the lateral midline of *nas-37* adults (Fig. 6C,D). Immunofluorescence images of the DPY-7 cuticular collagen demonstrated that the structures of the circumferential DPY-7 annuli were disrupted along the lateral cuticle in *nas-37* L4 larvae and adults (Fig. 6E,F), but not in the L1, L2 or L3 stages.

NAS-37 induces refractile ring formation in *Haemonchus contortus* cuticles

The molting defects of *nas-37* mutants suggested that NAS-37 plays a specific role in ecdysis, the opening of the cuticle to allow the worm to escape. A zinc-containing metalloprotease has been purified from the exsheathing fluids of the parasitic nematode *H. contortus* (Gamble et al., 1989b). This purified protease is able to induce the formation of a refractile ring 20 μm from the anterior tip of isolated cuticles in vitro (Gamble et al., 1989b). This ring is indistinguishable from cuticle changes induced during exsheathment in vivo (Gamble et al., 1989a). While the molecular identity of this protease is unknown, the *H. contortus* ortholog of NAS-37 is an excellent candidate for this protease. Gamble et al. (Gamble et al., 1989b) found that *H. contortus* exsheathing fluid could crossreact with cuticles isolated from four other trichostrongyles nematodes. If there is sufficient conservation between the molting processes in *C. elegans* and the exsheathment system in *H. contortus*, it is possible that the two proteases still retain some degree of crossreactivity to each other's cuticle substrates. To test whether this is the case, we tested whether purified NAS-37 protein could induce refractile ring formation in isolated *H. contortus* second-molt cuticles. We found that purified recombinant NAS-37 at 10 μg/ml could induce refractile ring formation resembling the in-vivo *Haemonchus* exsheathment (Fig. 7). Because neither collagenase nor trypsin is able to induce refractile ring formation (Gamble et al., 1989b), and because NAS-37 specifically causes refractile rings without noticeably affecting the cuticle as a whole, it is likely that NAS-37 shares specific substrates with the endogenous *H. contortus* exsheathing metalloprotease.

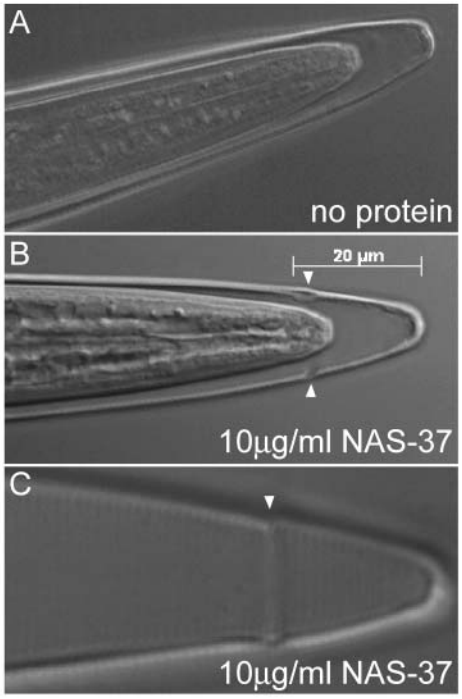

Fig. 7. NAS-37 induces refractile ring formation in isolated *H. contortus* second-molt cuticles. A DIC view of isolated *H. contortus* second-molt cuticles incubated with no protein (A) or 10 μg/ml purified NAS-37 protein (B,C) shows that NAS-37 can induce refractile ring (arrowheads) formation, 20 μm distal to the anterior tip.

Discussion

NAS-37 plays a critical role in *C. elegans* ecdysis. The expression of NAS-37 in Hyp5 and accumulation of protein at the tip of the nose of the animal is consistent with the ecdysis defects seen in mutant animals. The abundant expression of NAS-37 from the excretory duct cell is consistent with this role in ecdysis and molting (Bird and Bird, 1991). Likewise, pharyngeal, rectal, amphid and vulval expression patterns are consistent with the loosening of cuticle in these regions. Further, the ability of NAS-37 to crossreact with parasitic nematode cuticles suggests that the cuticle targets of NAS-37 during nematode exsheathment and ecdysis have been evolutionarily conserved.

A degradative function for NAS-37 is consistent with the known function of the related class V Astacin metalloprotease *nas-34*. *nas-34* is also known as *hch-1*, because mutations in this gene have been found to cause delayed *h*at*ch*ing from the eggshell (Hishida et al., 1996). Because these mutations can be suppressed by exogenously applied proteinases, it has been suggested that this gene encodes a *C. elegans* enzyme responsible for degrading eggshell proteins at hatching. Our results are consistent with a similar degradative role for NAS-37 during ecdysis.

It is interesting that NAS-37 protein is present in the anterior cuticle up to 4 hours before ecdysis, even though the actual opening of the anterior cuticle occurs in only a few minutes. Astacins require proteolytic processing to remove an inhibitory N-terminal pro-peptide, either through autoproteolytic activation or through processing by other proteases (Yiallouros et al., 2002). It is not known whether nematode astacins, including NAS-37, are able to auto-activate, or require processing by other proteases. It is possible that NAS-37 is secreted into the old cuticle in an inactive form (ether as a zymogen or complexed with an inhibitor) and activated only by a second protease secreted at ecdysis. Alternatively, NAS-37 may be acting continuously on the anterior cuticle during the entire 4-hour period, weakening it in preparation for mechanical disruption by movements of the pharynx and nose during ecdysis. We cannot conclusively distinguish between these possibilities. However, our observation that recombinant NAS-37 showed proteolytic activity demonstrates that NAS-37 can auto-activate to some degree, and may favor the model that NAS-37 is active the entire 4-hour period.

The expression of *Pnas-37::GFP* in almost all hypodermal cells at the start of new cuticle deposition suggests that in addition to opening the anterior of the cuticle, NAS-37 may play other minor roles in cuticle processing. Although this expression pattern suggests that NAS-37 might be involved in

apolysis (that is, in the separation of the cuticle from the hypoderm), no defects in apolysis were observed in *nas-37* mutants. It is possible that NAS-37 functions during apolysis but is redundant with other secreted proteases. Hypodermal expression is also consistent with a role for NAS-37 in collagen maturation. We did observe minor defects in collagen organization in the cuticle. Such a role is consistent with yet another class V Astacin metalloprotease in *C. elegans*, *nas-35* (also known as *dpy-31* and as *toh-2*). Because of specific genetic interactions with collagen mutants, *nas-35* has been proposed to be a procollagen-C-proteinase, involved in the proteolytic maturation of procollagen into its mature, functional form (Novelli et al., 2004). Similarly, NAS-37 may mature specific collagens. However, the collagen defects seen in *nas-37* mutants are mild relative to mutations that are known to affect collagen synthesis specifically (Thein et al., 2003). Additionally, if NAS-37 were necessary for all collagen maturation, then we would expect to observe expression during cuticle deposition at all stages, including the embryo. No expression is observed in the hypoderm during the formation of the L1 cuticle during embryogenesis. Moreover, there is no apparent expression of NAS-37 in the adult hypoderm even though the animal continues to grow and secrete collagen. Cuticular defects are not observed in earlier larval stages or in the non-lateral hypoderm of L4 larvae or adults. Thus, we favor an interpretation that the defects we see in collagen deposition are caused indirectly by defects in apolysis or ecdysis. Further, the cuticle defects are mild, and protein accumulation in the hypoderm is relatively small, suggesting only a minor role for NAS-37 in these regions.

In conclusion, we have identified an Astacin-class secreted metalloprotease that is required for the final step of nematode molting, ecdysis, but that may play supporting roles in the earlier steps of apolysis and cuticle synthesis. In addition, we have shown that ecdysis and exsheathment processes are likely to share conserved cuticular targets, and that orthologous genes in parasitic nematodes may be involved in the critical exsheathment process that initiates the infective stage of these animals.

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