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are discussed.

Antony P. Page*, Gillian McCormack and Andrew J. Birnie.

Institute of Comparative Medicine
Faculty of Veterinary Medicine
University of Glasgow
Bearsden Road
Glasgow
G61 1QH

Tel: 44 141 3301997
Fax. 44141 330560

* Corresponding Author a.page@vet.gla.ac.uk
Abstract

The nematode Caenorhabditis elegans represents an excellent model in which to examine nematode gene expression and function. A completed genome, straightforward transgenesis, available mutants and practical genome-wide RNAi approaches provide an invaluable toolkit in the characterization of nematode genes. We have performed a targeted RNAi screen in an attempt to identify components of the cuticle collagen biosynthetic pathway. Collagen biosynthesis and cuticle assembly are multi-step processes that involve numerous key enzymes involved in post-translational modification, trimer folding, procollagen processing and subsequent cross-linking stages. Many of these steps, the modifications and the enzymes are unique to nematodes and may represent attractive targets for the control of parasitic nematodes. A novel serine protease inhibitor was uncovered during our targeted screen, which is involved in collagen maturation, proper cuticle assembly and the moulting process. We have confirmed a link between this inhibitor and the previously uncharacterized bli-5 locus in C. elegans. The mutant phenotype, spatial expression pattern and the over-expression phenotype of the BLI-5 protease inhibitor and their relevance to collagen biosynthesis are discussed. (171)

Keywords: Cuticle, collagen, C. elegans, kunitz, nematode, enzyme.
1. Introduction

The nematode cuticle is a multilayered, flexible exoskeleton that affords protection from adverse environmental conditions including the host’s immune response. This structure and the moulting process have been most extensively studied in the free-living model nematode *Caenorhabditis elegans* (Singh and Sulston, 1978); (Kramer, 1997); (Johnstone, 2000); (Page, 2001). The cuticle is synthesized during late embryogenesis, then shed and re-synthesized at each larval stage through a process known as moulting. This process involves extensive tissue remodelling and is assisted by numerous proteases (Lustigman, 1993); (Davis et al., 2004); (Brooks et al., 2003). The major component (80%) of this extracellular matrix (ECM) is collagen. Collagens are ubiquitous trimeric structural proteins with a characteristic Glycine-X-Y tripeptide repeat, where X is commonly proline and Y is hydroxyproline. In addition to collagens, a novel highly cross-linked insoluble class of protein called cuticlin is associated with the cuticle (Sapio et al., 2005). The outermost layer, the epicuticle is lipid-rich and this in turn is overlaid by the glycoprotein-rich surface coat (Page and Winter, 2003).

The cuticle collagen gene family in *C. elegans* encodes 154 members, 21 of these when mutated result in a range of informative body morphology defects Dumpy (Dpy), Roller (Rol), Blister (Bli), Squat (Sqt), Ray abnormal (Ram) and Long (lon). Likewise mutations in the structural cuticlin genes result in morphological defects, predominantly of the Dpy class (Sapio et al., 2005). In addition to the collagen and cuticlin-related mutants a smaller number of morphogenetic mutants are associated with the collagen biosynthetic pathway. Collagen biosynthesis is a complex, multi-step process in both vertebrates and invertebrates (Figure 1). These modifications occur both intra- and extracellularly and involve chaperones and numerous modifying, folding and processing enzymes, and mutations in some members produce profound body morphology phenotypes.
The first important co-translational modification of procollagen is prolyl 4-hydroxylation which allows the proper folding into a thermally-stable form (Figure 1). The *C. elegans* *dpy-18* locus encodes a critical α subunit of the multi-component enzyme prolyl 4-hydroxylase (Winter and Page, 2000). These enzymes associate with the β subunit protein disulphide isomerase (PDI) to form active, soluble ER-resident enzymes. Null mutants of *dpy-18* are medium Dpy and the cuticle collagens exhibit reduced hydroxyproline content (Winter and Page, 2000). The combination of *dpy-18* and RNAi of a second α subunit *phy-2* (Winter and Page, 2000) or by crossing with a *phy-2* deletion mutant (Friedman et al., 2000), both result in embryonic lethality. Embryonic death was also noted following RNAi of the *pdi-2* β subunit-encoding gene (Winter and Page, 2000).

Unique active complexes between these 3 subunits were detected, both *in vitro* and *in vivo*; namely, mixed PHY-1/PHY-2/(PDI-2)\(_2\) tetramers and PHY-1/PDI-2 and PHY-2/PDI-2 dimers (Myllyharju et al., 2002).

The next important step in vertebrate collagen folding that must precede trimer formation is the correct registration of the monomers. Collagen trimerization has principally been reported to be initiated from the C-terminus, and may be set up via PDI catalysed disulfide bond formation (Figure 1). In support of this contention, the *C. elegans* collagens have C-terminal conserved cysteine clusters. In addition to the role in proline hydroxylation it is proposed that PDI-2 may be involved in this oxidative registration step.

The trimerization of imino-rich (~25% proline and hydroxyproline) collagen is rate-limited by the slow *cis-trans* proline isomerization, and requires the assistance of peptidyl prolyl cis-trans isomerase (PPIase) enzymes, of the cyclophilin (CYP) and FK506 binding protein (FKB) class (Figure 1). These genes constitute large redundant families in the worm including several ER-resident isoforms that may perform this function (Page et al., 1996); (Page, 1997); (Bell et al., in press).
Various chaperones, including SEC-23, have been implicated in the transit of procollagen from the ER into the secretory pathway (Roberts et al., 2003). The transport steps are thought to coincide with the pro-collagen N- and C-terminal processing events, that change the solubility properties and precede the mutlimerization and crosslinking of the mature insoluble collagens (Figure 1). The Kex2, furin-type serine protease encoding gene bli-4 is essential for post-embryonic viability in C. elegans (Peters et al., 1991). All cuticle collagens possess a highly conserved N-terminal cleavage site for BLI-4, a site experimentally demonstrated to be essential for the proper processing of ROL-6 and SQT-1 collagens (Yang and Kramer, 1994). Null mutants of bli-4 are embryonically lethal, whereas partial loss-of-function mutants were viable but Bli and Dpy (Peters et al., 1991); (Thacker et al., 1995), and recent RNAi screens targeting bli-4 also describe a moult defect (Kamath et al., 2001). The C-terminal processing of vertebrate fibrillar collagens is an essential step, and is carried out by zinc metalloprotease of the astacin, BMP class (bone morphogenic protein) (Canty and Kadler, 2005); (Pappano et al., 2003). The majority of nematode collagens have very short C-terminal non Gly-X-Y domains and until recently this processing step was not considered to be important for their maturation. Isolation and characterization of mutations in the astacin-encoding gene dpy-31 result in temperature sensitive severe Dpy, Emb and Let phenotypes in the worm (Novelli et al., 2004). This enzyme is hypodermally expressed and is required for normal cuticle collagen secretion. Suppressor screens identified the essential cuticle collagen SQT-3 to be a major substrate for this enzyme, and highlighted its cleavage site adjacent to the C-terminal tyrosine cross-linking site, demonstrating that dpy-31 encodes an essential procollagen C-peptidase (Novelli et al., 2004), (Figure 1).

The final steps in collagen maturation and cuticle synthesis are the structural cross-linking events. The cuticle collagens and cuticlin are covalently cross-linked via unusual di- and tri-tryrosine cross-links. These non-reducible cross-links impart the characteristic
strength and integrity to the cuticle (Page, 2001) and differ from the hydroxylysine-derived crosslinks of the vertebrate collagens (Myllyharju and Kivirikko, 2001). The major cuticle cross-linking enzyme is a dual oxidase enzyme encoded by the bli-3 locus (Edens et al., 2001); (Simmer et al., 2003), (Figure 1). This large enzyme has a signal peptide, a membrane-bound peroxide generating domain and a functionally-active peroxidase domain (Edens et al., 2001). RNAi (Edens et al., 2001) or single point mutations (Simmer et al., 2003) in this enzyme result in weakened cuticles that lack tyrosine cross-links, with resulting adult worms devoid of cuticle struts and exhibiting Dpy and Bli phenotypes. Characterization of this critical pathway is helping to uncover many nematode-specific features that may prove to be fruitful in future nematode control strategies. In addition to forward genetic screens to identify factors that affect body morphology and cuticle synthesis, numerous genome-wide RNA interference studies have identified novel genes and indeed have confirmed the significance of previously identified components of the collagen biosynthetic machinery (Kamath et al., 2003); (Simmer et al., 2003); (Frand et al., 2005). We have carried out a selective RNAi screen to identify cuticle collagen biosynthetic pathway factors using a tagged adult specific collagen strain, COL-19::gfp (Thein et al., 2003). This targeted approach focused on predicted collagen-associated enzymes and their inhibitors and uncovered, amongst other targets, a serine protease inhibitor F45G2.5 and linked it to the previously uncharacterized morphological defect bli-5. The identification and characterization of the BLI-5 kunitz protease inhibitor and its predicted role in the cuticle collagen biosynthesis pathway is described.

2. Materials and Methods

2.1 Strains. CB518 (bli-5), DR96 (unc-76) and N2 (wild type) C. elegans strains were obtained from the C. elegans Genetics Center. Worms were cultured and manipulated following standard C. elegans methods (Sulston and Hodgkin, 1988).
2.2 COL-19::gfp RNAi screens. The construction of the TP12 (COL-19::gfp) integrated strain and TP18 (CB518/TP12) strains are described elsewhere (Thein et al., 2003). Briefly, the annotated genome (www.wormbase.org) was screened for predicted collagen biosynthetic enzymes, together with expected inhibitors. These primary hits were either retrieved from the RNAi feeding library (Kamath et al., 2003) or cloned independently into the feeding vector L4440. TP12 COL-19::gfp nematodes were then allowed to feed on the RNAi bacteria and viewed at regular intervals under epifluorescence to observe the effect on the tagged collagen. For F45G2.5, a 550bp cDNA fragment was generated by PCR (primers bli5F, gcggatccaatgacctggaag and bli5R, gcgctgcagtcagaaaagatgaaagttggac) and cloned into the RNAi feeding vector L4440. Details of feeding experiments are described in detail elsewhere (Thein et al., 2003).

2.3 Microscopy. Worms were mounted on agar pads viewed under DIC or epifluorescence optics on a Zeiss Axioscope 2 and images were captured on an Axiocam MRm camera using Axiovision software. TEM samples were fixed in 2.5% gluteraldehyde then 1% osmium tetroxide in 0.1M phosphate buffers. Samples were dehydrated and infiltrated in LR-White (Page et al., 1992) and viewed on a Zeiss 902 transmission electron microscope.

2.4 bli-5 gene cloning. The bli-5 genomic and cDNA transcripts were cloned into a Topo vector (Invitrogen) prior to sequencing, using the above bli5F and R primers on genomic and cDNA template derived from bli-5 (e518) mutant worms using standard methods (Page et al., 1996).

2.5 bli-5 rescue. Two bli-5 rescue constructs were employed in this study, the first was a full-length copy of the cosmid clone F45G2 that was provided by Alan Coulson (Sanger...
The cosmid was co-injected at 10ng/µl with a marker for transformation, *dpy-7::gfp*, at 5ng/µl. The second construct was generated by PCR using genomic DNA as a template with the following primers; RescueF, gcgtgcagcgtctgacgagcgtgggcg and RescueR, gcggatcctgacgatgttagttcttcac. The 4,184bp fragment comprised the putative promoter (1,776bp), genomic coding domain and 3’UTR and was cloned into Topo and co-injected at 2ng/µl with the *dpy-7::gfp* marker at 5ng/µl. Transgenic lines were selected based on fluorescence and examined for repair of phenotype of the CB518 mutant background.

2.6 bli-5 spatial expression. A 2,310bp genomic insert encompassing the putative promoter to the second exon of F45G2.5 was generated by PCR using the following primer pair; RescueF and PromoR, gcgggatccgtcaggcatttctggagttatg. The insert was digested with *Pst* I and *Bam* HI and cloned into the reporter vector pPD96:04 (Addgene), then injected into DR96 worms at 5ng/µl together with the *unc-76* rescue plasmid at 5ng/µl. Rescued transformants (3 independent lines) were stained for β-galactosidase activity following published methods (Winter and Page, 2000) and different lines and stages were examined microscopically.
2.7 bli-5 Overexpression. The following primers were used to clone a 609bp cDNA copy and 1,991bp genomic copy of the bli-5 gene; HsF gcgggtaccatggtatctatccataattc and HsR, gcggagctctcagaaaagatgaaagttg, using either cDNA or genomic DNA as a template. The insert was digested with Kpn I and Sac I and ligated into similarly digested hypodermal heat shock vector pPD49:78 (Addgene). The constructs were co-injected at 25ng/µl together with the unc-76 rescue plasmid (100ng/µl) into DR96 (unc-76) worms. Mixed stage rescued transgenic lines (4 independent lines) were heat shocked at 33°C for 5 hours, allowed to recover at 20°C overnight and examined for associated phenotypes.

3. Results

3.1 RNAi of F45G2.5 leads to a COL-19 disruption pattern that is similar to that of bli-5 mutants. The TP12 COL-19::gfp strain displays a uniform annular and alae distribution of fluorescence in the wild type adult cuticle (Figure 2A) and has proved to be a powerful tool in the identification of cuticle collagen biosynthetic pathway components (Thein et al., 2003). Crossing with uncharacterized morphological mutants has also been highly informative with respect to their characterization and potential identification (Thein et al., 2003). Crossing this integrated marker strain with bli-5 (e518) mutant alleles demonstrates COL-19 disruption in both the dorso/ventral annulae and the lateral alae (Figure 2B). RNAi screens of potential collagen biosynthetic pathway components identified a kunitz type serine protease inhibitor encoded by F45G2.5 (Figure 2 C) to have a comparable COL-19 disruption pattern to bli-5 mutant alleles. In addition to the aberrant fluorescent phenotype, F45G2.5 RNAi also produced blister and moult defective phenotypes (data not shown). These observations and the close genetic map position of F45G2.5 and the bli-5
locus on the end of linkage group III (Figure 3) made the encoded protease inhibitor a good candidate for the bli-5 locus. bli-5 mutant alleles were originally isolated in early morphological screens (Brenner, 1974) but previously remained uncloned. One major phenotype of this mutation took the form of blisters in the adult cuticle particularly around the head region. One of the main distinguishing features that sets the adult cuticle apart from the larval counterparts is the presence of electron dense medial struts that separate the external (cortical) and internal (fibrous and basal) layers of the cuticle (Figure 2D). This medial layer is proposed to be fluid filled and the regular position of the struts corresponds exactly to the position of the annulæ when viewed in freeze fractured cuticle preparations (Kramer, 1997). In contrast to wild type worms (Figure 2D), the cuticle of bli-5 (e518) mutant nematodes is virtually devoid of struts, and those present were incomplete when viewed by TEM (Figure 2E). The lack of struts often corresponded to the presence of blisters that are characterized by vesicle-filled swellings between the basal and cortical cuticle layers (Figure 2F).

3.2 Cloning of the F45G2.5 gene from bli-5 (e518) reveals a single point mutation and phenotype rescue confirms their predicted association.

To determine if indeed the kunitz protease inhibitor corresponded to bli-5, the gene was cloned and sequenced from both cDNA and genomic DNA isolated from the bli-5 (e518) mutant worms (Figure 3), and the cosmid that contains F45G2.5 was tested in transformation rescue experiments with this mutant allele (Figure 4). The F45G2.5 open reading frame encodes a 202 amino acid protein with a signal peptide, followed by a nematode specific EB domain and a kunitz-type trypsin inhibitor domain (Figure 3). A homologue of this gene has been partially cloned from the parasitic nematode Ostertagia ostertagi (BQ457535) and shares 60% identity over 156 residues that include the EB and kunitz domains. Cloning of this gene from both genomic and cDNA templates revealed a
single point mutation TCA- TTA in the highly conserved EB domain, resulting in a single
serine to leucine change (Figure 3). The association was further confirmed by
transformation rescue of bli-5 (e518) mutant worms, with the cosmid F45G2 (Figure 4) and
with a 4,184 bp genomic rescue construct comprising the putative promoter, coding
sequence and putative 3’UTR of the F45G2.5 gene (data not shown). Co-expression of the
dpy-7::gfp marker corresponded to F45G2.5 transformed worms that were no longer
blistered, but wild type in appearance (Figure 4 A & B). These experiments validated the
link between F45G2.5 and the bli-5 mutant locus and this gene will subsequently be
referred to as bli-5.

3.3 Localization of bli-5 is consistent with its predicted role in cuticle collagen
maturation.

The putative promoter region incorporating the first 2 exons of bli-5 was
“translationally” fused to LacZ, and was co-transformed with an unc-76 rescue plasmid
into DR96 nematodes. Rescued transformants were selected, maintained and stained with
β-galactosidase to examine the spatial expression of the bli-5 transcript. bli-5 is
abundantly expressed in the larval (Figure 5A) and adult hypodermis, the hermaphrodite
vulva (Figure 5B) and the excretory cell and duct (Figure 5C). These localizations are
consistent with an enzyme inhibitor playing a role in cuticle collagen biosynthesis, cuticle
remodelling and the moulting process.

3.4 Over-expression of BLI-5 results in moulting, cuticle and vulval defects.
The full length cDNA and genomic bli-5 genes were cloned into a hypodermally
expressed heat shock construct, co-injected into DR96 nematodes with a rescue plasmid
and resulting rescue lines were maintained and analysed following heat shock (Figure 6).
Heat shock of genomic and cDNA lines produced identical phenotypes, namely moulting and
protruding vulva defects (Figure 6 A-D). Control heat shock of wild type nematodes did not confer a phenotype (data not shown). These results are consistent with the hypothesis that BLI-5 is regulating an enzyme that is involved in cuticle synthesis, collagen processing and the general moulting process.
The free-living nematode *Caenorhabditis elegans* has long been proposed as a genetic and experimentally amenable model nematode system (Politz and Philipp, 1992); (Page, 2001); (Page and Winter, 2003); (Gilleard, 2004). The full genome sequence of *C. elegans* was deciphered in 1998 (Consortium, 1998) and numerous genome-wide RNAi screens (Kamath et al., 2003); (Simmer et al., 2003); (Frand et al., 2005) have since been performed to elucidate a function to the 20,000 encoded genes. We have exploited this system in an attempt to investigate further the enzymology of the nematode cuticle collagen assembly (Page and Winter, 2003); (Figure 1). In addition to examining known components of the collagen biosynthetic pathway (Page and Winter, 2003); (Winter and Page, 2000); (Eschenlauer and Page, 2003) we have set up RNAi-based screens with a collagen marker strain (Thein et al., 2003) to look for novel factors involved in this pathway. The identification and characterization of one such target, namely *bli-5* is described in this report.

The *bli-5* gene encodes an unusual serine protease inhibitor that has many features that link it to the collagen biosynthetic pathway. RNAi of F45G2.5 produces blister, moult (Kamath et al., 2003); (Frand et al., 2005) and COL-19 disruption defects (Figure 2), phenotypes that mirror the closely associated *bli-5* mutant locus (Figure 2). Through mutant allele cloning and mutant phenotype rescue experiments we successfully confirmed the link between F45G2.5 and *bli-5* (Figure 3 & 4). BLI-5 is a secreted serine protease inhibitor with an EB domain and a kunitz-type pancreatic protease inhibitor domain. A homologue of this unusual inhibitor is present in the veterinary parasite *Ostertagia ostertagi* (accession number BQ457535). Kunitz-type serine protease inhibitors have been described from many species, including *Drosophila melanogaster* (Kramerova et al., 2000); (Kress et al., 2004) and from parasitic nematodes including *Ancylostoma caninum*.
(Hawdon et al., 2003) and *A. ceylanicum* (Milstone et al., 2000). Indeed, the *A. ceylanicum*
h�인지자 also localized to the sub-cuticle of the adult stages (Chu et al., 2004). In
addition, a class of inhibitors called the papilins that also contain a kunitz domain have
likewise been implicated in controlling the function of the invertebrate procollagen N-
peptidase ADAMTS (Kramerova et al., 2000); (Kramerova et al., 2003).

The presence of the EB domain (Pfam identifier PF01683) is intriguing since this
domain is completely nematode specific. EB domains characteristically have 8 conserved
cysteines that are predicted to form 4 disulphide bridges and are usually found in
association with kunitz domains. The functional significance of this domain was
confirmed during the sequencing of the *bli-5 (e518)* mutant allele, revealing the presence of
a point mutation in this domain that changed a hydrophilic serine to a hydrophobic
leucine residue (Figure 3). Such a change may be predicted to alter the binding properties
of this domain to its protein partner and hence lead to the resulting mutant phenotype.
This serine was also conserved in the *O. ostertagi* inhibitor (Figure 3).

Together with the BLI-5 cuticle-related phenotypes (Figure 2); the predicted role of
kunitz-type inhibitors in extracellular matrix remodelling and moulting in insects
(Kramerova et al., 2003), (Kress et al., 2004); the BLI-5 spatial expression pattern in the
hypodermal and excretory tissues (Figure 5); and the BLI-5 overexpression effects in the
cuticle (Figure 6), all point to a role for this inhibitor in the cuticle collagen biosynthetic
pathway.

What is the specific role of BLI-5 in vivo? This is currently unknown, but the following
model may be relevant. The inhibitor BLI-5 may be interacting with, and controlling the
protease activity of the BLI-4 blisterase kex2-like enzyme, or indeed a functionally related
regulatory enzyme. This is a plausible assumption, as both *bli-4 (e937)* and *bli-5 (e518)*
mutations and RNAi of their transcripts ([www.wormbase.org](http://www.wormbase.org)) result in moulting defects
and blister phenotypes. Moulting and blister phenotype are also a feature of BLI-5 over-
expression. In support of this assertion, kunitz-type serine protease inhibitors also control
tissue remodelling enzymes that are involved in the moulting process of *Drosophila* (Kress
et al., 2004). Interestingly, BLAST analysis of BLI-5 in addition to hitting kunitz-type
inhibitors also hits papilins and more intriguingly, kex-like proprotein convertases (data
not shown) and may support the hypothesis that BLI-5 and BLI-4 can interact directly.

BLI-4 is a well-characterized kex2-like protease in *C. elegans* (Peters et al., 1991); (Thacker
et al., 1995); (Thacker et al., 2000) that has homologues in parasitic nematodes (Poole et al.,
2003). The *bli-4* gene generates 9 alternative protein isoforms of the blisterase enzyme that
are expressed in the hypodermis, vulva and the neurones (Thacker et al., 2000). The
majority of mutant alleles are embryonic lethal, however *bli-4 e937* affects a limited subset
of these forms and results in the blistering phenotype of the adult cuticle (Thacker et al.,
1995). The main function of BLI-4 is predicted to be as an N-terminal procollagen
processing enzyme (Yang and Kramer, 1994); (Kramer, 1997); (Thacker et al., 2000), and all
cuticle collagens possess the characteristic N-terminal basic RXXR recognition site of kex2-
like enzymes. The cuticle collagen disruption pattern of BLI-5, the shared phenotypes and
the shared tissue expression patterns between BLI-5 and BLI-4 are all consistent with an
association. In addition to procollagen, numerous other proteins possess the RXXR
processing site, including known moulting and collagen processing enzymes. The NAS-37
astacin metalloprotease (Davis et al., 2004) and the procollagen C-peptidase DPY-31
(Novelli et al., 2004), also an astacin metalloprotease, are both activated following
processing via an RXXR site. An additional, or indeed an alternative function BLI-5 may
therefore be in the fine control of BLI-4 and/or another subtilisin-like protease that is
performing this critical enzyme activation step.

In this paper we review some of the recent data regarding the nematode cuticle
collagen biosynthetic pathway. In addition, we highlight the relatively simple application
of the *C. elegans* model system, in combination with RNAi and the well-annotated genome
data (www.wormbase.org) to rapidly assign a function to a previously uncharacterized gene and provide a link to a previously uncloned morphogenetic mutant. Similar reverse genetic approaches will help identify additional nematode-specific genes that are involved in nematode-specific processes, such as cuticle assembly and moulting, and may ultimately help identify potential drug and/or vaccine targets of relevance to the control of parasitic nematodes.

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linking activity and is involved in the maintenance of body morphology. J. Biol. Chem. 278, 4227-4327.


**Figure Legends**

**Figure 1**

Schematic representation of the critical modification steps in the biosynthesis of nematode cuticle collagens and assembly of the nematode cuticle (see accompanying text for a full description of listed enzymes).

**Figure 2**

The COL-19 cuticle collagen and gross cuticle morphology are disrupted in a similar manner in bli-5 (e518) mutants and following F45G2.5 RNAi in a wild type background. A, Wild type expression pattern of COL-19::gfp in the wild type adult cuticle (TP12 strain). B, Aberrant COL-19::gfp expression in bli-5 (e518) mutant nematodes. C, Aberrant COL-19::gfp expression following F45G2.5 RNAi in TP12 wild type strain. A-C, arrows define annulae and arrowheads the lateral alae, scale bar represents 10µM. D, TEM of wild type cuticle, depicting regularly spaced medial struts (arrowed). E, TEM of bli-5 (e518) mutant nematodes revealing comparative lack of struts. F, TEM of bli-5 (e518) mutant nematodes depicting a surface blister separating the basal and cortical layers of the cuticle (arrowed). D-F scale bar represents 1 µM. All images depict adult nematodes.

**Figure 3**

Genomic and physical relationship between the bli-5 locus and F45G2.5. The cosmid clone F45G2 maps at the end of linkage group III in the vicinity of the bli-5 locus. The open reading frame F45G2.5 encodes a transcript with 4 exons that encodes a 202 amino acid protein with a signal peptide (Sp), and nematode specific EB domain and a kunitz-type serine protease inhibitor domain. Sequencing of the genomic and cDNA copies of F45G2.5 from bli-5 (e518) mutants identified a single TCA-TTA (serine to leucine) point mutation in
the conserved EB domain. The same regions from *C. elegans* (N2) and *Ostertagia ostertagi* (O. ost) are shown for comparison.

**Figure 4** (Top panel)

Cosmid rescue of bli-5 (e518) mutant locus. The cosmid clone F45G2 was co-injected with dpy-7::gfp marker and transgenic worms were assessed microscopically. A, DIC field of 2 rescued and 1 non-rescued worms (arrowhead). B, Fluorescence image of same field revealing correlation of fluorescence with rescue of blister phenotype, in comparison to the non–rescued (blistered) non-fluorescent worm (arrowhead).

**Figure 5** (Bottom panel)

Spatial expression of the bli-5 transcript. Nematodes were transformed with the bli-5 promoter reporter construct, transgenic lines were selected and populations were fixed and stained for β-galactosidase activity. A, L3 larvae with predominant hypodermal seam cell expression (arrow). B, Adult hermaphrodite vulval expression (arrow). C, Adult excretory cell and excretory duct expression (arrow).

**Figure 6**

Overexpression of BLI-5 under a hypodermal heat shock promoter results in moult and vulval defects following heat shock. A, L2 larvae encased in unshed L1 cuticle, tip of head (black arrow) and tip of tail (white arrow) positions are indicated. B, Unshed L3 cuticle (black arrow) retained on head of L4 larvae. C, Unshed L4 cuticle on the head of and adult worm (black arrow), blisters also observed on cuticle (white arrow). D, Protruding vulva (arrow) on adult hermaphrodite.
Figure 3

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