RESEARCH ARTICLE

The *hedgehog*-Related Gene *qua-1* Is Required for Molting in *Caenorhabditis elegans*

Limin Hao,[1] Krishanu Mukherjee,[1†] Samuel Liegeois,[3†] David Baillie,[4] Michel Labouesse,[3] and Thomas R. Bürglin[1,2*]

The *Caenorhabditis elegans* genome encodes ten proteins that share similarity with Hedgehog through the C-terminal Hint/Hog domain. While most genes are members of larger gene families, *qua-1* is a single copy gene. Here we show that orthologs of *qua-1* exist in many nematodes, including *Brugia malayi*, which shared a common ancestor with *C. elegans* about 300 million years ago. The QUA-1 proteins contain an N-terminal domain, the Qua domain, that is highly conserved, but whose molecular function is not known. We have studied the expression pattern of *qua-1* in *C. elegans* using a *qua-1*::GFP transcriptional fusion. *qua-1* is mainly expressed in hyp1 to hyp11 hypodermal cells, but not in seam cells. It is also expressed in intestinal and rectal cells, sensilla support cells, and the P cell lineage in L1. The expression of *qua-1*::GFP undergoes cyclical changes during development in phase with the molting cycle. It accumulates prior to molting and disappears between molts. Disruption of the *qua-1* gene function through an internal deletion that causes a frame shift with premature stop in the middle of the gene results in strong lethality. The animals arrest in the early larval stages due to defects in molting. Electron microscopy reveals double cuticles due to defective ecdysis, but no obvious defects are seen in the hypodermis. Qua domain-only::GFP and full-length QUA-1::GFP fusion constructs are secreted and associated with the overlying cuticle, but only QUA-1::GFP rescues the mutant phenotype. Our results suggest that both the Hint/Hog domain and Qua domain are critically required for the function of QUA-1. *Developmental Dynamics 235:1469–1481, 2006.*
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Key words: *Caenorhabditis elegans*; *hedgehog*; Hint; molting; cuticle; *qua-1*

Accepted 20 January 2006

INTRODUCTION

Molting is a complex developmental process in which a new exoskeleton is produced and the old one is shed. It is characteristic of the clade Ecdysozoa, which includes arthropods, tardigrades, onychophorans, nematodes, nematomorphs, kinorhynchs, and priapulids (Aguinaldo et al., 1997). This critical developmental transition is accompanied by characteristic morphological changes. In *Drosophila*, molting is regulated by pulses of steroid hormone, 20-hydroxyecdysone, and the regulatory cascade involves the ecdysone receptor (EcR), which is a transcription factor of the nuclear hormone receptor group, and two other nuclear hormone receptors (NHRs), DHR3 and βFTZ-F1 (Kageyama et al., 1997; Lam et al., 1997, 1999; White et al., 1997).

In *C. elegans*, the cuticle is a collagenous extracellular matrix (ECM) synthesized by the underlying hypodermis that surrounds the entire body

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Grant sponsor: Swedish Foundation for Strategic Research (SSF); Grant sponsor: Genome Canada/BC; Grant sponsor: Ministère de la Recherche, France.
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DOI 10.1002/dvdy.20721
Published online 24 February 2006 in Wiley InterScience (www.interscience.wiley.com).

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(Johnstone, 2000). During its life cycle, *C. elegans* synthesizes the ECM five times, once in late embryogenesis and four times at the end of each larval stage before molting. Molting in nematodes consists of three major steps: (1) separation of the old cuticle from the hypodermis; (2) formation of new cuticle arising from the apical surface of the hypodermis; and (3) shedding of the old cuticle. However, ecdysone, which is the major molting hormone in *Drosophila*, does not appear to exist in *C. elegans*, and analysis of the *C. elegans* genome does not reveal a homologue of the EcR (Sluder et al., 1999). So far, the regulatory mechanisms of *C. elegans* molting are not yet understood, although about a dozen genes have been identified that lead to molting defects when mutated. These genes can be classified into five categories based on their possible functions: (1) proteases that are used to degrade the old cuticle, including a cathepsin Z-like cysteine protease (*Ce-cpz-1*) (Hashmi et al., 2004), and two metalloproteases, *nas-36* and *nas-37* (Davis et al., 2004; Suzuki et al., 2004); (2) transcriptional regulators, including *nhr-23* (Kostrouchova et al., 1998, 2001), *nhr-25* (Asahina et al., 2000; Gissendanner and Sluder, 2000), and *let-19* (Wang et al., 2004); (3) enzymes involved in cholesterol metabolism such as *let-767* (Kuervers et al., 2003) or cholesterol transporters such as *lrp-1* (Yochem et al., 1999); (4) molecules involved in secretion and extracellular transport such as *sec-23* (Roberts et al., 2003) and Ce-Vps-27 (Roudier et al., 2005); (5) others, such as the angiotensin converting enzyme-like non-peptidase *acn-1* (Brooks et al., 2003).

A recent comprehensive analysis of *C. elegans* molting by genome-wide RNAi screening discovered 159 genes including annotated transcription factors, secreted peptides, transmembrane proteins, and extracellular matrix enzymes essential for molting (Frand et al., 2005). By analyzing the possible functions of these genes, a model has been proposed to outline the regulation of molting where endocrine and possibly neuroendocrine cues trigger molting in *C. elegans*, stimulating epithelial cells to remodel the exoskeleton near the end of each larval stage. In response to the endocrine cue, transcriptional cascades involving NHRs, particularly NHR-23, alter gene expression to amplify the signal to molt. A large group of extracellular matrix proteins and secreted enzymes contribute to the new cuticle or regulate release of the old one (Frand et al., 2005). In addition, a recent functional genomic study of *C. elegans patched* (*ptc*), *patched-related* (*ptr*), and *hedgehog*-related genes revealed that several of the *hedgehog*-related genes including *qua-1* are involved in molting (Zugasti et al., 2005).

Here we characterize *qua-1*, a gene that causes molting defects when disrupted. *qua-1* was first identified in a search for *hedgehog* (*hh*) homologs in the *C. elegans* genome (Aspöck et al., 1999). Although there is no bona fide *hh* homolog in *C. elegans*, its genome encodes ten proteins that have sequence similarity with the Hint domain of Hh. The Hint domain is the C-terminal autoprocessing domain of Hh that shares sequence similarity with inteins (Beachy et al., 1997). C-terminal of the Hint domain is the sterol recognition region (SRR), which is required for cholesterol addition (Beachy et al., 1997). Here, we refer to the complete C-terminal domain, i.e., the Hint and the SSR domain, as the Hog domain (Fig. 1).

We have previously analyzed the N-terminal domains of the *hedgehog*-related genes, and classified them into distinct gene families: *warthog, groundhog*, and *ground-like* (Aspöck et al., 1999; Bürglin and Kuwabara, 2006). Only one gene, previously referred to as M110 (Aspöck et al., 1999), was found as a single copy. In analogy to the other animal-based gene names (*hedgehog, warthog, groundhog*) that mimic the bipartite nature of the protein structure, we, in consultation with other research groups, have named M110 *qua-1*, short for *quahog*. In the present report, we analyze the sequence similarity of the *qua-1* genes in different nematode species and the expression pattern and mutant phenotype of *qua-1* in *C. elegans*. We also show that the expression of *qua-1* undergoes cyclical changes during larval development. Further, both the Qua domain and the Hog domain of QUA-1 are secreted and associated with the cuticle.

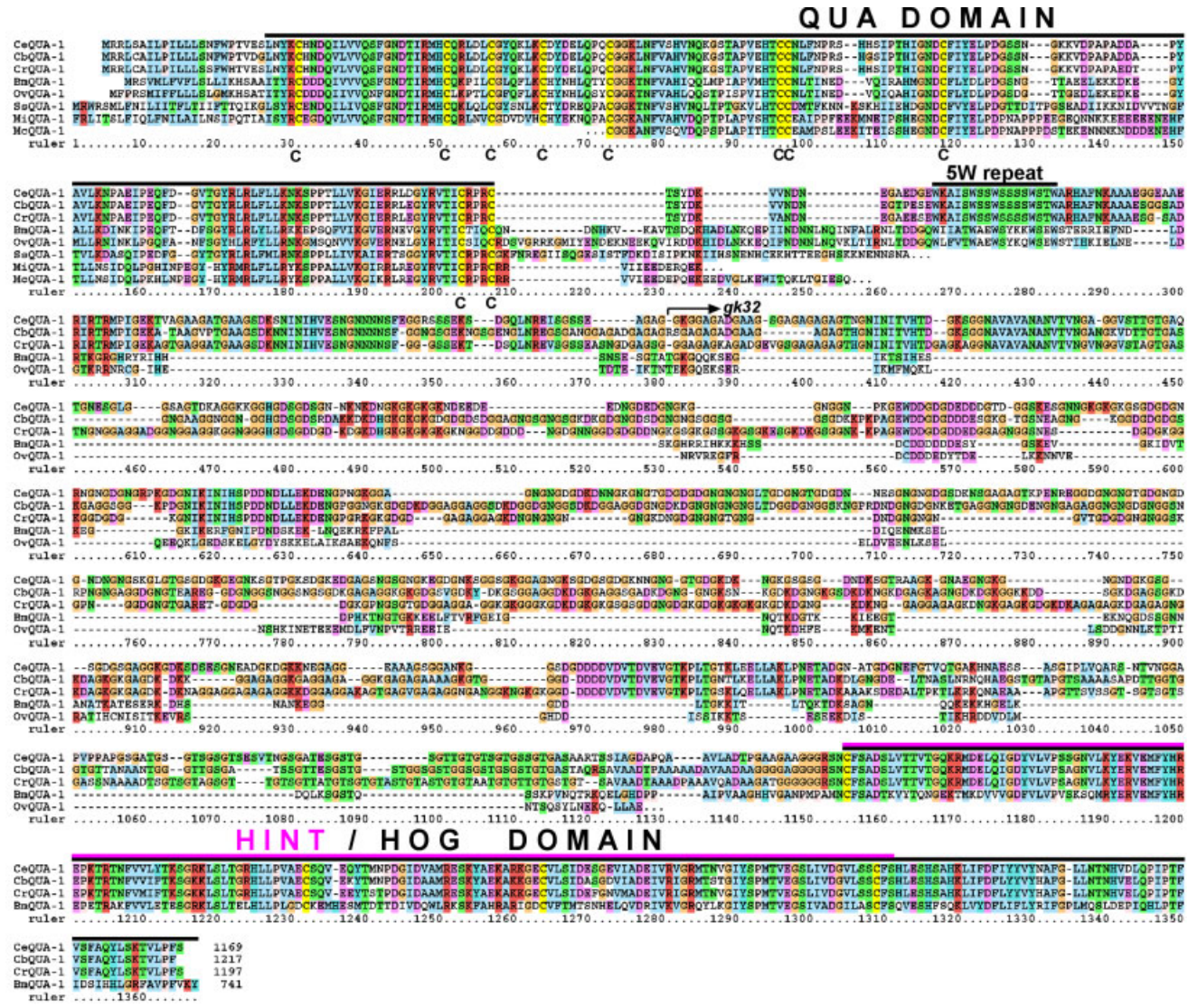
RESULTS

Structural Analysis of *qua-1* Genes

T05C12.10, a.k.a. M110 (Aspöck et al., 1999), has been named *qua-1* (pronounced kwo), and the gene family is referred to as *quahog*. The *C. elegans* QUA-1 protein sequence was used as query for performing tblastn searches against genomic and EST databases at NCBI. ESTs with sequence similarity were recovered from several nematodes, i.e., *Meloidogyne incognita, Meloidogyne chitwoodi, Strongyloides stercoralis*, and *Onchocerca volvulus*. We sequenced one of the ESTs from *O. volvulus* completely. The genomic sequence of *C. briggsae* (Stein et al., 2003) contains an ortholog of *qua-1*, CBG00717; however, we had to correct introns and the C-terminal end of

Fig. 1. Sequence alignment of Quahog proteins. Multiple sequence alignment of QUA-1 protein sequences from different nematode species (Bm, *B. malayi*, Cb, *C. briggsae*, Ce, *C. elegans*, Cr, *C. remanei*, Mc, *M. chitwoodi*, Mi, *M. incognita*, Ov, *O. volvulus*, Ss, *S. stercoralis*). The Qua and the Hog domains, as well as the 5W repeat, are marked by black lines, and the Hint domain is marked by a pink line. All proteins are predicted to have a signal sequence for protein export, and the predicted cleavage site for most proteins is right at the point where the Qua domain starts at the hydrophobic Leu or Ile. The ten cysteine residues of the Qua domain are indicated by "C." Dashes indicate gaps introduced to maintain the sequence alignment. The start of the frameshift of deletion *gk32* is marked by a line and arrow. OvQUA-1, MiQUA-1, McQUA-1, and SsQUA-1 are partial sequences. Note that the alignment of *B. malayi* QUA-1 through the repetitive region of the *Caenorhabditis* QUA-1 sequences is not definitive, the alignment varies markedly depending on parameters used; this is due to the fact that there is basically no sequence similarity.

Fig. 2. A: Intron-exon structure of four full-length QUA-1 proteins. The species codes are the same as in Figure 1. The yellow polygons indicate introns conserved from *B. malayi* to *Caenorhabditis*, and the pale yellow polygons indicate introns conserved within *Caenorhabditis*. **B:** Genomic structure of *qua-1*. The *qua-1* gene structure is shown on top, the different domains are colored as indicated in the inset legend. The extent of the deletion *gk32* is indicated, and the three GFP fusion constructs are shown at the bottom.



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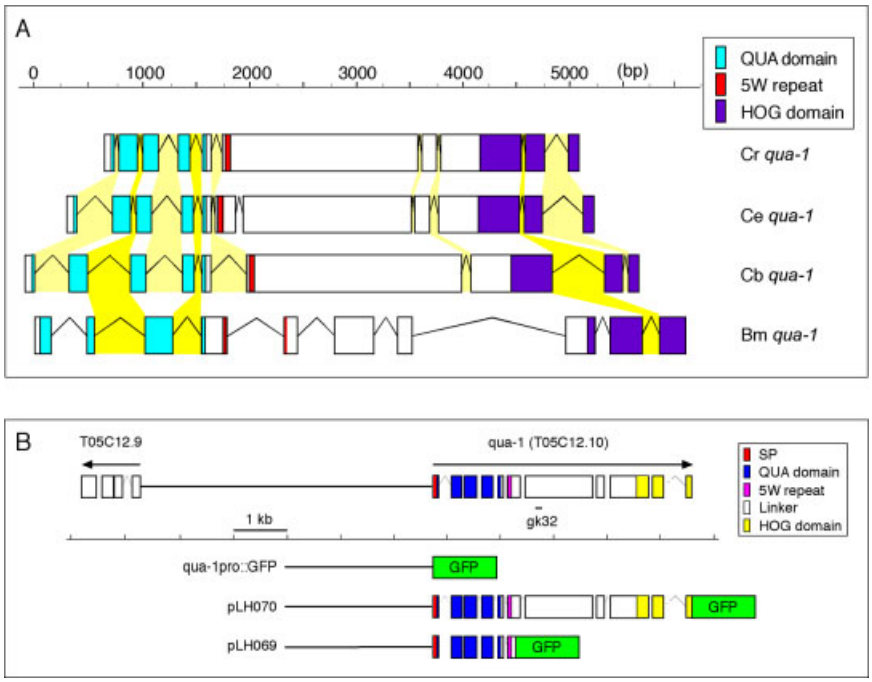

Fig. 2.

the ORF prediction by hand, since the last exon was missing. The genomic sequence coding for the *qua-1* ortholog in *C. remanei* was assembled from the trace reads at the Genome Sequencing Center at Washington University Medical School. Likewise, the *qua-1* orthologous sequence from *Brugia malayi* was assembled from genome assemblies at The Institute for Genome Research. The ORFs for both *C. remanei* and *B. malayi* were predicted from the genomic sequence using gene prediction software and manual inspection in a dotplot program using the related sequences as guides. In conclusion, we compiled four full-length sequences and four partial sequences from eight different nematode species. Apart from the similarity in the C-terminal Hog domain, no sequences with similarity to QUA-1

TABLE 1. Amino Acid Composition in the Repeat Region of the Three *Caenorhabditis* spp. QUA-1 Sequences[a]

	C. elegans (%)	*C. briggsae* (%)	*C. remanei* (%)
No. of residues	727	776	755
Gly	29.7	33.0	33.2
Ala	9.2	12.0	12.5
Ser	9.2	7.4	6.6
Thr	6.2	5.7	7.2
Asn	11.3	9.7	8.5
Asp	9.8	11.6	10.9
Glu	5.4	2.7	2.5
Lys	7.6	8.2	9.4
Arg	1.7	1.5	1.1
Acidic	15.1	14.3	13.4
Basic	9.9	10.7	11.3
Charged	25	25	24.6
G/A/S/T/N	65.6	67.8	68

[a]The repeat region is defined as starting after the conserved FNK residues (C-terminal of the 5W repeat) up to, but not including the CF residues of the Hint/Hog domain.

were discovered outside of the nematode phylum.

Multiple sequence alignment of the eight QUA-1 protein sequences revealed several interesting motifs (Fig. 1). All sequences have a predicted signal peptide for secretion. Immediately following the signal sequence is a highly conserved domain, as previously suspected (Aspöck et al., 1999). This domain, named Qua, is about 170–180 amino acids in length and contains ten conserved cysteine residues, which probably are important for the structure and disulfide bond formation. The second highly conserved domain is the C-terminal Hog domain that is also found in Hh, Warthog, and Groundhog proteins. The autoproteolytic cleavage site with the residues Cys and Phe is also conserved in the Hog domain of the QUA-1 proteins. This site has been experimentally verified for Hh and WRT-1 in vitro (Porter et al., 1996).

Downstream of the Qua domain is a small motif, that we termed 5W repeat, which is characterized by having five conserved tryptophane residues. The repeat has the form WxxxxWxxWxxxxWxxW, and there are many serine residues at the intervening positions. Presently, we have found no other proteins with similarity to this highly unusual repeat. Between the 5W repeat and the Hog domain is a long "linker" region in the *Caenorhabditis* spp. QUA-1 proteins that is characterized by large stretches of repeated residues interspersed with smaller blocks only conserved within *Caenorhabditis* spp. The repeated stretches are especially rich in glycine (~30%, Table 1), but also contain repetitive elements comprised of other small residues, i.e., alanine, serine, threonine, and asparagine (Table 1). These small residues, including glycine, make up about 66% of all residues in that region. This region is also highly charged (25%, Table 1), with acidic residues being more common. The secreted portion of the *Caenorhabditis* spp. QUA-1 proteins (Qua domain up to the autocatalytic cleavage site) is consequently slightly negatively charged. The residues found in the repeat region occur also frequently in repetitive regions of other proteins, such as homeodomain transcription factors (Bürglin, 1994), and probably serve mainly as flexible linker regions. The corresponding region in *B. malayi* QUA-1 is much shorter and it also contains a lot fewer repeated residues. The secreted portion of *B. malayi* QUA-1 is slightly basic (+15), in contrast to the *Caenorhabditis* spp. QUA-1 proteins. The non-conserved nature of the linker region suggests that it is not highly critical for function.

The intron/exon structure comparison of the *qua-1* genes shows that most introns are conserved between the three *Caenorhabditis* spp. (Fig. 2A). There is more divergence to *B. malayi*, only 3 introns are unambiguously conserved (Fig. 2A). The intron/exon structure of *B. malayi* also shows that no major exon in the linker region between the Qua and Hog domains exists that could encode a repeated region.

To better understand the origin of the *qua-1* gene family, we performed phylogenetic analyses (Fig. 3). Neighbor joining analysis of the Hog domains shows that the Quahog Hog domains are more similar to each other than to other Hog domains. Furthermore, these Hog domains are more similar to Hog domains of Warthog and Groundhog proteins than to those of Hh proteins (Fig. 3C). We also performed a phylogenetic analysis of the Qua domain. The clades of the phylogenetic tree have a similar topology as an evolutionary tree of nematodes (Fig. 3A,B). This suggests that the *quahog* gene diverged and evolved in early nematode evolution, and that it subsequently became fixed and evolved at more constant rates in the different nematode taxa.

Expression Pattern of *C. elegans qua-1*

To study the expression pattern of *qua-1*, a transcriptional GFP fusion construct of *qua-1* was made by PCR and directly injected into worms to generate transgenic lines (Fig. 2B). *qua-1pro*::GFP was found to be expressed in the hypodermal cells covering the whole body from the tip of the nose to the tip of the tail spike, but not in the lateral hypodermal cells, i.e., the seam cells (Fig. 4A). Furthermore, expression was seen in the excretory duct and pore cells from threefold stage embryos to adults (Fig. 4E). However, in adults the GFP intensity appears weaker than in larvae. In L1 larvae, *qua-1* is expressed in two, sometimes four, cells of the anterior as well as the posterior of the intestine and a rectal epithelial cell (Fig. 4B,G). In addition, we observed transient expression in the P cells in L1 in the ventral side of the animal (Fig. 4F) and in a few sensilla support cells in the head (Fig. 4D). In adults, *qua-1pro*::GFP is transiently ex-

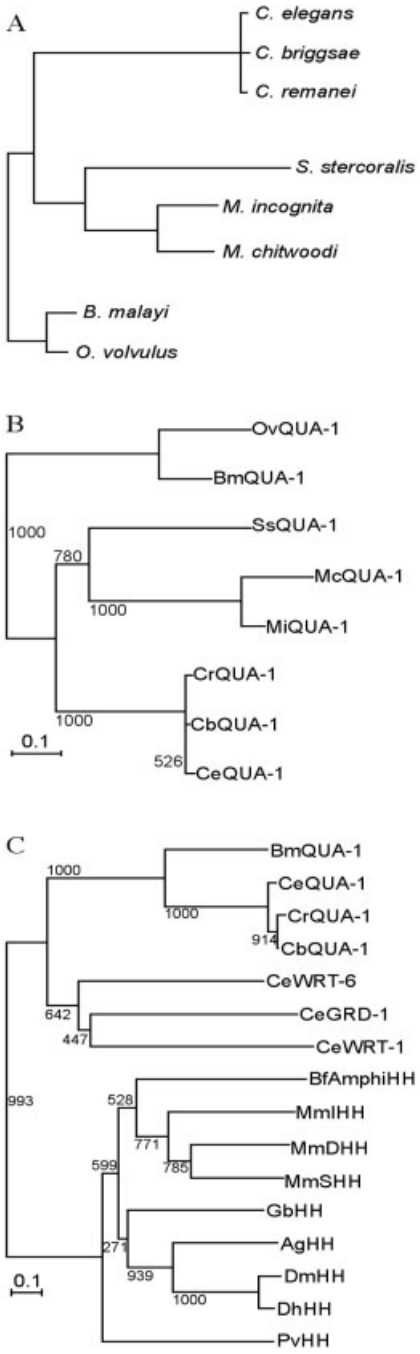

Fig. 3. Phylogenetic analyses of QUA-1 sequences. **A:** Evolutionary tree of the nematode species used in this report, adapted from Blaxter et al. (1998). **B:** Neighbor joining tree of the Qua domain, numbers indicate bootstrap values (1,000 trials). **C:** Neighbor joining tree of the Hog domain of QUA-1 proteins, and selected Warthog, Groundhog, and Hedgehog proteins. Species codes: Ag, *Anopheles gambiae*; Bf, *Branchiostoma floridae*; Dm, *Drosophila melanogaster*; Dh, *Drosophila hydei*; Gb, *Gryllus bimaculatus*; Mm, *Mus musculus*; Pv, *Patella vulgata*; other codes are the same as in Figure 1.

pressed in a few cells in the head that remain to be identified (Fig. 4C).

Deletion Mutant *qua-1(gk32)* Causes Molting Defects

A *qua-1* deletion mutant *(gk32)* was obtained from the *C. elegans* Gene Knock-out Consortium. The size of the deletion is only 173 bp and it is located in the repetitive area in the seventh exon (Figs. 1, 2B). The deletion causes a reading frame shift at the break point; hence, the protein terminates prematurely, ending with residues from another frame and a premature stop codon (not shown). The predicted protein product lacks the Hog domain, but has the signal sequence for export and the fully conserved Qua domain and the 5W repeat. *qua-1(gk32)* causes developmental arrests at the L1/L2 stage with a high penetrance (98%). This early arrest is in contrast to other molting mutants such as *lrp-1, nhr-23*, and *nhr-25* that exhibit molting defects in all four larval stages (Kostrouchova et al., 1998; Yochem et al., 1999; Gissendanner and Sluder, 2000). Observation of the arrested *qua-1* mutant larvae reveals that molting is defective. In many instances, the larvae are stuck in the old cuticle, which is full of liquid and vesicles (Fig. 5C,D). In many cases, the body cuticle has peeled off, but the cuticle lining the pharynx has not detached (Fig. 5A). When the pharyngeal cuticle fails to dissociate completely, parts of the cuticle can be seen linked to the mouth (Fig. 5B). Constrictions of the cuticle in the head and in the body are also observed when the old cuticle is still attached to the body (Fig. 5E,F). In summary, all of the observed phenotypes are typical Mlt phenotypes that are also found in many other Mlt mutants. The Mlt phenotypes of *qua-1(gk32)* are consistent with the RNAi phenotypes that have been observed in large-scale RNAi screens (Kamath et al., 2003; Simmer et al., 2003; Frand et al., 2005; Zugasti et al., 2005).

We repeated the *qua-1*(RNAi) feeding experiment and observed that the worms can have problems during ecdysis at any larval stage when RNAi treatment starts at the L1 stage. When RNAi treatment starts at the L2 or L3 stage, the worms can arrest with molting defects at the L3 or L4 stage (data not shown). This indicates that the QUA-1 protein produced at earlier stages is not sufficient to rescue later defects.

qua-1(gk32/+) heterozygote animals are viable, and no Mlt phenotypes are observed. In a few instances, we observed bulges in the body region, or abnormal tail development (Fig. 5G,H).

Both Qua Domain and Hog Domain of QUA-1 Are Secreted and Associated With Cuticle

QUA-1 is predicted to have a signal peptide sequence for secretion (Fig. 1). In order to visualize the location of the QUA-1 protein and to perform rescue experiments of *qua-1(gk32)*, we made two fusion constructs, one where only the Qua domain was fused to GFP (pLH069), and one where full-length QUA-1 was fused to GFP (pLH070) (Fig. 2B). When we injected QUA::GFP (pLH069) into *gk32*, we were not able to obtain rescue of the lethality and the Mlt phenotype. We also injected the construct into wild type N2 animals and observed dominant phenotypes. A portion of the transgenic worms displayed Mlt phenotypes (Fig. 6G,H). This effect was concentration dependent; higher concentrations of the transgene gave rise to higher percentages of Mlt phenotypes. In pLH069 transgenic worms, QUA::GFP is localized to the surface of the body (Fig. 6A), the excretory duct and pore (Fig. 6B), the vulva (Fig. 6C), and the anus (Fig. 6D). The GFP signal is strongest in the head region and weakest in the central body region (Fig. 6A). The accumulation of QUA::GFP in the head region is reminiscent of that of the metalloprotease NAS-37 (Davis et al., 2004). QUA::GFP (pLH069) is located in four quadrants along the body, reminiscent of the muscle quadrants. In fact, the GFP location overlies the muscle quadrants (Fig. 6F,F'). Circumferentially, the GFP pattern is composed of fine rings, which we presume coincide with the annuli of the cuticle (Fig. 6F–H). Taking advantage of the dominant Mlt phenotype of the QUA::GFP (pLH069), we find that

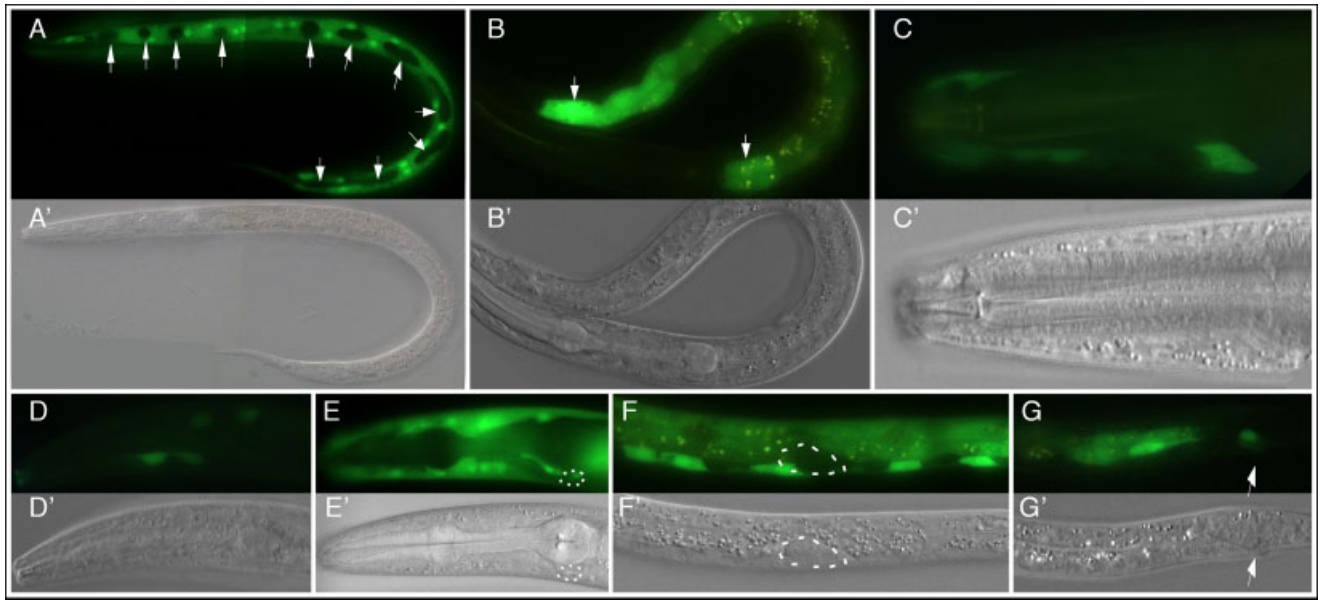

Fig. 4. Expression pattern of *qua-1pro*::GFP. *qua-1* is mainly expressed in all the hypodermal cells from late embryogenesis to adults, but not in the seam cells (**A**, white arrows), and in the excretory duct cell (**E**). In L1, it is also found to be expressed in the intestinal and rectal epithelial cells (G, white arrow points to the anus), and much stronger in the first two intestinal cells and the last four intestinal cells (**B, G**). Occasionally the *qua-1pro*::GFP is seen to be expressed in P cells (**F**, gonad is shown with a white circle). In adults, *qua-1pro*::GFP is expressed transiently in a few cells with processes, which might be the socket cells (**C, D**). Anterior is left and dorsal is up. A' to G' are DIC images of the corresponding fluorescent images of A to G, respectively. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

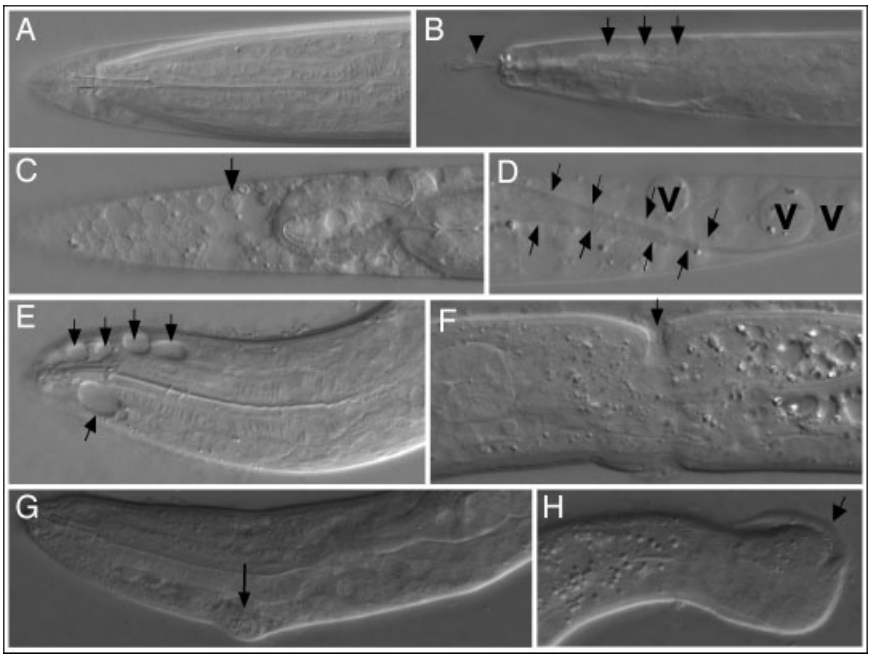

Fig. 5. Phenotype of the *qua-1(gk32)* deletion mutant. *qua-1(gk32)* is lethal and all the worms arrest at the L1 stage due to molting defects (**A, C, D**). It is often observed that the cuticle lining the pharynx is more difficult to shed than the body cuticle (A). A plug (arrow) is seen at the mouth (**B**). The buccal cavity is detached and many cells in the pharynx are consequently damaged (C, arrow points to one cell). The cuticle cocoon is usually full of liquid and vesicles (V) (D, the arrows outline the tail in the old cuticle). In the head, the constrictions of the cuticle at the anterior end can be seen (B and **E**, arrows). In the body the old cuticle is still attached to the body so that it forms constrictions (**F**, arrow). *qua-1* (+/−) is not lethal, but a few worms display deformed body shapes (**G,H**, arrow points to a bulge in G and the spike tail in H). Left is anterior and dorsal is up.

QUA::GFP (pLH069) is associated both with the detached cuticle and the newly formed cuticle (Fig. 6G, H). Furthermore, QUA::GFP is located in vesicles in the cytoplasm of hypodermal cells, indicating that cuticle-associated QUA::GFP is secreted by the underlying hypodermis (Fig. 6E,E'). In the full-length QUA-1::GFP (pLH070) construct, the Hog domain is tagged by GFP (Fig. 2B). This transgene is able to rescue the lethality and Mlt phenotype of the *gk32* allele. No dominant phenotypes are observed with pLH070 in a wild type N2 background. Full-length QUA-1::GFP (pLH070) localizes also to the cuticle-like QUA::GFP (pLH069) (Fig. 6I,J, data not shown). This is somewhat surprising, because the GFP marker is fused to the Hog domain. One might expect the Hog domain not to be found in the cuticle, since the Hint domain of Hh has apparently no function after the autoproteolytic cleavage process—thought to occur in the cis-Golgi region of the secretory pathway (Maity et al., 2005)—is complete. It should be noted though that the Hh Hint domain is secreted and not internally degraded, albeit as a highly soluble form (Lee et al., 1994).

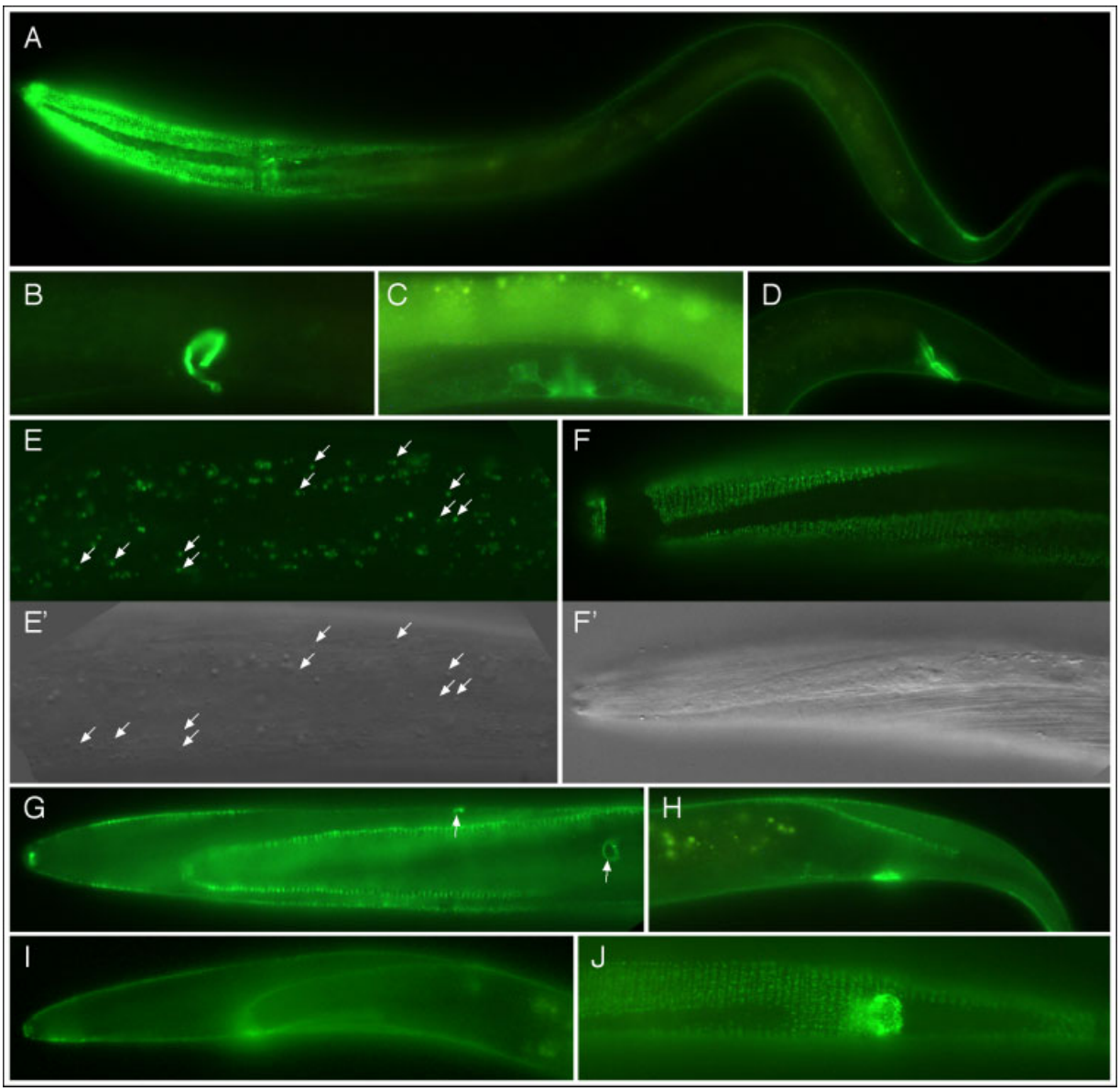

Fig. 6. The Qua domain is secreted and associated with the cuticle. **A:** L1 larva. QUA::GFP (pLH069) is present on the entire body surface and accumulates in the head region. QUA::GFP (pLH069) is secreted and associated with the cuticle overlying the excretory duct and pore cells (**B**), the vulval cells (**C**), and the rectal epithelial cells (**D**). **E:** L2 larva. QUA::GFP (pLH069) is located in secretory vesicles in the hypodermal cells. **F:** QUA::GFP (pLH069) localizes to the cuticle overlying the four muscle quadrants. QUA::GFP (pLH069) is associated with both the old and newly synthesized cuticle in the head (**G**, arrows point to the cuticle of the excretory duct and pore cells) and tail region (**H**). The GFP tag of QUA-1::GFP is associated with both the old and new cuticles while molting (**I**), and is associated with the cuticle in a pattern similar to QUA::GFP (pLH069) in F (**J**). A–J are fluorescent images, E' and F' are the corresponding DIC images of E and F. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Cycles of *qua-1* Transcription Levels

Several genes involved in molting have been shown to cycle expression levels through the larval molting cycles during larval development (Davis et al., 2004; Hashmi et al., 2004; Frand et al., 2005). To test whether the transcript levels of *qua-1* are also subject to cyclical changes, we conducted a time-course recording of the fluorescent signal in live transgenic worms carrying the transcriptional GFP fusion construct *qua-1pro*::GFP. We recorded two molting events for each larva examined. By recording many worms at different stages, we were able to obtain the entire overview of the transcript level changes of *qua-1*. A representative observation of one animal from 2.5 hr before the L2 molt to the L3 molt is shown in Figure 7A. Prior to and during the L2 molt, the *qua-1pro*::GFP expression levels are high. After the L2 molt, the *qua-1pro*::GFP levels go down and reach the lowest level at around the middle of the inter-molt (about 5.5 hr). Subsequently, GFP levels start to increase again and reach a maximum 3

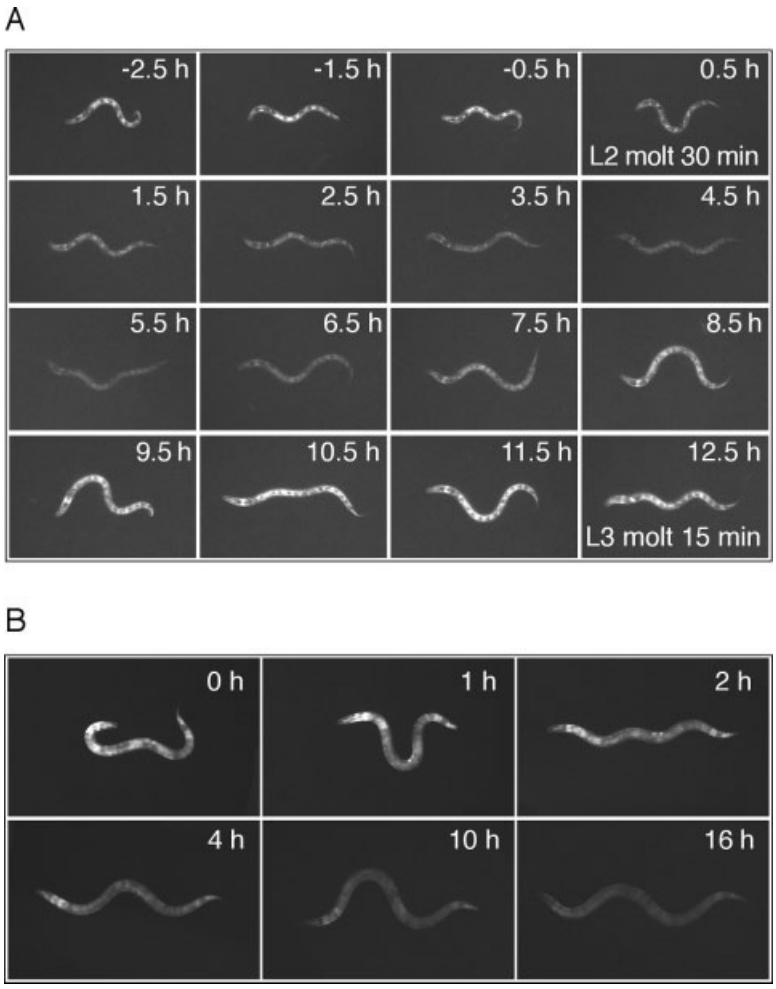

Fig. 7. Fluctuation of transcript levels of *qua-1* during larval development as monitored using the *qua-1pro*:GFP construct. **A:** The GFP fluorescent signal in a live transgenic larva is recorded in intervals of 1 hr starting from late L1 until the L2 molt. The time point at the L1 molt is defined as zero. The strongest signal is seen 3 hr before each molt, and the weakest signal is observed in the middle between molts. **B:** The GFP fluorescent signal in a live transgenic young adult is recorded in 1-hr intervals. After the L4 molt, GFP fluorescence levels decrease and are then maintained at a very low level.

hr later, which is maintained until the L3 molt. This cycle repeats during the next larval stages. After the L4 molt, *qua-1pro*::GFP levels in young adults decrease and stay at very low levels (Fig. 7B).

Electron Microscopy of Cuticle Defects

To see whether the *qua-1(gk32)* mutation affects the hypodermis or the cuticle, we performed electron microscopy (EM) on cross-sections of arrested *qua-1(gk32)* animals. Because *qua-1(gk32)* mutants are primarily arrested after the L1 molt, we used wild-type L1 and L2 larvae as controls. Arrested *qua-1(gk32)* larvae displayed a double layer of cuticle in places where the underlying cuticle was produced, but the overlying cuticle failed to be shed (Fig. 8B). Furthermore, both the remaining L1 and the L2 *qua-1(gk32)* cuticles were consistently clearer and more irregular (Fig. 8D) compared to control cuticles (Fig. 8A,C). Figure 8A shows the alae of a wild-type L1 larva, with one major protrusion and two smaller lateral structures. In arrested *qua-1(gk32)* animals, the alae from the remaining L1 cuticle displayed a flat and irregular protrusion (Fig. 8B). The L1 alae were also often mislocalized with respect to the seam cells, probably because the L2 animal can move within its L1 shell (see muscles under the alae in Fig. 8D). We did not observe any obvious morphological defects in the hypodermis of *qua-1(gk32)* mutants (data not shown). In particular, although QUA-1 protein is distributed in a pattern reminiscent of the muscle-epidermis-cuticle anchoring structure known as fibrous organelles (Fig. 6F, F'), which raised the possibility that QUA-1 might be involved in anchoring the epidermis to the cuticle, we did not observe epidermis-cuticle detachment defects such as those reported in *mua-3* mutant larvae (Bercher et al., 2001).

DISCUSSION

According to the new molecular phylogeny, nematodes are now classified as ecdysozoa (Aguinaldo et al., 1997), which includes arthropods. The presence of bona-fide *hh* genes in both arthropods and deuterostomes confirms that a *hh* gene existed already before the separation of protostomes and deuterostomes. Thus, the most parsimonious explanation for the lack of *hh* but the presence of *hh*-related genes, such as *qua-1*, in nematodes is that the latter have evolved from an ancestral *hh* gene in early nematode evolution (Bürglin and Kuwabara, 2006). The fact that the *qua-1* genes are highly conserved from *B. malayi* to *C. elegans*, two species that are thought to have shared a last common ancestor more than 300 million years ago, indicates that the evolution of *quahog* from *hh* or an *hh* derivative must have happened early in nematode evolution. Like the *hh*, *warthog, groundhog*, and *ground-like* genes (Aspöck et al., 1999), the *quahog* genes encode a highly conserved amino-terminal domain, in this instance the *quahog*-specific Qua domain. While it has been possible to discern some small common motif within the Wart, Ground, Ground-like, and Hedge domains (Aspöck et al., 1999), it seems that this motif is not well conserved in the Qua domain (Fig. 1).

We have observed that the QUA-1 protein is secreted and associated with the cuticle both in N2 and *qua-1(gk32)* mutant backgrounds (Fig. 6), indicating that the functional site of the protein is most likely in the ECM. The *C. elegans* cuticle is composed of many collagen molecules, which have

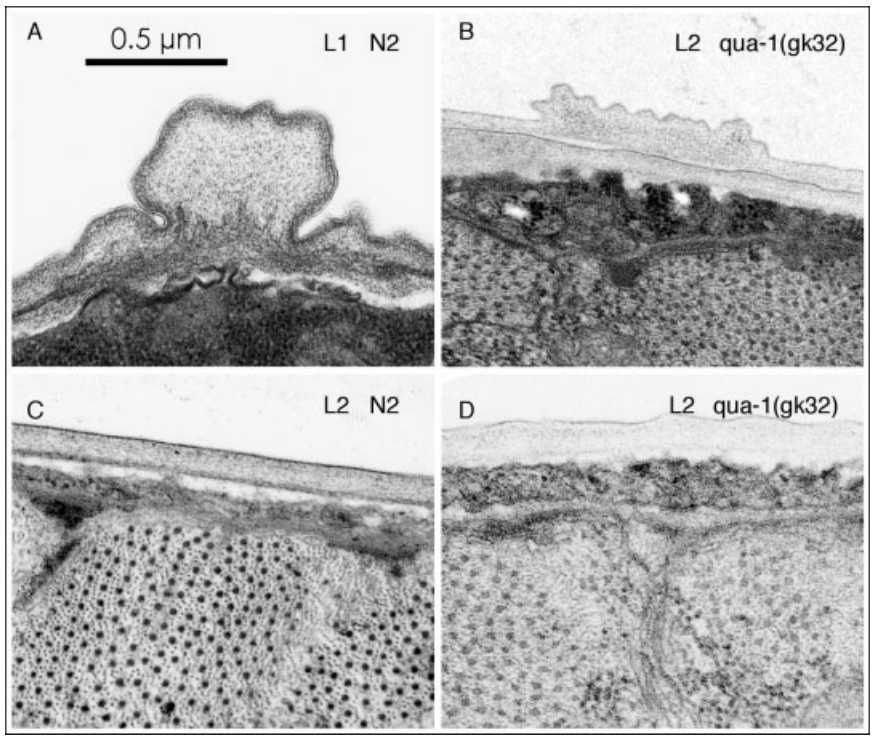

Fig. 8. Electron microscopy of cross-sections of *qua-1(gk32)* and N2. **A:** Alae structure of a wild type L1 larva. **B:** Alae structure of a *qua-1(gk32)* L2 larva. **C:** Hypodermis and cuticle of a wild type L2 larva. **D:** Hypodermis and cuticle of a *qua-1(gk32)* L2 larva.

trimeric glycine repeats. Although the repeat region in the QUA-1 protein is glycine rich, the glycine repeats in QUA-1 do not show the right conservation and spacing, since they are often also dimeric (GxGxG), rather than trimeric (GxxGxxG), and there is a distinct lack of proline residues. Furthermore, this repeat region is lacking in *B. malayi* QUA-1. Hence, this region probably does not interact with collagens in a collagen-typical fashion. However, there are ten conserved cysteine residues in the Qua domain that may form intramolecular disulphide bonds as seen in many extracellular molecules, and the Qua domain and the 5W motif may interact with cuticle components.

Hh protein is known to be modified by cholesterol in animals. The Hint/Hog domain of Hedgehog undergoes autocatalytic cleavage and confers a cholesterol to the secreted N-terminal portion (Beachy et al., 1997). Likewise, the *C. elegans* Warthog protein WRT-1 has been shown to undergo autoprocessing in *Drosophila* cultured cells (Porter et al., 1996). The QUA-1 proteins also have a conserved Hog domain, indicating the possibility that these proteins are secreted and undergo the same kind of processing. Our observation that the full-length QUA-1::GFP fusion as well as the Qua-domain GFP fusion localizes to the cuticle shows that even if the protein is autoproteolytically cleaved, both protein moieties are secreted. However, this secretion is not controlled by the Dispatched homolog CHE-14, since we observed no changes in location of QUA-1::GFP in the background of *che-14(e1960)* (data not shown). But whether the QUA-1 protein is autoprocessed in the cis-Golgi like Hh (Maity et al., 2005) or only later in the cuticle, and modified by cholesterol or some other sterol remains to be seen (Mann and Beachy, 2000). The *gk32* deletion causes a frameshift in the middle of the protein that leads to a premature stop that removes the Hog domain, but leaves the Qua domain and the 5W repeat intact. We surmised that *gk32* might not be a null mutation, although it is possible that the *gk32* mRNA is degraded by a surveillance system. In order to test the function of the Qua domain alone, we generated a fusion construct containing the Qua domain and the 5W repeat fused to GFP. This construct failed to rescue *qua-1(gk32)*, unlike a full-length QUA-1::GFP construct. This highlights the critical function of the Hog domain. However, the Qua domain construct produces strong dominant molting defects in a wild type background, demonstrating that the well-conserved Qua domain is also essential for function. The Qua domain-only protein is also located in the cuticle, like full-length QUA-1, suggesting that the dominant negative phenotypes are due to the improperly processed Qua domain that may interfere with QUA-1 multimerization, or compete for QUA-1 interaction sites in cuticle components. Thus, the cooperation of both domains is necessary for proper molting.

Electron microscopic analysis revealed that *qua-1* mutant larvae can assemble a new cuticle, although both the old and the new cuticles were generally clearer and more irregular and alae formation was abnormal. Therefore, QUA-1 could constitute a structural component of the cuticle. Our observation that RNAi feeding at L1 or L2 causes arrest at the L3 or L4 molts shows that synthesized QUA-1 is not recycled and retained for the next molt. In fact, most of the QUA-1 protein is probably lost with the shed cuticle. In *qua-1*(RNAi) feeding experiments, we also observed that the animals that are trapped in the old cuticle continue to develop other organs such as gonads (data not shown), suggesting that *qua-1* does not affect the timing of other aspects of development. The shedding defects raise the possibility that QUA-1 is also involved in the shedding process. Since the GFP-tagged Hog domain of the full-length QUA-1::GFP construct is localized to the cuticle, it is possible that the proteolytic activity of the Hog domain could act on other extracellular proteins and contribute to the shedding process. While we do not know how QUA-1 functions molecularly, our EM results suggest that it is less likely that QUA-1 plays a role in signaling like Hh. In Drosophila, the membrane-embedded receptor for Hh is Patched. In *C. elegans*, two *patched* genes, *ptc-1* and *ptc-3*, as well as 24 *patched*-related genes exist (Kuwabara et al., 2000). RNAi analysis of these genes revealed that several, no-

tably *ptc-3, ptr-4, -16, -18*, and *-23* also cause strong Mlt phenotypes when knocked down (Zugasti et al., 2005). It is feasible that QUA-1 interacts directly with some of these Ptc or Ptr proteins, which may be involved in facilitating secretion of QUA-1. However, we cannot exclude yet that QUA-1 may also propagate a molting signal, perhaps via some of these Ptc and Ptr molecules.

In summary, *qua-1* is a unique gene that presently exists in nematodes only. It encodes a secreted protein with two major conserved domains, a C-terminal Hog domain with presumed autocatalytic protease activity and an N-terminal Qua domain with unknown function. *qua-1* is expressed mainly in the hypodermis and disruption of the *qua-1* gene leads to strong molting defects. Our analyses have shown that both the Hog domain as well as the Qua domain are essential for proper molting. The conserved nature of this gene suggests that it plays a role in molting in other nematodes as well and might, consequently, be a useful drug target.

EXPERIMENTAL PROCEDURES

Sequence Analyses of *qua-1* Genes

TBlastn searches (Altschul et al., 1997) of genomic as well as EST databanks were performed at NCBI (www.ncbi.nlm.nih.gov/BLAST/) using T05C12.10 (QUA-1) as query. ESTs recovered were: SWOv3MCAM30H08, SWOv3MCAM21D03 (acc. no. AW257707, AI444860) for *O. volvulus*; ri62g01.y1 (acc. no. CB931749) for *M. chitwoodi*; rd30d09.y1 (acc. no. BQ613344) for *M. incognita*; kq58h02.y1, kq19a09.y1, kq23h04.y1 (acc. no. BF014893, BG226231, BE579200) for *S. stercoralis*. The EST clones SWOv3MCAM30H08 and SWOv3MCAM21D03 were obtained from Susan Haynes and Dr. Steven Williams. They were excised according to the Stratagene in vivo excision protocol (http://www.stratagene.com/manuals). However, we only recovered an insert for SWOv3MCAM30H08 (1.3 kb), which was completely sequenced (MWG, The Genomic Company, Munich, Germany). This clone was found to be not full-length at the 5′ end as well as at the 3′ end, the latter presumably due to internal priming of the oligo-dT primer. The full-length sequence of SWOv3MCAM30H08 was combined with SWOv3MCAM21D03, which is partially overlapping. The EST sequence of SWOv3MCAM21D03 contains several undetermined residues (N). In the overlap region with SWOv3MCAM30H08, these residues are usually an A, thus we replaced the undetermined residues with A in the SWOv3MCAM21D03 EST sequence. Thus, the first 210 residues of the combined transcript presented here (submitted as acc. no. AY775955) may still contain mistakes.

The genomic region of *C. briggsae* (Stein et al., 2003) was retrieved from Wormbase (www.wormbase.org). The genomic *C. briggsae* sequence data were produced by the Sanger Institute (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/c_briggsae) and the Genome Sequencing Center at Washington University Medical School (http://genome.wustl.edu/blast/briggsae_client.cgi) (Stein et al., 2003). The predicted ortholog of *C. elegans* QUA-1 in *C. briggsae*, CBP00142, appeared to lack the C-terminal end. The genomic region was then compared to the genomic region of *C. elegans* using the dotplot program PPCMatrix (Bürglin, 1998) and an extra exon was thus uncovered at the 3′ region. Comparison of the intron/exon structures of *C. elegans, C. briggsae*, and *C. remanei* (Fig. 2A) revealed two erroneous introns in exon 6 of *C. briggsae*. The corrected *C. briggsae* QUA-1 protein prediction is shown in Figure 1. The sequence of *C. remanei* QUA-1 was assembled from trace reads as follows. Tblastn searches against *C. remanei* reads were performed at the Genome Sequencing Center at Washington University Medical School (http://genome.wustl.edu/blast/client.pl). Vector sequences were removed by hand and sequences were assembled partially using MSE in the EMBOSS suite (emboss.sourceforge.net) (Rice et al., 2000). Final assembly was performed using The CAP EST Assembler at IFOM (http://bio.ifom-firc.it/ASSEMBLY/assemble.html). More than 6,700 bp of genomic sequence was assembled, covering the complete ORF of *C. remanei qua-1*. Gene prediction was carried out using FGENESH+ at www.softberry.com. The gene prediction for *C. remanei* had to be manually corrected through the repetitive region using PPCMatrix by comparing to *C. elegans* and *C. briggsae* (*C. remanei qua-1* submitted as acc. no. AY775956). For *B. malayi qua-1*, tblastn searches were performed at The Institute of Genome Research (TIGR, www.tigr.org). This sequencing effort is part of the International Brugia Genome Sequencing Project and is supported by an award from the National Institute of Allergy and Infectious Diseases, National Institutes of Health. Two contigs were found that covered most of the QUA-1 ORF. A small gap in these contigs was fortuitously covered by a small fragment of DNA sequenced as an EST, i.e., clone kb60c04.y1 (acc. no. CB338757), although this clone is not a cDNA, but rather a piece of contaminating genomic DNA. Gene prediction for *B. malayi qua-1* was carried out as above and submitted (acc. no. AY775954). ORF analysis through the region showed that there is no long ORF through the region that corresponds to the repetitive regions in the *Caenorhabditis* spp. genes, confirming that *B. malayi qua-1* does not have the long repetitive stretches in the center (Fig. 2A). The signal sequences for protein export were predicted using the SignalP Web server (http://www.cbs.dtu.dk/services/SignalP/) (Bendtsen et al., 2004).

For multiple sequence alignment, the protein sequences were aligned using ClustalX (Thompson et al., 1997) and MAFFT (http://www.biophys.kyoto-u.ac.jp/%7Ekatoh/programs/align/mafft/) (Katoh et al., 2002). For phylogenetic analyses, sequences were trimmed to either the Qua domain or the Hog domain and analyzed using Neighbor joining in ClustalX (Thompson et al., 1997). Bootstrap values for 1,000 trials are shown.

Nematode Maintenance and Culture

Standard procedures for nematode and genetic manipulation were followed and all genetic characterizations were performed at 20°C (Lewis and Fleming, 1995).

Constructs and Expression Pattern Analysis of *qua-1*

The putative promoter region of *qua-1*, a sequence from the start codon on upstream with length of 2,880 bp was amplified by PCR and the PCR product was fused with GFP by fusion PCR (Hobert, 2002; McKay et al., 2003). The primers used are T05C12.1-A, 5′GACACGTGTTTCAAAATGTTTCAT3′, T05C12.1-A*, 5′CATTTTTGTATGCCCAATTTTTGT3′, T05C12.1-B, 5′AGTCGACCTGCAGGCATGCAAGCTGCCGCCGGATCTTAAATATAG3′, 95-67-C, 5′AGCTTGCATGCCTGCAGGTCGACT3′, 95-67-D, 5′AAGGGCCCGTACGGCCGACTAGTAGG3′, 95-67-D*, 5′GGAAACAGTTATGTTTGGTATATTGGG3′. The start codon was mutated to guarantee that the translation always starts at the GFP. Consequently, the expression from the *qua-1pro*::GFP transcriptional fusion is a GFP without signal peptide for secretion. The construct was microinjected into *dpy-5(e907)* mutants using *dpy-5* as marker by standard techniques (Mello et al., 1991) to produce strain BC12591 (McKay et al., 2003). For constructing pLH069 and pLH070, the *qua-1* promoter (same as above) was firstly cloned by PCR into pPD114.108 at the PstI/NcoI sites, and then either the Qua domain coding region or the full-length *qua-1* genomic region were amplified by PCR from cosmid T05C12 and inserted into it at the NcoI/NotI sites following the promoter. The stop codon had been removed so that the coding sequence was in frame with the GFP fusion. These constructs together with *rol-6* as marker were injected into N2 or *qua-1(gk32)* to test their rescue capability. The plasmid pLH069 caused a Mlt phenotype in the N2 background. It was impossible for us to obtain a stable transgenic line when the plasmid concentration was higher than 10 ng/μl. The transgenic worms carrying 5 ng/μl pLH069 produced less Mlt worms than those carrying 10 ng/μl pLH069, although we did not score them.

Characterization of *qua-1(gk32)*

qua-1(gk32) was isolated by the *C. elegans* Reverse Genetics Core Facility in Vancouver. The deletion is 173 bp long and results in a frame shift in the seventh exon causing a premature stop. Since the mutant is lethal, it was balanced using inversion mIn1 of chromosome II (Edgley and Riddle, 2001), which yielded strain VC42. Starting with VC42, we outcrossed the mutant to N2 eight times and then balanced the mutant again by crossing with DR2078 (*mIn[dpy-10(e128) mIs14]/bli-2(e768) unc-4(e120)*) which carries an inversion covering the *qua-1* locus (Edgley and Riddle, 2001); the outcrossed strain is called TB1700. The phenotype of strain TB1700 was characterized by picking ten heterozygotes and singling them onto individual plates. They were transferred onto fresh plates every 10–14 hr until they stopped laying eggs and their progeny were scored and observed. The TB1700 worms were also washed off from plates and placed onto 2% agarose pads for DIC microscopy. Taking advantage of the balancer inversion fragment carrying two GFP constructs that stained both pharyngeal muscle cells and intestine, the heterozygotes and homozygotes could be distinguished easily.

Microscopy and Image processing

Images were captured using a Zeiss Axioplan2 imaging microscope equipped with digital CCD cameras (Zeiss AxioCam and Hamamatsu ORCA ER with a Snapper DIG-16 framegrabber). Acquisition software used was Openlab 3.1.5 (Improvision). The images were further processed and assembled with Adobe Photoshop 7.0 and Freehand 10.3.9.

For manual time-lapse recording of transgenic GFP animals, one worm was picked at a time and transferred to a fresh plate. Images were captured at 1-hr intervals using a dissecting fluorescent microscope (Olympus SZX12) equipped with a Hamamatsu ORCA ER camera. Blue excitation illumination was adjusted to a low level to avoid damaging the animal. Each time-lapse recording session lasted about 16 hr so that two molts could be observed. Room temperature was 21°C during recordings.

Electron Microscopy

N2 adults were allowed to lay eggs for 3 hr. L1 and L2 larvae were picked 20 and 30 hr after egg laying, respectively. *qua-1(gk32)* larvae segregating from the heterozygous strain TB1700 were picked on the basis of their L1-L2 molting defect, after checking that they were still alive (moving). N2 and *qua-1(gk32)* larva were sectioned and fixed for at least 24 hr, with 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer pH 7.2 at 4°C. Worms were post-fixed for 5 hr with 2% osmium tetroxide in the same buffer at 4°C. Animals were dehydrated in graded alcohol, and then embedded in Epon. Ultra-thin sections of 70 nm were contrasted with uranyl acetate and lead citrate. Sections were observed with a Philips CM12 electron microscope, operating at 60 kV. For both *qua-1(gk32)* and N2, at least four different animals were observed.

ACKNOWLEDGMENTS

We thank the *C. elegans* Reverse Genetics Core Facility in Vancouver for isolation of the *qua-1(gk32)* deletion and Tony Page for helpful discussion. Some nematode strains used in this work were provided by the Caenorhabditis Genetics Center, which is funded by the NIH National Center for Research Resources (NCRR). The two *O. volvulus qua-1* EST phage clones were kindly provided by Susan Haynes and Dr. Steven Williams. The *B. malayi* sequencing effort is part of the International Brugia Genome Sequencing Project and is supported by an award from the National Institute of Allergy and Infectious Diseases, National Institutes of Health. We thank Dr. John Plenefisch for providing the worm strain EE86. The plasmid containing *dpy-5* as coinjection marker was a gift from Dr. Ann Rose and Dr. Colin Thacker at the University of BC. The BC12951 strain construction is supported by Genome British Columbia and Genome Canada. This research is supported by the Swedish Foundation for Strategic Research (SSF) and Genome Canada/BC. The EM work was supported by a grant from the Ministère de la Recherche (ACI-program) to M.L.

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