MLT-10 Defines a Family of DUF644 and Proline-rich Repeat Proteins Involved in the Molting Cycle of Caenorhabditis elegans

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The molting cycle of nematodes involves the periodic synthesis and removal of a collagen-rich exoskeleton, but the underlying molecular mechanisms are not well understood. Here, we describe the mlt-10 gene of Caenorhabditis elegans, which emerged from a genetic screen for molting-defective mutants sensitized by low cholesterol. MLT-10 defines a large family of nematode-specific proteins comprised of DUF644 and tandem P-X-L-(S/T)-P repeats. Conserved nuclear hormone receptors promote expression of the mlt-10 gene in the hypodermis whenever the exoskeleton is remade. Further, a MLT-10::mCherry fusion protein is released from the hypodermis to the surrounding matrices and fluids during molting. The fusion protein is also detected in strands near the surface of animals. Both loss-of-function and gain-of-function mutations of mlt-10 impede the removal of old cuticles. However, the substitution mutation mlt-10(mg364), which disrupts the proline-rich repeats, causes the most severe phenotype. Mutations of mlt-10 are also associated with abnormalities in the exoskeleton and improper development of the epidermis. Thus, mlt-10 encodes a secreted protein involved in three distinct but interconnected aspects of the molting cycle. We propose that the molting cycle of C. elegans involves the dynamic assembly and disassembly of MLT-10 and possibly the paralogs of MLT-10.

INTRODUCTION

The molting cycle is the hallmark of the ecdyszoan clade that encompasses more than 90% of animal species on earth (Aguinaldo et al., 1997). Molting of nematodes involves the synthesis and removal of collagen-rich extracellular matrices (ECM) and related points of cell–ECM adhesion. However, the signaling and enzymatic cascades that trigger and execute the molting process are not well understood. Dysfunctions of related processes in humans contribute to the metastasis of tumors and various disorders of skin, connective tissue, and ectodermal organs. These disorders include genetic collagenopathies, certain muscular dystrophies, Marfan syndrome, and baldness (Campbell, 1994; Krause and Foitzik, 2006; Page-McCaw et al., 2007; Blank and Boskey, 2008; Ramirez and Dietz, 2009).

The exoskeleton of nematodes, also called the cuticle, is a complex, multilayered ECM secreted by underlying epidermal-like cells and syncytia (Page and Johnstone, 2007). Collagens serve as major structural components of the cuticle, along with nematode-specific proteins called cuticulins (Cox et al., 1981; Johnstone, 1994; Sapio et al., 2005). Consequently, the Caenorhabditis elegans model has been very useful for studies of collagen biosynthesis (Page and Johnstone, 2007). Less is known about the biogenesis of other cuticle components, including the glycoproteins and lipids found in the surface coat and epicuticle, respectively.

During the process of molting, a new cuticle is synthesized underneath the old one and gradually displaces the preexisting structure from the hypodermis. The outer layer of the cuticle is secreted first, and the annular furrows found there correspond to transient invaginations in the apical membrane of the hypodermis (Page and Johnstone, 2007). Particular macromolecules are depleted from the old cuticle during the process of molting, and some scavenged components may, in principle, be incorporated into the new exoskeleton. Lateral attachments that anchor the cuticle to the underlying muscle basement membrane (BM) are also remade during molting.

Larvae eventually escape (ecdysce) from the old cuticle using a stereotypical set of behaviors. This sequence includes regurgitation of the anterior half of the pharyngeal cuticle, rotation on the long axis, contractions, and forward thrusts, in that order (Singh and Soulston, 1978). C. elegans larvae molt four times, once every 8–10 h under standard culture conditions. Molting takes about 2 h, but ecdysis takes only a few minutes.

The rapid molting cycle of C. elegans requires precise temporal and spatial control over the production and destruction of ECM macromolecules. Accordingly, many genes required for the completion of molting encode proteases and antiproteases involved in the synthesis or degradation of collagens and other ECM proteins (Hashmi et al., 2002, 2004; Davis et al., 2004; Suzuki et al., 2004; Frand et al., 2005; Page-

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et al., 2006; Partridge et al., 2008). Several genes in the general pathways for secretion and endocytosis are also required for shedding larval cuticles (Roberts et al., 2003; Frand et al., 2005; Roudier et al., 2005). However, the molecular mechanisms that coordinate the assembly and disassembly of extracellular matrices with the progression through ecdysis are not well understood.

The C. elegans nuclear hormone receptors NHR-23 and -25 are also required for the removal of larval cuticles (Kostrouchova et al., 1998; Asahina et al., 2000; Gissendanner and Sluder, 2000; Kostrouchova et al., 2001). NHR-23 and -25 are homologous to the ecdysone-responsive DHR3 and FTZ-F1 factors of Drosophila melanogaster and also the ROR/RZR/Reverb and SF-1 receptors of mammals, respectively. The requirement for NHR-23 and -25 suggests that steroid hormones regulate the molting cycle of nematodes, similar to how pulses of the steroid hormone ecdysone trigger molting and metamorphosis in insects (Thummel, 1996). NHR-23 and -25 are expressed in hypodermal cells and synctyia and regulate the expression of particular cuticle collagen and matrix modification enzymes (Kostrouchova et al., 2001; Davis et al., 2004; Chen et al., 2004; Silhankova et al., 2005). However, the targets of these receptors germane to the molting cycle have not been fully described in any system (Riaud et al., 2010).

Large gene families whose members function synergistically were likely to evade detection in any previous RNAi-based screens for molting-defective larvae (Frand et al., 2005). We therefore conducted a forward genetic screen for mutations unable to fully shed old cuticles. Here, we describe the isolation and characterization of the C. elegans mlt-10 gene, which defines a large family of proteins involved in the epissodic synthesis and removal of cuticles during postembryonic development.

MATERIALS AND METHODS

Genetic Analysis

The culture and genetic manipulation of C. elegans were performed using standard methods (Epstein and Shakes, 1995). Low-cholesterol (LC) nematode medium (NGM) was prepared with SeaKem GTG agarose (FMC, Rockland, ME) rather than agar and no added cholesterol. Escherichia coli OP50 were washed with M9 buffer before seeding LC plates. Bacterial-mediated RNA-interference (RNAi) was performed as described (Frances et al., 2000). All primers were sequenced to determine the expanse of the region amplified from RB1962, cloned into pCR-XL-TOPO (Invitrogen, Carlsbad, CA), and subsequently was fed OP50 for two hatches larval cuticles (Roberts et al., 2005). We therefore conducted a forward genetic screen for molting-defective larvae (Frand et al., 2005). To construct translational fusion genes, the mlt-10 gene and regulatory sequences were amplified from N2 genomic DNA using primers AFA47 and AFA48 and Phusion High-Fidelity Polymerase (Finnzymes). The PCR product was cloned into pCR-Blunt II-TOPO and subsequently into pPD129.36 (Fire Lab, Stanford University School of Medicine) and E. coli strain HT115(DE3).

The mlt-10p::gfp-pest gene was generated as previously described (Frand et al., 2005). To construct translational fusion genes, the mlt-10 gene and regulatory sequences were amplified from N2 genomic DNA using primers AFA47 and AFA48 and Phusion High-Fidelity Polymerase (Finnzymes). The PCR product was cloned into pCR-Blunt II-TOPO (Invitrogen). The NotI site was generated in pAF599 in-frame between the codons specifying Ala30 and Val31 of MLT-10. An NotI cassette containing the mCherry gene was created by cloning the mCherry gene into the cloning vector pPD114.35 (Fire Lab, Stanford University School of Medicine) and E. coli strain HT115(DE3).

Microscopy and Cell Biology

Nematodes were anesthetized using sodium azide and mounted on 2% agarose pads. Images were captured using a Zeiss Axioscope (Thornwood, NY) with an attached Hamamatsu Orca ER camera (Bridgegewater, NJ) and Velocity software (Improvision, Lexington, MA). For confocal work, we used a Zeiss LSM 5 PASCAL microscope equipped with Axiocam software (Zeiss). All images were prepared for publication using Adobe Photoshop and Adobe Illustrator (San Jose, CA).

RNA was isolated from C. elegans as described (Pasquinelli et al., 2000). We used the ZR-RNA kit (Ambion, Austin, TX) to remove any contaminating genomic DNA and the RETROscript kit (Ambion) with random primers to synthesize cDNA. The mlt-10 cDNAs were amplified using primers sets AFA47/AFA48 or AFA47/AFA50. The mlt-10 cDNA was amplified using primers sets AFA47/AFA48 and AFA47/AFA50. The resulting plasmid (pAF599) contained a single C to A transition, at the base corresponding to nucleotide 10407 of cosmid CO98. A new NotI site was generated in pAF599 using the Phusion Site-Directed Mutagenesis kit (Finnzymes, Espoo, Finland) with primers AFA49 and AFA50. The plasmid was cloned into pCR-Blunt II-TOPO and subsequently into pPD129.36 (Fire Lab, Stanford University School of Medicine) and E. coli strain HT115(DE3).

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RESULTS

Isolation of mlt-10 in a Forward Genetic Screen

Nematodes cannot synthesize cholesterol de novo (Hieb, 1968; Chitwood, 1999). C. elegans must therefore acquire sterols from the culture medium in order to produce various steroid hormones essential for development and reproduction (Crowder et al., 2001; Merris et al., 2003; Matyas et al., 2004; Motola et al., 2006). Wild-type larvae cultured without...
cholersterol can rely on sterols stored by their mothers to develop to the adult stage. However, the progeny of animals cultured on low-cholesterol media arrest development as larvae, often trapped in partly shed cuticle: the molting-defective or Mlt phenotype (Figure 1A: Yochem et al., 1999). We used low cholesterol to sensitize a forward genetic screen for mutants unable to fully shed old cuticles (Figure 1B). Briefly, we isolated 17 mutants that require exogenous cholesterol, in addition to any maternal stores of cholesterol, to complete larval development. Larvae descended from 13 of the 17 mutants showed the Mlt phenotype under standard culture conditions (data not shown).

The mg364 allele of mlt-10 emerged from our screen. We analyzed the phenotypes associated with mg364 after outcrossing several times to remove any unlinked mutations. Only 0.3% (n = 317) of mlt-10(mg364) larvae developed to the adult stage when cultured on low-cholesterol media, compared with 100% (n = 211) of wild-type larvae (Figure 1C). As previously reported, mutations in the C. elegans ncr-1 gene also blocked development on low-cholesterol plates (Sym et al., 2000; Li et al., 2004). The ncr-1 gene is homologous to the human Niemann-Pick type C1 disease gene, which is important for the proper transport and storage of cholesterol (Smith and Levitan, 2007).

Under standard culture conditions, 56% (n = 466) of homozygous mlt-10(mg364) animals became trapped in a larval cuticle (Figure 2A). Roughly the same fraction of animals became trapped in each one of the four molts. Larvae incarcerated in a molt typically perished. An additional 22% of mg364 larvae arrested development but did not appear trapped in the cuticle upon inspection by light microscopy. The surviving mg364 animals developed slowly, with a generation time of ~90 h at 20°C, compared with 65 h for wild-type animals. The Mlt phenotype was also observed in 16% (n = 365) of heterozygous mlt-10(mg364)/mlt-10(+) animals, indicating that mg364 is semidominant. Together, these results show that mlt-10(mg364) causes a strict blockade of the molting cycle.

**The mlt-10 Gene Corresponds to C09E8.3**

To identify the mlt-10 gene, we mapped the mg364 mutation to a 3.4 map unit region on the left end of chromosome II by standard methods (Wicks et al., 2001). Because the mg364 mutation was semidominant, we reasoned that inactivation of mlt-10 might restore the ability of mg364 mutants to shed old cuticles. We therefore systematically and individually inactivated the annotated genes in the map interval by bacterial-mediated RNAi (Timmons et al., 2001; Kamath et al., 2003). After the inactivation of C09E8.3, the vast majority of mlt-10(mg364) larvae developed into healthy adults (Figure 2B). Sequencing C09E8.3 identified a C-to-T transition in mg364 mutants that specifies the substitution H590Y in the predicted MLT-10 protein. In addition, an extrachromosomal array containing C09E8.3 amplified from mg364 mutants (mgEx701, Figure S1) rendered wild-type larvae unable to shed old cuticles. To confirm the identity of the gene, we isolated and characterized six additional alleles of mlt-10 by screening for intragenic suppressors of mg364. The new alleles included four distinct missense mutations and two splice-site mutations in C09E8.3 (Table S3). Together, these
results show that mlt-10 corresponds to C09E8.3 and that mg364 is a gain-of-function (GOF) allele.

Figure 3A shows the structure of the mlt-10 gene, as confirmed by the sequence of 76 distinct cDNAs curated by Wormbase (www.wormbase.org). In addition to the point mutations described above, we obtained a variety of mlt-10 alleles from public resources. These alleles included cxTi9515, tm3331, and ok2581, reagents kindly provided by Laurent Segalat (University of Lyon), the C. elegans Gene Knockout Consortium, and the National Bioresource Project of Japan, respectively (Table S3). The cxTi9515 allele is an insertion of the Mos1 transposon in the 5’/H11032 UTR of mlt-10 (Bessereau et al., 2001; Martin et al., 2002). The mutation severely decreased the level of mlt-10 expression, as indicated by RT-PCR (Figure S2). The mlt-10(ok2581) insertion/deletion causes a frame-shift in exon 3 that introduces an early stop codon. The ok2581 insertion/deletion encodes a frameshift in exon 3 and part of exon 8. Transcripts of mlt-10 were shorter and less abundant in ok2581 mutants than wild-type animals (Figure S2). Sequencing those cDNAs indicated the use of an atypical splice acceptor site for exon 8 and the related introduction of a premature stop codon. As we shall describe, we analyzed the phenotypes associated with these loss-of-function (LOF) alleles of mlt-10 after out-crossing the corresponding strains several times to remove any unlinked mutations.

**MLT-10 Defines a Family of DUF644 and Proline-rich Repeat Proteins**

MLT-10 is the first characterized member of a large family of annotated proteins comprised of the domain of unknown function (DUF) 644 and distinctive tandem repeats rich in prolines and hydroxy amino acids (Figure 3, B and C, Figures S3 and S4). DUF644 contains many lysine residues and other basic or acidic amino acids. The repeats found at the C-terminus of MLT-10 have the consensus sequence P-X$_2$-L-(S/T)-P, where X is any hydrophobic residue except glycine. MLT-10 also contains an annotated secretory signal sequence and four putative acceptor sites for N-linked glycans, features characteristic of secreted proteins.

In principle, the biosynthesis of MLT-10 and the paralogs of MLT-10 may involve several posttranslational modifications, including but not limited to 1) disulfide bond formation, 2) the addition of O-linked glycans, 3) cleavage of dibasic sites in the nonrepetitive region by subtilisin/Kex2-like proteases, 4) cross-linking of glutamine and lysine residues by transglutaminase, and 5) the hydroxylation of some proline residues in the repetitive region. These particular modifications occur during the biosynthesis of collagens and other ECM proteins of nematodes (Fetterer and Rhoads, 1990; Lustigman et al., 1995; Thacker and Rose, 2000; Edens et al., 2001; Page et al., 2006).

We identified 13 paralogs of mlt-10 in the fully sequenced genome of C. elegans and named those genes mltn-1 through mltn-13 for (mlt-ten-related; Figure 3C and Table S4). Previous high-throughput analysis indicated that most if not all of the C. elegans mltn genes are expressed (Wormbase). The MLT-10 paralogs include mltn-1/Y52B11A.7, mltn-2/Y39D8B.7, mltn-11/W06G6c.7, mltn-12/C53B4.8, and mltn-13/F15A8.7, as well as three genes on cosmid W0B28 (II:13,916,260–13,930,421), two genes on cosmid F19H8 (II:14,616,033–14,626,346), and three genes on cosmid Y39D8B (V:364,951–379,673). Nota-
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likely, several of the gene models described in Table S4 include exons not depicted in the current release of Wormbase (WS204). In particular, the gene annotated as Y39D8B.1 in WS204 represents an in silico fusion of the predicted paralogs mltn-6 and mltn-7, formerly annotated as Y39D8B.2 and Y39D8B.1, respectively, in WS100. The fused gene depicted in WS204 specifies an atypical protein containing two copies of DUF644 but only one set of proline-rich repeats. The existence of three clusters of paralogs in the genome suggests that multiple gene duplication events contributed to the evolution of the mltn family. Many homologues of MLT-10 were also identified in the genomes of other species of nematodes and the annotated proteomes of parasitic nematodes including Onchocerca volvulus and Brugia malayi, which cause River Blindness and lymphatic filariasis in humans, respectively (Figure 3C and S4).

Figure S4 displays an alignment of the P-X$_2$-L-(S/T)-P repeats of the predicted paralogs and selected homologues of MLT-10. Most of these proteins contain about 30 repeats interspersed with only a few charged residues, with the exception of MLT-13/F15A8.7. Repeats similar to those of MLT-10 were found in several annotated but uncharacterized and otherwise unrelated proteins of eukaryotes, suggesting a widespread and ancient function for the P-X$_2$-L-(S/T)-P$_1$, sequence. We identified such proteins in the sequenced genomes of D. melanogaster, H. sapiens, or Saccharomyces cerevisiae. Thus, MLT-10 defines a large family of proteins that is well conserved only in nematodes.

Either the Loss or the Gain of mltn-10 Function Hinders the Removal of Larval Cuticles

We used genetic analysis to investigate the role of the mltn-10 family in development and better define the nature of the mltn-10(mg364) allele. A variety of mltn-10(lof) mutants developed from the L1 to the adult stage more slowly than wild-type animals, but did not display a terminal Mlt phenotype (Figure 2B). We therefore closely examined larvae partially synchronized around the time of the L2-to-L3 molt, using a compound microscope to detect buccal caps. The caps are comprised of old cuticle and temporarily seal the buccal cavity during the process of molting. Caps were observed on the majority of mltn-10(tm3331) and mltn-10(ok2581) mutants (Figure 2C), compared with 20% (n = 76) of wild-type larvae. When cultured on low-cholesterol media, 70% (n = 13) of mltn-10(tm3331) mutants and 92% (n = 12) of mltn-10(ok2581) mutants failed to shed the fourth larval cuticle, compared with 29% (n = 14) of wild-type animals (Figure 2D). Together, these results suggest that mltn-10 is needed for the efficient removal of larval cuticles, even though mltn-10 is not essential for development under standard culture conditions.

To further evaluate the function of the mltn-10 family, we used RNAi to systematically and individually knock down the 13 annotated paralogs of mltn-10 in wild-type larvae; mltn-10(lof) mutants; and rrf-3 mutants, which are more sensitive to dsRNA (Simmer et al., 2002). None of these animals became terminally trapped in cuticle (Table S5). Also, the
MLT-10 is involved in the molting cycle

The mltn-13/f15a8.7 gene. Thus, the paralogs of mltn-10 appear individually dispensable for the removal of larval cuticles, possibly due to functional redundancy among the large gene family. A thorough investigation of any such redundancy awaits the availability of null alleles in all thirteen paralogs of mltn-10.

As a complementary approach to investigate the mltn-10 family, we used high-copy arrays to increase the dosage of mltn-10 and selected paralogs of mltn-10. The array mgEx699[mltn-10] rendered 38% (n = 457) of larvae unable to shed old cuticles, and RNAi of mltn-10 suppressed that phenotype (Figure 2B). As expected, wild-type mltn-10 appeared less toxic than mltn-10(mg364) after the microinjection of equivalent amounts of DNA (data not shown). High-copy arrays that contained mltn-4 and mltn-7 also conferred the mltn phenotype. High-copy arrays that contained mltn-3 and mltn-11 caused embryonic and larval lethality prohibitive to the propagation of transgenic nematodes (data not shown). Thus, increased expression of mltn-10, mltn-4, or mltn-7 can prevent the removal of larval cuticles. Activities of the other mltn genes will be tested in future work.

Our screen for intragenic suppressors of mltn-10(mg364) identified additional mutations that affect the proline-rich repeats of MLT-10. As previously stated, mg364 specifies the substitution H590Y. H590 is conserved among the annotated orthologues of MLT-10 and is one of only five charged residues present in the repetitive region (Figure S4). The intragenic suppressors mgk477, mgk434, and mgk435 specified the substitutions G597R, W688R, and G597E, respectively. None of these missense mutations blocked expression of the mltn-10(mg364) gene (Figure S2). However, each of the corresponding substitutions introduced a charged residue into the repetitive region. The mechanism of suppression may therefore involve the rectification of MLT-10(H590Y) or destabilization of the otherwise toxic protein. The mgk477 and mgk435 mutations affected splice sites of mltn-10 and reduced expression of the gene (Figure S2). The sequence of mltn-10(mg416) cDNAs indicated correct splicing. In contrast, the sequence of mltn-10(mgk436mg364) cDNAs indicated the use of an atypical splice acceptor site for exon 8 and the related introduction of a premature stop codon upstream of the proline-rich repeats (Table S3). In this case, diminished expression of the repetitive region likely accounts for the suppression of mg364. Taken together, these findings indicate that dysfunction of the repetitive region of MLT-10 blocks the removal of larval cuticles. Moreover, the data suggest that electrostatic interactions involving the P-X2-L-(S/T)-P repeats influence the utility of MLT-10. As we shall discuss, we hypothesize that MLT-10(H590Y) interferes with the function of multiple members of the MLT-10 family, as does increased expression of wild-type MLT-10.

The mltn-10 gene is expressed in the hypodermis during larval development

To determine the spatial and temporal expression pattern of mltn-10, we fused the promoter of mltn-10 to the gfp-pest gene, which encodes a variant of green fluorescent protein (GFP) that is rapidly degraded in vivo (Li et al., 1998; Frand et al., 2005). The mltn-10::gfp-pest fusion gene was expressed in the major body hypodermal syncytium (Hyp7), the dorsal and ventral ridges of the hypodermis, hypodermal cells in the head and tail, and the pharyngeal myoepithelium, but not the lateral seam cells (Figure 4A). In L4-stage animals cultured at 25°C, GFP was first detected in the anterior hypodermis ~3.5 h before ecdisis. The fluorescence spread throughout Hyp7 and intensified for about 3 h. The fluorescence dissipates at the end of lethargus and was barely detectable 1 h after ecdisis (Figure 4A). A similar pulse of expression of GFP accompanied all four molts and faithfully recapitulated expression of the endogenous mltn-10 gene, as previously described (Frand et al., 2005). GFP was also expressed in epidermal cells of pretzel-stage embryos, which synthesize the cuticle for the first larval stage (data not shown). We conclude that most hypodermal cells and syncytia express mltn-10 whenever a new exoskeleton is made.

We previously reported that nhr-25 and -23 drive super-numerary bouts of expression of mltn-10 in let-7 family mutants that continue molting after the fourth larval stage (Hayes et al., 2006). Here, we asked if nhr-25 and -23 also regulate expression of mltn-10 during larval development. We used RNAi to knockdown nhr-25 or -23 in mgk439[mltn-10::gfp-pest] larvae. The hypomorph allele nhr-25(ku217RNAi) was used to sensitize animals to RNAi of nhr-25 (Chen et al., 2004). Sets of larvae were repeatedly monitored for expression of GFP over an 8-h time period encompassing the L4-to-adult molt. The mid-L4-stage larvae selected for this experiment were active at the start, but became lethargic and attempted to shed the L4-stage cuticle by the end. Only 40% (n = 49) of nhr-25(ku217RNAi) animals expressed the GFP reporter during this time, compared with 100% (n = 22) of control animals. Moreover, the fluorescence associated with GFP was dim and ephemeral in nhr-25(ku217RNAi) mutants, compared with wild-type animals. None (n = 24) of the nhr-23(RNAi) animals expressed any detectable GFP, as previously reported (Frand et al., 2005). RNAi of nhr-25 or -23 also greatly reduced the abundance of mltn-10 messages in late L4 larvae, as indicated by RT-PCR (Figure 4B). Thus, NHR-25 and -23 promote expression of mltn-10 during the larval molting cycle.

To detect the MLT-10 protein in vivo, we constructed two distinct, full-length translational fusion genes between mltn-10 and fluorescent reporters (Figure S1). The mCherry tag was inserted at the C-terminus of MLT-10. GFP was inserted downstream of the predicted signal sequence. The corresponding arrays conferred many of the same phenotypes as mgEx699[mltn-10], confirming expression of the MLT-10 fusion proteins.

During the process of molting, the MLT-10::mCherry and GFP::MLT-10 fusion proteins were detected in vesicle-like objects near the apical surface of the epidermis (Figure 5, A and B). Cherry was also readily detected in other structures, whereas GFP was possibly due to proteolytic processing of the N-terminal fusion protein.

In young adults, the MLT-10::mCherry fusion protein was also detected in coelomocytes, distinctive scavenger cells that endocytose material from the pseudocoelom (Grant and Sato, 2006). Cherry was detected in the coelomocytes of ~22% (n = 176) of aadEx16[mltn-10::mCherry] animals. Fusion proteins secreted from the apical surface of the hypodermis may have mixed with coelomic fluids in this particular background, because the mltn-10::mCherry array was associated with gaps in the syncytial epidermis, as we shall describe. Alternatively, some MLT-10::mCherry may have been released from the basolateral surface of Hyp7 or the pharyngeal myoepithelium. In either case, uptake by coelomocytes verified that MLT-10::mCherry was secreted, as coelomocytes do not express the mltn-10 gene. Collectively, these observations suggest that MLT-10 is released from the hypodermis to the surrounding matrices and fluids during the process of molting.

The MLT-10::mCherry fusion protein was also detected in strands and loops near the surface of transgenic animals (Figure 5D). These structures were observed in 27% (n = 123) of mixed-stage larvae, but were most prominent in...
animals completing the fourth molt. The strands ranged from 5 to 30 μm in length and appeared to be positioned above the hypodermis. The formal possibility that these structures result from the nonspecific aggregation of mCherry cannot be eliminated at this time. Nonetheless, these observations are consistent with the model that MLT-10 assembles into oligomeric complexes in vivo. A complete description of any such complexes awaits the availability of anti-MLT-10 antibodies.

Either the Loss or the Gain of mlt-10 Function Impinges on the Exoskeleton

The molting cycle involves both the synthesis and the removal of cuticles, and these processes are likely interconnected. We therefore examined several aspects of the exoskeleton to more fully define the phenotypes associated with particular mutations in mlt-10. To determine the effect of MLT-10(H590Y) in the absence of unshed cuticles, we examined mlt-10(mg416mg364) double mutants, rather than mlt-10(mg364) single mutants, in many experiments. To evaluate the barrier function of the exoskeleton, we treated larvae with the DNA-binding dye Hoechst 33258, as previously described (Moribe et al., 2004). The mlt-10(tm3331), mlt-10(ok28581), mlt-10(mg364), and mlt-10(mg416mg364) mutants were all more permeable to Hoechst than wild-type larvae (Figure 6). In this regard, mlt-10(mg364) larvae resembled bus-8(e2887) mutants, which have more porous cuticles because of a defect in the glycosylation of secreted proteins (Partridge et al., 2008). The increased permeability of mlt-10(mg416mg364) mutants to Hoechst was directly attributable to expression of the MLT-10(H590Y) protein, because mlt-10(mg416) single mutants were not permeable to the dye. High-copy mlt-10(+) and mlt-10::mCherry arrays were also associated with increased permeability of the cuticle (data not shown). As a complementary approach, we tested the ability of animals to survive chronic osmotic stress. The survival rate of various mlt-10 mutants was significantly lower than that of wild-type animals on media containing 300 mM NaCl (Figure 6F). Thus, mutations of mlt-10 decrease the utility of the exoskeleton as a barrier to the environment.

To detect a cuticle collagen in vivo, we used a translational fusion gene between col-19 and gfp, kindly provided by Anthony Page (University of Glasgow). In wild-type animals, COL-19::GFP is incorporated into the circumferential annuli and longitudinal alae of the adult exoskeleton (Thein et al., 2003). In contrast, COL-19::GFP macromolecules were disorganized in many mlt-10(tm3331) and mlt-10(ok2581) animals (Figure 7). Expression of MLT-10(H590Y) or MLT-10::mCherry also interfered with the assembly of COL-19::GFP, particularly above the lateral hypodermis (Figure S5). The severity of disorganization of COL-19::GFP varied among isogenic mlt-10 mutants, perhaps because of compensatory changes in the expression of other MLTN proteins. We conclude that both LOF and GOF mutations of mlt-10 affect the patterning of COL-19::GFP and possibly other cuticle collagens.
We also examined the morphology of the longitudinal alae on the adult exoskeleton. The alae of wild-type adults were continuous, straight, and comprised of three ridges (n = 28). In contrast, in many mlt-10(ok2581) and mlt-10(tm3331) mutants, segments of the alae were broken, branched, or comprised of four ridges (Figure 8). The alae were malformed in 24% (n = 80) of mlt-10(lof) mutants. The alae were similarly malformed in 72% (n = 22) of mlt-10(mg364) animals and the majority of mgEx699[mlt-10] adults. Atypical sausage-shaped structures were observed on the surface of some mlt-10(gof) mutants (Figure 8F). Thus, both LOF and GOF mutations of mlt-10 affect the morphology of the adult exoskeleton, which is synthesized during the final molt. Taken together, these ob-

Figure 5. Localization of the MLT-10::mCherry and GFP::MLT-10 fusion proteins. (A–D) Representative fluorescence and Nomarski micrographs show expression of the fusion proteins. (A) MLT-10::mCherry detected in vesicle-like objects (arrow) in the lateral epidermis of a late L4 larva molting to the adult stage. Alae are visible on the underlying cuticle (arrowhead). (B) GFP::MLT-10 detected in vesicles (arrow) in the lateral epidermis of a molting larva. A double cuticle covers the mouth (arrowhead). (C) Cherry detected in a coelomocyte (arrow) of a young adult. (D) MLT-10::mCherry found in strands (arrows) near the surface of a young adult. Scale bars, 10 μm.
Observations indicate that mlt-10 is involved in the synthesis of new cuticles, as well as the removal of old ones.

**Mutations of mlt-10 Affect Development of the Epidermis**

Molting requires the coordinated activity of epidermal cells and syncytia spread across the body. We therefore examined the status of the epidermis in mlt-10 mutants, focusing on the stem cell-like lateral seam cells, which produce the longitudinal alae. The seam cells divide asymmetrically early in the L1 stage (Soulston and Horvitz, 1977). Afterward, the anterior daughters fuse with the hypodermis, whereas the posterior daughters elongate and reconnect with one another. In this report, adherens junctions at the seam cell margins were detected using the AJM-1::GFP fusion protein and the seam cell nuclei were detected using a scm::gfp transcriptional fusion gene, as previously described (Hope, 1991; Mohler et al., 1998). As expected, bilateral rows of rectangular cells were observed in wild-type larvae late in the L1 stage (Figure 9A; Podbilewicz and White, 1994). In contrast, particular seam cells were misshapen and overlapped their sisters in mlt-10(tm3331) and mlt-10(ok2581) mutants (Figure 9B). Abnormal cells were observed in 6% (n = 278) of mlt-10(tm3331) mutants, but were not observed in any of 150 wild-type larvae. Oddly shaped seam cells were also present in 8.5% (n = 200) of mlt-10(mg416mg364) larvae at this stage of development (Figure 9, C and D).

In wild-type animals, the seam cells fuse with their sisters late in the L4 stage and thereafter cease to divide (Soulston and Horvitz, 1977). The resulting bilateral syncytia were malformed and contained extra nuclei in some mlt-10(mg416mg364) mutants, a phenotype suggestive of seam cell hyperplasia (Figure 9F). Moreover, the syncytial seam of many Ex[mlt-10::mCherry] and mlt-10(mg364) adults contained gaps lacking detectable AJM-1 at the cell margins (Figure S6 and data not shown). In addition, GFP was detected outside of the syncytial seam in the majority of mlt-10(lof) and mlt-10(416mg364) mutants expressing the scm::gfp and ajm-1::gfp fusion genes (Figure 9F). Preliminary studies using Nomarski microscopy and DAPI staining identified some sites of ectopic GFP expression as hypodermal nucleoli. Collectively, these findings show that mutations of mlt-10 affect several aspects of epidermal development, including dynamic changes in cell shape. Aberrant development of the seam cells likely contributes to the malformation of alae in mlt-10 mutants and possibly to defects in the molting cycle.

We obtained additional information about the status of the seam cells in mlt-10 mutants by staining larvae with the

**Figure 6.** Increased permeability of the cuticle in mlt-10 mutants. (A–D) Representative fluorescence micrographs of larvae stained with Hoechst 33258. All images were acquired with an exposure time of 50 ms. Scale bar, 10 μm. (E) The fraction of larvae with nuclei stained by Hoechst 33258. Values represent the average of two independent experiments; error bars, SEM. Asterisks indicate a significant difference from wild-type animals (p < 0.05). (F) Early L1 stage larvae of the indicated genotypes were collected and cultured on high-salt or standard NGM plates for 3 d at 20°C. Values represent the average of two independent experiments; error bars, SEM.
lec tin wheat germ agglutinin (WGA) conjugated to the fluorescent dye Alexa Fluor 488 (Figure S7). Similar probes are regularly used to detect the surface glycans of *C. elegans* (Politz *et al.*, 1990), and mucin-type glycans are major ligands of WGA in this system (Natsuka *et al.*, 2005). WGA bound to the margins of the seam cells in *mlt-10*(mg364) mutants but not in wild-type larvae (Figure S7), suggesting the unnatural exposure or accretion of particular glycans. Those glycans may influence the adhesiveness and shape of the seam cells. Widespread ligands of WGA were also de-

**Figure 7.** Disorganization of a cuticle collagen in *mlt-10* mutants. (A–D) Representative confocal fluorescence micrographs show COL-19::GFP in the adult exoskeleton. Arrows point to disorganized assemblies of COL-19::GFP flanking the longitudinal alae. Scale bar, 10 μm.

**Figure 8.** Malformation of the adult-specific alae in *mlt-10* mutants. (A–F) Representative Nomarski micrographs show the adult exoskeleton. Arrow, abnormalities in the alae including gaps, branches, and regions with four ridges. Arrowhead, an atypical structure in the cuticle. Each region of interest was digitally magnified 2.5-fold for display in the inset. Scale bar, 10 μm.
tected in the shed cuticles (molts) of mlt-10 mutants and wild-type larvae. Moreover, ligands of WGA were concentrated at the anterior end of partly shed cuticles on mlt-10(lof) and mlt-10(mg364) mutants (Figure S7). Such mucus has not yet been observed on wild-type larvae, but could, in theory, provide natural lubrication at the moment of ecdysis.

**Additional Phenotypes of mlt-10 Mutants**

A variety of other phenotypes were associated with the mlt-10(mg364) mutation, including the presence of large, seemingly fluid-filled spaces and vacuoles in the body; aberrant shape of the body; uncoordinated movement; and improper development of the gonad and vulva. Some of these phenotypes may be attributable to abnormalities in the exoskeleton and epidermis. Notably, mlt-10(mg364) adults produced only 6 ± 10 (n = 18) progeny, whereas wild-type animals produced 262 ± 26 (n = 9) offspring. The cause of this sterility is not yet understood.

**DISCUSSION**

Here, we describe the isolation and characterization of the mlt-10 gene of *C. elegans*. MLT-10 is the first reported member of a large family of nematode-specific proteins characterized by DUF644 and tandem P-X-L-(S/T)-P repeats. The mg364 allele of mlt-10 emerged from a genetic screen on low cholesterol. The mutation specifies the substitution H590Y in the repetitive region of MLT-10 and is thought to interfere with the function of multiple members of the MLT-10 family. Nine additional alleles of mlt-10, including two intragenic deletions and four substitution mutations, as well as high-copy mlt-10 arrays, were used to analyze the role of mlt-10 in larval development.

Our findings suggest that MLT-10 is a secreted protein involved in the removal of old cuticles as well as the synthesis of new cuticles. In review, either the loss or the gain of mlt-10 function impede the shedding of old cuticles. Both LOF and GOF mutations of mlt-10 are also associated with abnormalities in the larval and adult exoskeletons. The syn-
thesis and removal of cuticles are almost certainly interconnected in nematodes, as newly synthesized cuticles displace the preexisting ones during the process of molting. Consistent with that view, deformities in the ultrastructure of the underlying cuticle have been observed in particular molting-defective mutants (Hao et al., 2006). Moreover, certain enzymes involved in the biosynthesis of collagens and other ECM proteins are needed for the removal of larval cuticles, including the glycosyltransferase BUS-8, the peroxidases BLI-3 and MLT-7, and the protease BLI-5 (Edens et al., 2001; Davis et al., 2004; Frand et al., 2005; Page et al., 2006; Partridge et al., 2008; Thein et al., 2009; Stepek et al., 2010). In theory, progress through ecdysis might depend on physiological feedback on the status of the new exoskeleton. Consistent with that idea, animals forced to ecdyse before completion of a new cuticle perish, probably due to osmotic shock (Ruada and Bessereau, 2006).

Mutations of mlt-10 also affect the development of epidermal cells and syncitia that synthesize the exoskeleton. Notably, several transcription factors required for proper development of the seam cells are also necessary to remove larval cuticles, including NHR-25 and the GATA factors ELT-5 and -6 (Asahina et al., 2000; Gissendanner and Sluder, 2000; Koh and Rothman, 2001; Chen et al., 2004; Silhankova et al., 2005). We hypothesize that some abnormalities in seam cell development observed in nhr-25 mutants relate to the deregulation of mlt-10. Further, we speculate that MLT-10 directly or indirectly affects cell–ECM interactions important for epidermal development.

Using a transcriptional fusion gene, we show that mlt-10 is transiently expressed in hypodermal cells and syncitia whenever the exoskeleton is remade. The conserved nuclear hormone receptors NHR-23 and -25 are required for the periodic expression of mlt-10. Consequently, the amount of mlt-10 expressed at any time in development probably relates to the abundance of steroid hormones that bind these receptors. Transcriptional control by NHR-23 and -25 may coordinate the expression of MLT-10 with the production of particular collagens, matrix modification enzymes, and signaling molecules involved in the molting cycle.

Two full-length, translational fusion proteins were used to evaluate the distribution of MLT-10. Both the MLT-10::mCherry and the GFP::MLT-10 fusion proteins were detected in putative vesicles in the lateral hypodermis during molting. The MLT-10::mCherry fusion protein was also detected in strands near the surface of animals. After completion of the molting cycle, Cherry was detected in coelomocytes. Collectively, these findings suggest that MLT-10 is secreted from the hypodermis to the surrounding matrices and fluids during the process of molting.

Proline-rich repeats are found in several well-characterized extracellular proteins, including collagen, elastin, and flagelliform silks (Bhattacharjee and Bansal, 2005; He et al., 2007; Matsushima et al., 2008; Savage and Gosline, 2008; Wise and Weiss, 2009). After comparing MLT-10 with these proteins, we predict that three main factors contribute to the secondary structure of the C-terminal region of MLT-10: 1) steric repulsion among proline residues, 2) attraction among hydrophobic residues, and 3) hydrogen bonding among hydroxy amino acids. One possibility is that the P-X_{S/T}-P repeats of MLT-10 promote the assembly of oligomeric complexes, perhaps including other MLTN proteins. The structure of any such complexes would likely be distinct from collagen, because the P-X_{S/T}-P repeats contain very few glycine residues. An alternative possibility is that the C-terminal region of MLT-10 lacks regular secondary structure and mostly provides extension or flexibility. In either case, we expect the tertiary structure of MLT-10 to allow segregation of the hydrophobic P-X_{S/T}-P repeats from the hydrophilic DUF644.

We propose that the molting cycle involves the dynamic assembly and disassembly of oligomeric MLT-10 complexes. In principle, the MLT-10(H590Y) substitution mutation might interfere with intermolecular interactions among multiple members of the MLTN family, by altering the electrochemical properties of the repetitive region or forming inappropriate di-tyrosine cross-links. Interference with multiple paralogs could account for the severe phenotypes caused by the mlt-10(mg364) mutation. Excessive production of MLT-10 might also disrupt interactions between MLT-10 and the MLTN proteins. Notably, many disease-associated substitution mutations in human collagens interfere with the assembly of collagen fibrils (Blank and Boskey, 2008). Studies of these particular dominant-negative mutations in collagens have greatly enriched our understanding of ECM remodeling in human development and disease.

Our working model is that MLT-10 serves as an instructive or structural component of the cuticle and thereby influences the assembly or disassembly of collagens. Alternatively, MLT-10 might promote the trafficking of collagens through the secretory pathway of the hypodermis. A similar function has been proposed for the membrane-spanning proteins CUTI-1 and TSP-15, which are required for shedding larval cuticles (Moribe et al., 2004; Fritz and Behn, 2009). A third possibility is that MLT-10 and the paralogs of MLT-10 serve as monomeric lubricants that help dislodge old cuticles from the hypodermis. As lubricants, the MLTN proteins might also facilitate particular behaviors used to escape old cuticles, including longitudinal rotation. Further research is needed to fully define the function of MLT-10.

The increased dependence of mlt-10 mutants on exogenous cholesterol suggests a reduced capacity to acquire or utilize sterols. We therefore speculate that mutations of mlt-10 directly or indirectly reduce the function of other proteins linked to sterols and essential for shedding cuticles. Those proteins include the hedgehog-like protein QUA-1, several homologues of Patched, and the low-density lipoprotein receptor-like protein LRP-1 (Yochem et al., 1999; Zugast et al., 2005; Hao et al., 2006). LRP-1 is expressed on the apical surface of the hypodermis (Yochem et al., 1999), which may represent an important site of sterol uptake in nematodes (Fleming and Fetterer, 1984).

Our ongoing investigation of the MLT-10 family may directly benefit the development of new drugs for filarial diseases currently affecting over 140 million people living primarily in tropical regions. The compounds currently in use target ion channels and cytoskeletal proteins that are conserved between nematodes and mammals, and these compounds can be toxic to humans. In contrast, DUF644 is well conserved only in nematodes. Surface glycoproteins also comprise major antigens of parasitic nematodes (Blaxter et al., 1992) and may include multiple homologues of MLT-10. The homologues of MLT-10 are therefore attractive targets for drug development.

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