MUP-4 is a novel transmembrane protein with functions in epithelial cell adhesion in *Caenorhabditis elegans*

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Tissue functions and mechanical coupling of cells must be integrated throughout development. A striking example of this coupling is the interactions of body wall muscle and hypodermal cells in *Caenorhabditis elegans*. These tissues are intimately associated in development and their interactions generate structures that provide a continuous mechanical link to transmit muscle forces across the hypodermis to the cuticle. Previously, we established that *mup-4* is essential in embryonic epithelial (hypodermal) morphogenesis and maintenance of muscle position. Here, we report that *mup-4* encodes a novel transmembrane protein that is required for attachments between the apical epithelial surface and the cuticular matrix. Its extracellular domain includes epidermal growth factor-like repeats, a von Willebrand factor A domain, and two sea urchin enterokinase modules. Its intracellular domain is homologous to filaggrin, an intermediate filament (IF)-associated protein that regulates IF compaction and that has not previously been reported as part of a junctional complex. MUP-4 colocalizes with epithelial hemidesmosomes overlying body wall muscles, beginning at the time of embryonic cuticle maturation, as well as with other sites of mechanical coupling. These findings support that MUP-4 is a junctional protein that functions in IF tethering, cell–matrix adherence, and mechanical coupling of tissues.

**Introduction**

Cell–cell and cell–extracellular matrix (ECM)* interactions are mediated by junctional complexes such as hemidesmosomes and adherens junctions. These junctions are necessary for multicellular animals to maintain the integrity of individual cells and to integrate tissue functions. Transmembrane proteins at junctional complexes, such as integrin and dystroglycan, link ECM components to intracellular cytoskeletal networks of microfilaments, microtubules, and intermediate filaments (IFs). These interactions are regulated, and defects in components of junctional complexes can affect the integrity and function of the cells, or the coordinated functions of tissues, including accommodation of mechanical forces and the assembly of ECM (Campbell, 1995; Vidal et al., 1995; Henry and Campbell, 1998; Fuchs and Yang, 1999).

This paper investigates the contributions of the *mup-4* gene to the muscle and epithelial tissue functions in *C. elegans*. Body wall muscle and the hypodermis are an excellent model to study coordinated tissue interactions. The hypodermis is a specialized epidermis that secretes a complex matrix, the cuticle, which is modified throughout the life of the worm (Cox et al., 1981; Sulston et al., 1983; Cox and Hirsh, 1985; Priess and Hirsh, 1986; Waterston, 1988; Costa et al., 1998; Hresko et al., 1994, 1999; Johnstone, 1994, 2000; Kramer, 1997; Moerman and Fire, 1997; Williams-Masson et al., 1997, 1998). During midembryogenesis, the hypodermis migrates to enclose the ovoid embryo, which then elongates three- to fourfold (threefold stage) (Sulston et al., 1983; Williams-Masson et al., 1997; Chin-Sang and Chisholm, 2000). Commensurate with embryo elongation are specific interactions between the hypodermis and body wall muscle cells, which lie in two dorsal and two ventral strips extending the length of the animal just below the hypodermis (Figs. 1 and 2 A) (Waterston, 1988; Hresko et al., 1994). These hypodermal regions are greatly flattened with elaborate IF structures, appearing similar to vertebrate...
epithelial tonofilaments. IFs are the most diverse of the cytoskeletal components and function as structural integrators of the cytoplasm to resist and transmit mechanical stress (Bartnik and Weber, 1988; Fuchs and Cleveland, 1998; Houseweart and Cleveland, 1998; Fuchs and Yang, 1999). The hypodermal IFs are associated with dense membrane plaques and fibers extending into the ECM. These structures (also called fibrous organelles) are analogous to vertebrate hemidesmosomes (Francis and Waterston, 1991), cell–matrix junctions that provide adhesive spotwelds between the IF cytoskeleton and the ECM. IFs are regulated by IF-associated proteins (IFAPs), and specific IFAPs provide linkages at desmosomes (cell–cell junctions) and hemidesmosomes (Houseweart and Cleveland, 1998; Jones et al., 1998; Borradori and Sonnenberg, 1999); however, the dynamics of IFs and IFAPs in development are not well understood. In C. elegans embryos, it is known that the development of the hypodermal hemidesmosomes requires interactions between muscle and hypodermal cells, and thus represents a dynamic junctional complex that coordinates tissue functions, including the transmission of muscle force to the cuticle (Francis and Waterston, 1991; Hresko et al., 1994, 1999; Plenefisch et al., 2000).

The mup-4 gene (muscle position defective) has essential functions in the C. elegans embryonic epidermis: mup-4 embryos arrest development either with their hypodermis failing to enclose the embryo, or during the threefold stage with defects in hypodermal cell organization and muscle cell positions (Fig. 2, B and C; Gatewood and Bucher, 1997). We now show that MUP-4 is a novel transmembrane protein composed of extracellular domains with EGF-like repeats, a von Willebrand factor A (vWFA) domain, two sea urchin enterokinase (SEA) modules, and an intracellular domain with homology to the vertebrate IFAP filaggrin. Analyses of the localization of MUP-4 and the ultrastructural lesions in mutants support the hypothesis that MUP-4 functions to tether IFs and to adhere the hypodermis to the apical ECM, thus providing a mechanical link that transmits muscle forces to the cuticle.

Results

Cloning of mup-4

mup-4 was previously mapped to a 0.02 map–unit interval on LG III (Gatewood and Bucher, 1997), and within this region resided a partial ORF with multiple EGF-like repeats (cosmid K07D8; cDNA group, CELK00028; Fig. 3 A) (Wilson et al., 1994). We examined mup-4 alleles for polymorphisms in this candidate and found that mup-4(s2426) is a 0.8-kb Df (Materials and methods; Fig. 3 A). Sequence analysis of cloned PCR fragments demonstrated that nucleotides 2476–3318 were deleted (K07D8, sequence data available at EMBL/GenBank/DDBJ under accession no. L16679), and any protein product from mup-4(s2426) would be truncated: the Df begins at amino acid 1295 (see below) within the EGF repeat just before the first SEA domain, and results in the addition of RESREQKEVV-GYWNPQFHFXTREAKCCCRASQCVH* after amino acid 1295. Consistent with this molecular analysis, mup-4(s2426) appears null (Materials and methods).

Supporting this polymorphism result, introduction of either fosmid H14A12 or PCR products (some with a green fluorescent protein [GFP] tag) as transgenes rescues mup-4 mutants (Fig. 3 A; Table I). Although the fosmid has two other predicted genes (C07H6.5 and C07H6.4), neither of these genes is present in other rescuing fragments, and cosmids leftward of this locus fail to rescue mup-4 (Gate-
amino acid signal peptide at the NH₂ subclass including 27 unusual repeats, 14 with residues served residues, the 28 EGF-like repeats fall into different (classification according to Herz et al., 1988). Based on con-
times, that is similar to the class B repeats of LDL and EGF motif is a cysteine-rich motif (Fig. 4, legend), repeated 28 follow by three distinct motifs. The first class of lar domain, composed of transmembrane protein (Fig. 3 B). The predicted extracellu-
encoding a predicted product of 2,104 amino acids. Comparison of the cDNA and genomic sequences shows
that mediate Ca²⁺ binding, and one of the EGF-Notch B.1 class. Except for a unique cysteine repeat 5, MUP-4 EGF-
like repeats show high similarity to a C. elegans homologue mua-3 (Bercher et al., 2001), with the next highest similarity often being to the Notch and Fibrillin CB-EGFs. EGF-like domains have been shown to bind other extracellular proteins (Rees et al., 1988; Engel, 1989).

A single vWFA module occurs after the eighth EGF-like repeat (Fig. 5 A). Consensus sites for Mg²⁺ binding (Lee et al., 1995), disulfide bridging, and glycosylation—three modifications typical of the vWFA domain—are found in MUP-4. Biochemical studies suggest that this domain binds to proteins such as collagen and regulates the organization of other matrix proteins (Sasaki et al., 1987; Titani and Walsh, 1988; Colombatti et al., 1993).

The final extracellular motif is the twice-repeated SEA module, separated by EGF repeat 23 (Fig. 5 B). The SEA module is associated with glycosylated proteins (Bork and Pathy, 1995), and consistent with this, MUP-4 has consen-
sus sites for glycosylation.

After the 28th EGF-like repeat is a single pass hydrophobic segment bounded by charged residues (amino acids 1858–1882) (Argos and Rao, 1986), followed by an intracellular domain of 213 amino acids. This domain has consensus sites for phosphorylation and is homologous to an IFAP, human filagrin (Fig. 5 C). Residues 1882–1950 are 28% similar, and the remainder are 40% similar to human filagrin. This homology is striking since this class of IFAPs is typified by abundant phosphorylation sites and a high content of histidine, serine, glutamic acid, glycine, alanine, and arginine, often with little identifiable linear sequence homology (Steinert et al., 1981; Harding and Scott, 1983; Patthy, 1995), and consistent with this, MUP-4 has consen-
sus sites for glycosylation.

MUP-4 encodes a novel transmembrane protein with a modular structure Comparison of the cDNA and genomic sequences shows that mup-4 has 20 exons (Fig. 3 A; cDNA, under EMBL/ GenBank/DDBJ accession no. L16679). mup-4 transcripts are transspliced to SL1, and the mature mRNA is 6739 nt encoding a predicted product of 2,104 amino acids.

Conceptual translation reveals that MUP-4 is a large transmembrane protein (Fig. 3 B). The predicted extracellu-
lar domain, composed of 1,855 amino acids, has a 15–amino acid signal peptide at the NH₂ terminus (von Heijne, 1986) followed by three distinct motifs. The first class of motif is a cysteine-rich motif (Fig. 4, legend), repeated 28 times, that is similar to the class B repeats of LDL and EGF (classification according to Herz et al., 1988). Based on con-
served residues, the 28 EGF-like repeats fall into different subclasses including 27 unusual repeats, 14 with residues

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wood, 1996). Finally, introduction of dsRNA (Fire et al., 1998) of this ORF causes a Mup phenotype (Table II, A and B) identical to mup-4 mutants (Fig. 2, compare B and C with D). These data establish that this ORF is mup-4.
Distribution of MUP-4 in wild-type (WT) and mutant embryos

To determine the cellular localization of MUP-4, we examined threefold stage embryos expressing a mup-4::gfp transgene (Table I); since the transgene constructs rescue mup-4(mg36) lethality, functional MUP-4::GFP must be expressed in the correct locations. GFP fluorescence was abundant in circumferential annular rings overlying muscle of threefold embryos (not shown). The antipeptide antibody raised to the intracellular domain showed similar staining (Fig. 6, A and B). In contrast, we never observed this pattern in mutants (Fig. 6, C–F); although mutant and wild-type (WT) embryos also exhibit diffuse patterns of antibody reactivity. These data are consistent with a function in hypodermal cell–matrix junctions specifically over muscle cells.

Figure 4. Alignment of 28 cysteine-rich motifs in MUP-4. Dark highlights indicate identical amino acids, and light highlights indicate conservative changes. The EGF motif is comprised of a characteristic pattern of six cysteines and forms a globular domain stabilized by disulfide bridges (Davis, 1990). A consensus for the MUP-4 EGF-like repeats is shown (Herz et al., 1988). The consensus specific to the Ca\(^{2+}\)-binding EGF subclass (EGF-CB) to which Mup-4 repeats 2, 3, 4, 7, 14, 15, 16, 18, 19, 20, 21, 25, 26, and 27 belong (according to Rees et al., 1988), and a consensus shared by the MUP-4 EGF-like repeats are also shown. The first 27 repeats appear as a hybrid of EGF-like classes, as defined by their spacing of six cysteines and like B.1, whereas after the fifth cysteine, it is more like B.2. In places where an intervening number of residues is less variant, the number most often found is listed. The position of each EGF-like motif in the amino acid sequence is shown in the last column. The juxtaposition of EGF-like domains is close, most being separated by only a few amino acids, with the exception of 67 amino acids between repeats 9 and 10. Other larger spacings reflect the interspersed vWFA and SEA domains (see Figs. 3 B and 5, A and B).
GFP and MUP-4 antibodies also localize to other regions where cells show mechanical attachments to the hypodermis including (a) the inner surface of the pharynx where the radial pharyngeal muscle cells attach to the marginal hypodermal cells, which secrete a cuticle; (b) overlying anal and intestinal muscles (e.g., the B cell; Fig. 7, A and B); (c) overlying vulval and uterine sex muscles; (d) male tail muscle attachment zones; and (e) the six mechanosensory neurons (Fig. 7 E) that mediate touch sensitivity through their attachment to the hypodermis (Chalfie and Sulston, 1981). Examination of WT animals also labeled with mAb MH27, which recognizes adherens junctions, shows that MUP-4 staining can extend beyond the muscle to the seam cell boundary (Fig. 7 D). In some mup-4::gfp-expressing lines, we often see high concentrations of protein around the hypodermal or touch neuron nuclei (Fig. 7 B; data not shown). These patterns support the mosaic analysis (Gatewood and Bucher, 1997) that MUP-4 functions in hypodermal rather than muscle cells. Furthermore, the observed pattern of MUP-4 is reminiscent of the pattern of the MH4 mAb, which recognizes IFs of hemidesmosomes (Francis and Waterston, 1985, 1991).

The relationship between MUP-4 and IF localization was further examined by overlay of confocal images of double-labeled animals. This analysis demonstrated that their patterns largely
MUP-4 expression in larvae. (A) Lateral view of an N2 larva (L2) stained with a MUP-4 antibody. Arrows mark staining of MUP-4 in circumferential rings, arrowheads mark B cell perinuclear staining. (B) Lateral view of an L2 larvae MUP-4::GFP expression in an integrated mup-4::gfp line (EE82). Arrows mark staining in circumferential rings, arrowheads mark B cell perinuclear staining. Small arrow marks diffuse hypodermal cell staining in hypodermal cells overlying muscle. (C) Head of an L3 larva showing MUP-4::GFP fluorescence (EE73; arrow). (D) Confocal overlay of immunolocalization of MUP-4 (red) and MH27 and MHCA (green). MH27 demarcates seam cell boundary (arrow). (E–G) Confocal analysis of an L3 larva stained with MUP-4 and MH4 showing localization to circumferential rings (arrow) and the touch neuron (arrowheads). The confocal overlay (G) shows partial colocalization.

mup-4 functions during postembryonic development

The postembryonic expression of MUP-4 suggested later developmental functions not revealed by our genetic analysis. We tested this possibility by RNA interference (RNAi) (Table II). In support of the specificity of the assay, worms treated with mup-4 dsRNA segregate Mup progeny (Fig. 2 D, +) and injection of mup-4 dsRNA into a mup-4::gfp transgene line (EE86) caused dramatic reduction in GFP expression in all Mup animals (Table II D). In contrast, worms treated with mua-3 dsRNA segregate Mua progeny identical to mua-3(ar62) mutants and, when mua-3 dsRNA is injected into the mup-4::gfp transgene line, all Mua progeny showed normal mup-4::gfp levels (Fig. 2, E–G). These RNAi data support the identity of the mua-3 gene and that the RNAi assay is gene specific. We then soaked worms (P0 interference) in mup-4 dsRNA and found that MUP-4 is necessary in larvae: some larvae exhibited a phenotype within 12 h, and by 24 h, many worms were paralyzed and had displaced muscles (Fig. 2 D).

Ultrastructural analysis of mup-4 mutants

We used transmission EM to examine the ultrastructural defects underlying the muscle displacement and hypodermal disorganization in threefold mup-4 mutants. Micrographs of mutants demonstrated that on the dorsal surface the hypodermal cell membrane is cleanly separated from the cuticle (Fig. 8 A). Similarly, on the ventral bent surface, detachment of the hypodermis from cuticle is exhibited as giant folds of the cuticle that rise above the hypodermis, whereas the hypodermal membrane retains a straight outline (Fig. 8, A and B). We have never observed any evidence of either hypodermal cell lysis (i.e., membrane remnants associated with the cuticle), or a lesion between muscle and hypodermis (i.e., hypodermal cells associated with the cuticle with tearing at the basement membrane). In contrast, our control analysis of a mup-2 mutant for the muscle protein troponin T (TnT)-1, in which there is abnormal muscle contraction and localized muscle detachment (Myers et al., 1996; Mc Ardle et al., 1998), shows that the hypodermis is still attached to the cuticle (Fig. 8 C) in regions where the dorsal muscle is detached, as well as in curved ventral regions (Fig. 8 D). In contrast, all mup-4 mutants (n = 14) showed a lesion between the apical hypodermal membrane and the cuticle.

Discussion

We report that MUP-4 is a novel transmembrane protein with domains consistent with its function as an IFAP at epithelial junctions. mup-4 is expressed in the hypodermis (also see Gatewood and Bucher, 1997) and localizes to hypodermal cell–matrix boundaries, rather than cell–cell boundaries. This localization is coincident with hemidesmosome structures overlying body wall muscle, as well as at other sites of mechanical attachments of the hypodermis. MUP-4 localization develops in late threefold embryos, the stage when body wall muscle detachment occurs in mup-4 mutants. This localization is maintained throughout development and is required, as demonstrated by RNAi. These data, and the ultrastructural studies that demonstrate a defect in attachment between the apical hypodermis and the cuticle in mup-4 mutants, argue that MUP-4 provides a structural attachment between the epithelial cell IFs and the ECM and thereby transmits mechanical forces from the hypodermal tonofilaments to the cuticle (Fig. 1).

MUP-4 is a novel modular transmembrane protein

The MUP-4 modular structure is typical of ECM protein and matrix receptors, although aspects of this organization
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are novel. First, 27 EGF repeats are unusual: similar EGF-like repeats have only been identified in MUA-3 and in the 63-Kd sea urchin sperm membrane protein (Mendoza et al., 1993; Bercher et al., 2001). Second, a single class B.1 EGF motif, a class associated with signaling events, occurs next to the transmembrane domain of MUP-4; although the functional significance of this organization is not yet known, it is notable that this arrangement is observed in MUA-3, and three members of the low-density lipoprotein receptor superfamily: mammalian and C. elegans megalin (Herz et al., 1988; Yochem and Greenwald, 1993; Yochem et al., 1999) and C. elegans RME-2 (Grant and Hirsh, 1999). Third, although a nonnematode homologue of MUP-4 has yet to be found, some of the motifs found in MUP-4 have yet to be found, some of the motifs found in MUP-4 are observed together in other proteins (e.g., EGF and vWFA modules are found in cartilage matrix protein and integrin). The presence of EGF and vWFA modules supports the phenotypic data that MUP-4 has additional functions in matrix organization and ECM cell signaling (Sasaki et al., 1987; Titani and Walsh, 1988; Engel, 1989; Colombatti et al., 1993).

A key structural feature of MUP-4 is the presence of the filaggrin domain. The filaggrin and trichohyalin class of IFAPs regulate IF compaction to generate intracellular tonofilaments during vertebrate epithelial development (Steinert et al., 1981; Rothnagel and Rogers, 1986; O’Guin et al., 1992). In differentiating epithelial cells, a highly phosphorylated propeptide of multiple filaggrin repeats, profilaggrin, is localized in keratohyalin granules. Regulated dephosphorylation controls profilaggrin processing and subsequent keratin filament aggregation (Presland et al., 1992; Haydock et al., 1993; Resing et al., 1989; Kuechle et al., 1999). This dramatic rearrangement of keratin filaments generates a squamous epithelium that is resistant to mechanical forces. Although it is conceivable that the MUP-4 single filaggrin repeat of the intracellular domain is cleaved like profilaggrin, we did not identify an obvious cleavage site. Instead, the requirement of MUP-4 in cell–matrix attachment, and the filaggrin motif within MUP-4, supports a novel association of a filaggrin domain in a transmembrane junctional protein.

Ultrastructural studies support a MUP-4 function in adhesion

The hypodermal membrane and cuticle are separated in mup-4 mutants. In contrast, ultrastructural studies for mup-1 (Goh and Bogaert, 1991) and mup-2/TnT-1 (this study) mutants, which exhibit muscle detachment phenotypes grossly similar
to mup-4, showed normal hypodermal-cuticle disposition. Thus, detachment of the hypodermis from the cuticle is not an indirect consequence of muscle detachment, but rather a specific effect of the mup-4 mutation. Other defects observed in the EMs for all three of these mup mutations, e.g., nervous system and internal organ displacement, are likely a secondary consequence of muscle/hypodermal displacement. Interestingly, our RNAi analysis of mua-3 in the mup-4::gfp-expressing strain EE86 showed that the mup-4::gfp protein was associated with displaced muscle, consistent with a hypodermal cuticle detachment for mua-3 mutants as well (Bercher et al., 2001). The muscle and hypodermal disorganization in threefold Mups is due to loss of linkage between the epithelium and cuticle ECM. This phenotype is analogous to lesions between the cell and basement membrane found for mutants of functional components, including IFAPs (Jones et al., 1998) and cell–matrix receptors/adhesion proteins such as myotactin and dystroglycan (Henry and Campbell, 1998; Hresko et al., 1999).

mup-4 and mua-3 are homologues

mup-4 and mua-3 are homologues based on the high conservation and overall organization of EGF repeats, vWFA modules, and SEA modules (Bercher et al., 2001). For example, homologous EGF-like repeats are often ordered, the final EGF-like repeats are class B.1, and the EGF-like repeats before the vWFA domains are nearly identical. The major differences between the proteins are that MUA-3 has LDL class A repeats and nearly twice the number of EGF repeats as MUP-4. In addition, MUP-4 and MUA-3 intracellular domains have limited similarity (Fig. 5 C), although the amino acid charge distribution of the MUA-3 intracellular domain is similar to MUP-4 and filaggrins. Thus, MUA-3 may also interact with IFs (Steinert et al., 1981; Harding and Scott, 1983; Presland et al., 1992). The conserved protein structures and similar phenotypes suggest similar functions, although these functions are not redundant (see below).

MUP-4 function during development: a proposed function in IF association and hemidesmosome structure and function

Before the threefold stage, muscle is attached to the basement membrane, and microfilaments and microtubules in the hypodermis bear the internal forces of the embryo and the forces of muscle contraction during organismal elongation (Press and Hirsh, 1986; Costa et al., 1997). Hypodermal hemidesmosomes and basement membrane components associated with muscle attachment are organized by the twofold stage (Rogalski et al., 1993; Hresko et al., 1994, 1999; Williams and Waterston, 1994; Moerman and Fire, 1997), including myotactin, a transmembrane protein that has been proposed to function at the hypodermal cell–basement membrane interface (Fig. 1 B) (Hresko et al., 1999). During the late threefold stage, there are striking transitions: (a) myotactin localization specifically to IF structures; (b) cuticle maturation; (c) reorganization of the hypodermal actin and microtubule cytoskeleton; (d) transfer of muscular forces to the cuticle; and (e) localization of MUP-4 to IF structures. These cellular transitions are essential for the mechanotransduction of muscle contraction to the maturing cuticle and to bear the internal hydrostatic forces of the worm.

A structural role for MUP-4, beginning at this critical threefold stage, is supported by the protein structure, the localization of MUP-4 to IF structures in the hypodermis overlying muscle, loss of this localization in mutants, and a lesion between the hypodermis and cuticle in mutants. Intriguingly, our antibody studies show that MUP-4 localizes to attachment structures during the late threefold stage when the cuticle is being assembled. This raises the possibility that the MUP-4 extracellular domain may be activated by secreted cuticle components, thus facilitating IF binding through dephosphorylation of the filaggrin domain. Whether or not regulation of the phosphorylation state of the filaggrin domain occurs, MUP-4 might bind to existing tonofilaments (such as that recognized by MH4) and possibly contribute in maintaining IF localization (e.g., hemidesmosomes are eventually disrupted in myotactin mutants; Hresko et al., 1999). Alternatively, the intracellular domain of MUP-4 could compact and tether other IFs (Dodemont et al., 1994; Wilson et al., 1994; The C. elegans Genome Consortium, 1998). Although a regulatory role has yet to be tested, a role for MUP-4 in a mechanical linkage between the cytoskeleton and the ECM is strongly supported by our data.

Cell–ECM attachments exist at the apical and basal surfaces of the hypodermis, and the MUP-4, MUA-3, and myotactin proteins each appear to be components of the hemidesmosome complex that transverses the hypodermis (Fig. 1): each exhibits similar protein localization; each has a sequence composition consistent with being IFAPs; and mutants for each of these genes have a muscle detachment phenotype. Due to their grossly similar phenotypes, we initially hypothesized that MUP-4 and MUA-3 could be stage-specific isoforms (Bucher and Greenwald, 1991; Gatewood and Bucher, 1997; Pleanchef et al., 2000); however, mup-4 is also essential in larvae when mua-3 functions. Although mua-3 is expressed in embryos (Bercher et al., 2001), neither RNAi experiments, nor analysis of mua-3(ar62) and of mup-4 mua-3 double mutants (Gatewood and Bucher, 1997), revealed embryonic requirements. Thus, MUP-4 appears sufficient for attachments in embryos; but during larval stages when sarcomeres are added and muscle forces increase, both MUA-3 and MUP-4 are essential. The data do not yet discriminate whether these proteins function in either the same or molecularly distinct hemidesmosomes or apically versus basally, and the thinness of the hypodermis renders differentiating apical versus basal localization impractical, even by immunogold; however, the apical hypodermal lesions in mup-4 and mua-3 and basal lesions in myotactin mutants demonstrate different functional requirements of these genes. Continued studies of these proteins should provide new insights into how these IFAPs function in IF cytoskeletal regulation, matrix interactions, and the transmission of mechanical forces during development.

Materials and methods

Worm culture

Worms (Table III) were grown according to published methods (Brenner, 1974).

Analysis of mup-4(s2426)

(s2426) is an allele of mup-4 based on its map position, failure to complement mup-4(ar60), and segregation of Mups (Gatewood, 1996; Gatewood and Bucher, 1997; Stewart et al., 1998). Phenotypic analysis of mup-
4s(2426) used a chromosomally integrated array, jcIS1 IV (provided by J. Hardin and J. Simske, University of Wisconsin, Madison, WI), which expresses JAM-1 (the MH27 antigen) at epithelial cell boundaries (Mohler et al., 1998). We constructed the strain EE81 and examined mutants under Nomarski and fluorescence microscopy. Of 326 mutants examined, 262 showed a leaflike phenotype (data not shown). These phenotypes are similar to mutants with alleles, which are variably expressed and predicted null (unpublished data; Gatewood and Bucher, 1997). The increase in early arrest over the 10% seen previously may be related to mup-4(ar60) ncl-1(e1865) unc-36(e251)glp-1(q46); qDp3(III;f) EE86

upIs1 [mup-4::gfp(CeMup-4.99.1617); rol-6(pRF4)]

CS195

ncl-1(e1865) unc-36(e251)glp-1(q46) mua-3(ar62); qDp3(III;f)

CS234

mup-4(ar60) ncl-1(e1865) unc-36(e251)glp-1(q46) qDp3(III;f)

Sequence analysis

The 5’ end of the mup-4 cDNA was determined by sequencing the 4.08-kb EST clone, yk27d6 (Kohara, 1996). 5’ sequences were generated by PCR from an N2 cDNA library (superscript II reverse transcriptase; GIBCO BRL). PCR sense and antisense primer pairs (EMBL/GenBank/DDBJ accession nos. L11679 and K07D8) were as follows: BO208 and BO187; BO211 and BO210; BO214 and BO215. The 5’ boundary of the gene was determined using BO241 and primer BO244 to the spliced leader SL1 (Krause and Hirsh, 1987; Huang and Hirsh, 1989).


table i

| Name       | Genotype                                                                 |
|------------|--------------------------------------------------------------------------|
| N2         | wild type                                                                |
| BC4636a    | dpy-17(e164) mup-4(s2426) unc-32(e189); dP3I(III;f)                      |
| EE22       | mup-4(mg36) ncl-1(e1865) unc-36(e251); qP3I(III;f)                      |
| EE67       | mup-2(e2346ts; him-8(e1489)                                              |
| EE69       | Ex [mup-4::gfp(CeMup-4.99.1617); rol-6(pRF4)]                           |
| EE73       | mup-4(mg36) ncl-1(e1865) unc-36(e251); Ex [sur-5::GFP(pTG96_2) H14A12] |
| EE74, EE75 | mup-4(mg36) ncl-1(e1865) unc-36(e251); Ex [mup-4(CeMup-4.99.9)]         |
| EE81       | dpy-17(e164) mup-4(s2426) unc-32(e189); III; dP3I(III;f); jcIS1 IV      |
| EE82       | mup-4(mg36) ncl-1(e1865) unc-36(e251); Ex [mup-4::gfp(CeMup-4.99.1617)] |
| EE86       | upIs1 [mup-4::gfp(CeMup-4.99.1617); rol-6(pRF4)]                       |
| CS195      | ncl-1(e1865) unc-36(e251)glp-1(q46) mua-3(ar62); qDp3(III;f)             |
| CS234      | mup-4(ar60) ncl-1(e1865) unc-36(e251)glp-1(q46); qDp3(III;f)             |

mup-4 constructs for transgenic studies

Fosmid H14A12 and Cosmid C07H6 DNAs (The C. elegans Genome Consortium, 1998) were prepared by Cesium gradient purification (Sambrook et al., 1989). PCR fragments were generated with the expand long range PCR kit (Boeringer). CeMup-4.99.9 (10 kb) begins 1 kb upstream (BO232) from the AUG and ends 0.5 kb downstream (BO225) of the stop codon. CeMup-4.99.9 (14 kb) begins 5 kb upstream (BO268) from the AUG and ends 0.5 kb downstream (BO225) of the stop codon. CeMup-4.99.1617 (14 kb) begins 5 kb upstream (BO268) from the putative AUG and ends with a gfp reporter gene fused in frame at the mup-4 (BO270) COOH terminus. BO273 and BO269 were used to generate gfp coding and unc-34 untranslated sequences from pPD117.01 (provided by Andy Fire, Carnegie Institute of Washington, Baltimore, MD). BO270 and BO273 were hybrid primers containing mup-4 and reporter gene sequences. The two pieces were linked by combining 1 μl of each purified fragment (Qiagen PCR purification kit; QIAGEN) in a PCR reaction with outside primers BO268 and BO273. PCR fragments were pooled from several independent reactions, purified, and resuspended in 1× injection buffer.

Transgenic rescue and expression studies

DNAs were coinfected (20 ng/μl) with either pTC96_2 sur-5:gfp (50 ng/μl) (Yochem et al., 1998) or pRF4 rol-6(su1006) rol-6(80 ng/μl) (Mello et al., 1991; Mello and Fire, 1995) into EE22 or N2. EE22 progeny inheriting the free duplication qP3I are phenotypically WT, whereas those failing to inherit qP3I are dead (Mup) (Bucher and Greenwald, 1991; Gatewood and Bucher, 1997). In the case of the sur-5 marker, transgenic progeny were identified as GFP-positive animals and their progeny scored for uncoordinated (Unc, unc-36 phenotype) GFP-positive animals, indicating rescue by complementation (genotype: mup-4(mg36) ncl-1(e1865) unc-36(e251); Ex[sur-5::gfp; H14A12]). This was confirmed by picking Uncs and verifying that they segregated Mups and GFP-positive Uncs. For those coinjected with pRF4, the Rol phenotype was selected in addition to MUP-4::gfp and Unc. We observed many rescued F1 progeny (not shown) and analyzed six independent transgenic lines and two independent integrated lines (integrated according to Mello and Fire, 1995).

Table IV. Primers

| Name   | Sequence                          |
|--------|-----------------------------------|
| BO125  | ACTATCAAGTGGCCTACGGGAGGGCGGCTG   |
| BO126  | GGAAGAGCATCTCCCCAGACGCGCGCGCCTG  |
| BO187  | ATGTTCTTCATCTCTCCGGCCGTCC         |
| BO208  | ACGTTCAGATCCCCGCCGAGGCGGCGCGCG   |
| BO210  | CACACTTGTTTGTACGCCCGTGACG        |
| BO211  | GAGAGCTCTGTCCATATGGACGCGGCGGCG   |
| BO214  | TGATCTCGTGCTGATGGCGGCGGCGGCGG   |
| BO215  | TGCTTCGGTCCTAGTGAGCGGCGGCGGCGG   |
| BO225  | GCGCGGACCGCGGCGCGGCGGCGGCGGCGG   |
| BO232  | TTTGGGCGCGGCGGTGAGGGGATGAGTGAAGAGGACTC   |
| BO241  | CATTGGTATCCGACC                  |
| BO244  | GTTTATTAATCCAGGTTAAGGGCGG          |
| BO268  | TTTGGGCGCGGCGGTGAGGGGATGAGTGAAGAGGCTG       |
| BO269  | AAGCAGAAGAGAATTCTTTTGCTGCGGATAAAGAGGAGGCTGAGGGGAGG     |
| BO270  | TTTACTCCTTTTTTCTACCCGGGTACAAAGTACTTACTTCTGCTGTTT |
| BO273  | AAAAAGGCGGTTTGGG                           |
| BO300  | CAATGCGCGTAAAGCAGGCTACATGGCGTGGTTAAAAGATGAAAGGAGGCGGAGG   |
RNA-mediated interference

RNA was synthesized, using the RibomAX large scale RNA production system (Promega), and annealed (Fire et al., 1998). mup-4 and mu-3 dsRNAs were generated from yk27d6 (4.08 kb) and yk360c5 (2.4 kb), respectively. Young adult hermaphrodites, either N2 or EE6, were injected with either dsRNA (150 μg/ml) or 1X injection buffer alone, plated clonally, and transferred daily (Table II). For RNAi soaking experiments, staged worms were washed with M9 buffer. 25 μl of concentrated worms were aliquoted into siliconized tubes, to which 25 μl of either dsRNA (300 μg/ml) or 1X injection buffer were added and placed on a rotator at 22°C overnight and then plated. Individual hermaphrodites were transferred clonally 6 h later and then transferred daily (Table II).

MUP-4 antibody

The MUP-4 antibody was raised against the peptide, cysteine-linked-PRAKLARPLYGDEMGDD, from the unique intracellular domain (Fig. 5 C). The peptide and rabbit polyclonal antibody were contracted from Quality Control Biochemicals and affinity purified.

Staining

Phalloidin staining was as described (Gatewood and Bucher, 1997). For antibody staining, some embryos were harvested by treatment with a 1:10 dilution of commercial bleach in 1N NaOH and fixed (Miller and Shakes, 1995); however, the cuticle renders worms impermeable, beginning at the late thirdfold stage. Thus, analysis of mutants and some N2 worms was performed by the method described previously (Finney and Ruvkun, 1990; Miller and Shakes, 1995). Fixed worms were incubated with the following primary antibodies: MUP-4 rabbit polyclonal, 1:500; mouse mAb M4H to a C. elegans IF, 1:125; mouse mAb 5-6-11 to C. elegans myosin heavy chain A, 1:100; and rabbit polyclonal Living Colors™ (8367-1; CLON-TECH Laboratories, Inc.) to GFP, 1:200.

Microscopy

Fluorescence was analyzed either with a Leica DAS microskop DMR camera containing a Eastman Kodak Co. KAF-1400 chip, 1317 × 1035 resolution (Princeton Instruments), or as described previously (Gatewood and Bucher, 1997). Confocal images were gathered using a Zeiss Axiosvert 100M with a C-Apochromat 63× water-correction lens using LSM510 v2.02 software.

Electron microscopy

mup-4(ar60), mup-2(e2346ts) (Myers et al., 1996), and N2 worms were sequentially fixed in 2% OsO4 (3.5% glutaraldehyde and 2% OsO4 again) and 1% Mg2+ Na2 cacodylate buffer, pH 7.2–7.4 for 1 h at room temperature, with three washes in cacodylate buffer between fixatives. The worms were en bloc stained with 1% uranyl acetate in 70% ethanol overnight at room temperature. Worms were then washed in buffer, postfixed in 2% OsO4 in cacodylate buffer for 1 h at room temperature, and en bloc stained in saturated aqueous uranyl acetate for 1 h. Samples were embedded in Epon, and ultrathin sections were stained with 3% uranyl acetate in 50% ethanol, and with lead salts (Sato, 1985). 22 sectioned animals were examined in a Philips 410 EM: four were necrotic and disregarded; four appeared to be WT heterozygous siblings; and fourteen were mutants.

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