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Laminin α subunits and their role in *C. elegans* development

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SUMMARY

Laminins are heterotrimeric (α/β/γ) glycoproteins that form a major polymer within basement membranes. Different α, β and γ subunits can assemble into various laminin isoforms that have different, but often overlapping, distributions and functions. In this study, we examine the contributions of the laminin α subunits to the development of *C. elegans*. There are two α, one β and one γ laminin subunit, suggesting two laminin isoforms that differ by their α subunit assemble in *C. elegans*. We find that near the end of gastrulation and before other basement membrane components are detected, the α subunits are secreted between primary tissue layers and become distributed in different patterns to the surfaces of cells. Mutations in either α subunit gene cause missing or disrupted extracellular matrix where the protein normally localizes. Cell-cell adhesions are abnormal: in some cases essential cell-cell adhesions are lacking, while in other cases, cells inappropriately adhere to and invade neighboring tissues. Using electron microscopy, we observe adhesion complexes at improper cell surfaces and disoriented cytoskeletal filaments. Cells throughout the animal show defective differentiation, proliferation or migration, suggesting a general disruption of cell-cell signaling. The results suggest a receptor-mediated process localizes each secreted laminin to exposed cell surfaces and that laminin is crucial for organizing extracellular matrix, receptor and intracellular proteins at those surfaces. We propose this supramolecular architecture regulates adhesions and signaling between adjacent tissues.

Key words: Laminin, Basement membranes, Extracellular matrix, *C. elegans*, Cell adhesion, Cell polarity, Cell migration, Differentiation, Cell-cell signaling

INTRODUCTION

The laminin proteins form one of the major networks within basement membranes and are required during development. Five laminin α, three laminin β and three laminin γ subunits are known in vertebrates and over 12 heterotrimeric laminin isoforms are thought to be assembled (Burgeson et al., 1994; Iivanainen et al., 1995; Miner et al., 1995). In vertebrates and invertebrates, laminin isoforms are widely distributed and a variety of phenotypes have been associated with the disruption of different laminins. For example, laminin α2 mutations are found in some congenital muscular dystrophies (Helbling-Leclerc et al., 1995; Sunada et al., 1994; Xu et al., 1994); laminin α3, β3 or γ2 mutations are found in junctional epidermolysis bullosa, a skin blistering disease (Aberdam et al., 1994; Kuster et al., 1997; McGrath et al., 1995); and mutations of the laminin β2 chain gene disrupts neuromuscular and renal function (Noakes et al., 1995). Multiple defects are observed in mice that lack the laminin α5 chain and the animals arrest late in embryogenesis (Miner et al., 1998). Mutations in zebrafish indicate that laminin β and γ subunits are important for notochord development (Parsons et al., 2002). In *Drosophila*, the two laminin α subunits are required for embryonic viability; mutants have defects in the morphogenesis of heart, somatic muscle and trachea (Henchcliffe et al., 1993; Martin et al., 1999; Yarnitzky and Volk, 1995). Furthermore, there is evidence that one of the *Drosophila* laminin α subunits is required for the follicle cell/oocyte signaling that establishes the anteroposterior axis of the organism (Deng and Ruohola-Baker, 2000).

Although genetic studies have established the diversity and complexity of laminin functions in vivo, the manner by which laminins mechanistically regulate development is not well understood. Traditionally, laminin and basement membranes have been viewed at substrates that support cell adhesion and migration. However, the idea that the supramolecular organization of laminin itself has an instructive role has gained support (Colognato and Yurchenco, 2000). On the surface of

cells, laminins are known to bind several receptors and receptor-like molecules, including integrins, α/β-dystroglycan, and syndecans. One model predicts that laminin receptors anchor laminin and drive laminin polymerization on cell surfaces by causing the critical concentration for laminin self-assembly to be locally exceeded (Colognato et al., 1999). The formation of a laminin polymer appears to be necessary before other components are able to assemble into a basement membrane (Aurelio et al., 2002; Smyth et al., 1999). Polymerization further triggers the reorganization of the receptors within the plasma membrane and facilitates the reorganization of cytoskeletal components. It has been observed that on the surface of cultured myotubes, this reorganization drives laminin, laminin receptors and cytoskeletal components into a polygonal network (Colognato et al., 1999).

The receptor-facilitated laminin self-assembly model predicts that in vivo secreted laminin associates with receptors on exposed cell surfaces. Loss of laminin function is predicted to cause defective basement membrane assembly and spatial organization of receptor complexes and cytoskeletal components. The detailed description of the anatomy and cell lineages of *C. elegans* makes it a particularly attractive genetic system to examine these predictions. In particular, serial section electron microscopy has allowed every cell and cell contact to be described in the wild-type animal, allowing the genetic analyses of cellular development to be studied in remarkable detail (see www.wormatlas.org). Four members of the laminin family have been predicted in *C. elegans*: there are two α, one β and one γ, which are encoded by *epi-1*, *lam-3*, *lam-1* and *lam-2*, respectively (Hutter et al., 2000). We report that both laminin α subunits are secreted between the primary tissue layers and become localized in different patterns to exposed cell surfaces, consistent with a receptor-facilitated process. Mutations within each laminin α subunit gene cause abnormal cell-cell adhesions at regions associated with the localization of the subunit. Some cells fail to make the proper connections to adjacent tissues, while other cells inappropriately adhere to and invade neighboring tissues. Affected cells may fail to properly differentiate or migrate, suggesting widespread disruption of inductive interactions between adjacent tissues. Using electron microscopy, we observe missing or abnormal extracellular matrix, mispositioned adhesion complexes and disoriented cytoskeletal elements. For example, we observe on the surface of body wall muscle cells laminin organizes into a polygonal array and in mutants muscle cells may fail to properly adhere to the overlying epidermis. Muscle adhesion complexes and myofibrillar components are improperly positioned and in the epidermis the cytoskeleton is defective adjacent to where the muscle cells attach. Taken together, our results are consistent with the idea that laminin plays a crucial role in organizing a supramolecular architecture comprising extracellular matrix, receptors and cytoskeletal components, and that this architecture is important for regulating adhesion and signals between adjacent tissues.

MATERIALS AND METHODS

C. elegans strains and genetics

Animals were maintained as described (Brenner, 1974). Bristol strain N2 was used as wild type. The strains used in this study are as follows.

Chromosome I: *MT6550, lam-3(n2651)/dpy-5(e61)unc-75(e950)*; *PD9753, ccIs9753*; *PD4251, ccIs4251*.

Chromosome II: *SP756, unc-4(e120) mnDf90/mnC1*.

Chromosome IV: *GG23, emb-9(g23)*; *RW3600, pat-3(st564)/qC1*; *NG2324, ina-1(gm86)/qC1*; *NJ52, epi-1(rh27)*; *NJ244, epi-1(rh92)*; *NJ497, epi-1(rh152)*; *NJ569, epi-1(rh165)*; *NJ572, epi-1(rh191)*; *NJ590, epi-1(rh199)*; *NJ594, epi-1(rh200)*; *IM131, epi-1(rh233)*; *PD4251, ccIs4251*; *PD9753, ccIs9753*; *IM19, urIs13*.

Chromosome V: *IM336, nid-1(ur41)rhIs4(glr-1:GFP)*.

The *epi-1 (rh152)* allele was isolated by screening F2 progeny of mutagenized N2 animals for the presence of defective epithelial conversion of the gonad. This allele was three factor mapped to lie between *dpy-20* and *unc-5* on LGIV. To isolate additional alleles of *epi-1*, F2 progeny of mutagenized *dpy-13/mec-3* animals were screened for embryonic larval lethals or adult steriles linked to *dpy-13*. These mutants were examined for phenotypes that were similar but more severe than those of *rh152*. Each selected allele was tested and shown to fail to complement *rh152*. *epi-1(rh199)* and *epi-1(rh200)* were maintained from heterozygous mothers because the homozygotes are early lethal or sterile (Zhu et al., 2000).

Mutations in *lam-3* were isolated in a genome-wide screen for EMS-induced larval lethal mutations causing morphological defects (A.D.C., unpublished). Four mutations (*n2488*, *n2493*, *n2561* and *n2563*) confer similar defects in integrity of the pharyngeal basement membrane, as judged using Nomarski microscopy, and result in a fully penetrant lethal phenotype. All four mutations display linkage to chromosome one and fail to complement the reference allele *n2488*. *n2563* was mapped in the *lin-11 unc-75* interval: from heterozygotes of genotype *n2563/n566 e950*, 36 Lin non-Uncs were picked of which three segregated Lam (Pha/Let) worms; 2/2 Unc non-Lin recombinants segregated Pha/Let worms. Map data for other alleles were less extensive but consistent with this position.

Electron microscopy

Animals were immersion fixed using buffered aldehydes and then osmium tetroxide as described previously (Hall, 1995). Three or four animals were aligned within an agar block, then embedded and sectioned together. Serial thin sections were collected on slot grids and post-stained with uranyl acetate and lead citrate, and examined with a Philips CM10 electron microscope. To fix young L1s from RNAi experiments, animals were exposed to microwave irradiation during the primary fixation in buffered aldehydes, using a model 3450 oven (Ted Pella) at half power (Paupard et al., 2001). Subsequent fixation steps follow our normal protocols (Hall, 1995).

Molecular biology

RNA-mediated interference (RNAi) was performed as described previously (Guo and Kemphues, 1995; Rocheleau et al., 1997). RNA was prepared by in vitro transcription (Promega kit) using both T3 and T7 RNA polymerase, and the products were pooled. As cDNA templates, a cloned 0.4 kb *Pst*I fragment of *lam-3*, which encodes for G1, and a cloned 1.0 kb *Bam*HI fragment of *epi-1*, which encodes for G1 and part of G2, were used. N2 hermaphrodites were placed on separate plates 12-24 hours after injection, and allowed to lay eggs. These plates were examined every 24 hours for 3 days to determine the numbers of eggs that hatched and to which larval stage the animals would develop. To score *lam-3* and *epi-1* genetic null mutants, transheterozygous *lam3/dpy-5; unc-75* and *epi-1/mec-3* animals were placed on separate plates to lay eggs. Each parent was transferred to a fresh plate after 5, 10 and 15 hours. Development was scored as above. From the *lam-3* and *epi-1* heterozygous parents, one quarter of the progeny (227/863 and 324/1260, respectively) arrest as embryos or larvae. We inferred that these were homozygous for the laminin mutation as *dpy-5; unc-75* and *mec-3* homozygotes develop to the adult stage.

For sequencing of *epi-1* alleles, four sets of primers were designed to create PCR fragments that would span the entire *epi-1* genomic

region (12,371 bp) with 200 to 300 bp overlaps between fragments. Primer sets were designed to produce *Not*I and *Spe*I sites at either end of a fragment. For templates, genomic DNA from five to seven mutant hermaphrodites was prepared as described (Williams et al., 1992). Expand High Fidelity PCR enzyme (Roche) was used to generate the PCR fragments in order to minimize PCR based errors. The PCR fragments were gel purified, digested with *Not*I and *Spe*I, and ligated into *Not*I/*Spe*I-digested pBluescript SK(+) vector (Stratagene). Each product was completely sequenced. In all cases, at least two cloned fragments from two independent PCR reactions were sequenced.

Laminin αA was deduced from the analysis RT-PCR products in combination with sequence analysis of cDNA clones obtained from Y. Kohara (Gene Library Laboratory, National Institute of Genetics, Japan). PCR of reverse transcribed *C. elegans* RNA was performed using primers selected based on the genomic sequence of cosmids T22A3 and H10E24 (*C. elegans* genome sequence project). The *lam-3* mRNA sequence was deposited under Accession Number AF074902.

The promoter sequence for the *lam-3* reporter construct was PCR amplified from cosmid T22A3.8. The sequence starts at 2.6 kb 5′ of the predicted ATG start codon. The promoter sequence for the *epi-1* reporter construct was PCR amplified from cosmid K08C7.3 and starts at 2.8 kb 5′ of the predicted ATG start codon. Both fragments were cloned into the GFP bearing vector, pPD96.62 [provided by A. Fire (Carnegie Institution of Washington, Baltimore, MD)]. Transgenic strains expressing the GFP reporters driven by each promoter were generated by standard methods (Mello and Fire, 1995; Mello et al., 1991). A plasmid containing wild type *dpy-20* sequence was co-injected with the reporter construct into *dpy-20(e1282ts)* animals as an injection marker.

To detect *lam-3* and *epi-1* RNA, in situ hybridization was performed as described previously for detection of RNA in whole-mount *C. elegans* embryos (Seydoux and Fire, 1995). AP-anti-DIG antibody (Boehringer Mannheim, IN) was used for alkaline phosphatase (AP)-mediated detection. DAPI (1 mg/ml) was included in the staining solution to allow nuclei to be identified by epifluorescence microscopy.

Preparation of antisera and morphological analysis

To generate antisera against laminin αA, a plasmid construct was made by subcloning into the vector pQE (Qiagen, CA) an 800 bp cDNA fragment that contains the sequence encoding the G3 domain. To generate antisera against laminin αB, plasmid constructs were made by subcloning cDNA fragments encoding the G2 domain. The fusion proteins produced contain 6×HIS-tags and were purified according to the instructions of Qiagen and were used to immunize rabbits. Antisera against each fusion protein were also raised in chickens by Pocono Rabbit Farm & Laboratory (Canadensis, PA). Immune serum was affinity purified on columns coupled to the fusion protein to which they were generated. Immunostaining was performed as described for embryos (Wadsworth et al., 1996) and for larvae and adults (Finney and Ruvkun, 1990). Anti-rabbit and anti-chicken fluorescein- and rhodamine-conjugated secondary antibodies were used. The *epi-1(rh199)* mutant embryos and *epi-1(RNAi)* embryos lack detectable laminin αB antiserum staining and *lam-3(RNAi)* and *lam-3(n2561)* larvae lack detectable laminin αA antiserum staining (see Fig. S2 at http://dev.biologists.org/supplemental/). The following antibodies were also used to visualize tissues: MH4, a monoclonal antibody that recognizes an intermediate filament subunit (Francis and Waterston, 1991; Hresko et al., 1994); MH27, a monoclonal antibody that recognizes the adherens junction protein JAM-1 (Francis and Waterston, 1991; Leung et al., 1999; Mohler et al., 1998); anti-myotactin, formerly named MH46 (Hresko et al., 1999); anti-UNC-54, a monoclonal antibody that recognizes myosin heavy chain B (Miller et al., 1983); and MH25, a monoclonal antibody that recognizes PAT-3β integrin (Gettner et al., 1995). Images were obtained using a Zeiss LSM 410 Inverted Laser Scan microscope.

RESULTS

C. elegans laminin α genes

From the *C. elegans* genome sequence, two laminin α, one β and one γ subunits are identified (Zhu et al., 2000). Therefore, two laminin isoforms each containing a different α subunit are predicted to form in *C. elegans*. The *C. elegans* laminin α genes *lam-3* and *epi-1* encode for laminin αA and laminin αB, respectively (Fig. 1). A description of the isolation and sequencing of the *epi-1* gene and a sequence comparison of some alleles have been presented (Hutter et al., 2000). The laminin αB protein has a predicted structure that is similar to other reported laminin α subunits. We analyzed *lam-3* mRNA and found that it is derived from 13 exons and is 9450 nucleotides long plus a SL1 trans-spliced leader and a poly A tail. We predict that the *lam-3* mRNA sequence encodes a protein, laminin αA, that is similar to the laminin α1 and laminin α2 subunits found in other species, with the exception that that are only four LG domains instead of the usual five, and that there are 10 LE modules instead of the usual eight.

The laminin α subunits have different distribution patterns

To investigate the localization of laminin α subunits, chicken and rabbit polyclonal antibodies were raised against laminin αA and laminin αB fusion proteins. The antisera indicate that early expression of the α subunits occurs during gastrulation and that the subunits have distinct distributions as early organogenesis proceeds (Table 1). In *C. elegans*, gastrulation begins at the 28-cell stage as intestinal precursor cells move from the ventral surface into the interior (Sulston et al., 1983). As gastrulation proceeds, cell divisions give rise to a central cylinder of intestinal and pharyngeal precursor cells surrounded by body myoblasts and an outer cell layer. At this point cell proliferation largely ceases and the embryo, a spheroid of about 550 cells, begins to elongate. During this stage of morphogenesis, the organs develop into their mature

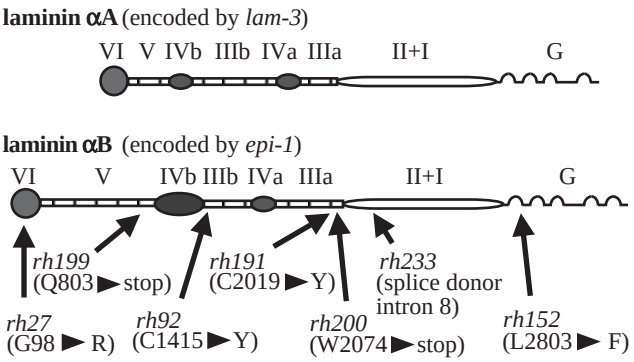

Fig. 1. Modular organization of *C. elegans* laminin αA and laminin αB. The N-terminal (LN, domain VI) and the internal globular (L4, domain IV) modules are represented by filled ovals. The rod-like epidermal growth factor-like (LE) repeats are indicated by rectangles. The coiled-coil forming domains are shown as open ovals. The C-terminal G-repeat (G) modules are indicated by half circles. The approximate locations of different *epi-1*mutations are shown (arrows). Laminin αB domains and the alterations of *rh27*, *rh92*, *rh199* and *rh200* have been previously presented (Zhu et al., 2000).

Table 1. Summary of laminin α subunit gene expression, protein distribution and membrane morphology

Tissue/cells	Basement membrane/ extracellular matrix*	Laminin α subunit gene expression		Laminin α subunit distribution	
		Embryo	Larvae	Embryo	Larvae
Sublateral nerves	Thin			αA	αA
Ventral nerve cord	Thin			αA	αA
Nerve ring	Thin			αA	αA
Epidermis	Thin	*lam-3*		αB	αB
Excretory canal	Thin			αA, αB	αA, αB
Pharyngeal epithelium	Very thick	*epi-1, lam-3*	*lam-3*	αA, αB	αA, αB
Pharynx	Very thick	*epi-1, lam-3*	*lam-3*	αA, αB	αA, αB
Pharyngeal-intestinal valve	Very thick			αA, αB	αA, αB
Intestine	Thin	*epi-1, lam-3*	*epi-1*	αA, αB	αA, αB
Muscles					
Body wall†	Very thick/thin†	*epi-1*	*epi-1*	αA, αB	αB
Vulval	Very thick/thin†	*epi-1*	*epi-1*	αB	αB
Intestinal	Very thick/thin†	*epi-1*		αB	αB
Anal depressor	Very thick/thin†	*epi-1*	*epi-1*	αB	αB
Male tail bursa, etc.	Very thick/thin†			αB	αB
Gonad					
Germ cells	Thin				αB
Gonad sheath	Thin		*epi-1*		αB
Uterine epithelium	Very thick/thin†		*epi-1*		αB
Spermatheca and valve	Thick		*epi-1, lam-3*		αA, αB
Distal tip cells	Thick		*epi-1*		αB
Oocytes	Thick				
Coelomocytes	ND				αA, αB

*Although these measurements are fixation sensitive, thin membranes are typically 20 nm across, including the 'lollypop' layer, thick membranes are typically 40 nm across and very thick membranes are typically 50-100 nm across (see Figs S4 and S5 at http://dev.biologists.org/supplemental/).

†Thickened basement membrane facing the epidermis/cuticle and a thin membrane elsewhere (see Fig. S5 at http://dev.biologists.org/supplemental/).

ND, not determined.

form and the embryo elongates about fourfold along its longitudinal axis.

Laminin αA is first detected between tissue layers near the end of gastrulation (Fig. 2A) and then becomes localized along the muscle cells as the embryo begins to elongate (Fig. 2B). By the threefold stage of elongation, staining along the muscle quadrants is weaker and becomes restricted to a band at the center of each quadrant, which colocalizes with the dorsal and ventral sublateral nerve tracts in the adult. Staining is intense around the pharynx, pharyngeal-intestinal valve, and intestine during morphogenesis (Fig. 2C). In larvae and adults, the antiserum stains the spermatheca strongly (Fig. 2D) and only weakly stains the pharynx, the intestine and the excretory canal (Fig. 2F). Laminin αA is also associated with the nervous system. During elongation and throughout the rest of development, αA is localized at the nerve ring, a bundle of ~100 axons that encircles the outside of the pharynx, at the right fascicle of the ventral nerve cord and at the sublateral nerves (Fig. 2C,E).

Laminin αA association with the nervous system is interesting because nerves are sandwiched between the epidermis and the overlying basement membrane. Using electron microscopy, the membrane overlying the nerves is indistinguishable from the adjacent membrane. To examine whether laminin αA is localized by interactions that involve neurons or whether it localizes to the nerve pathways regardless of whether the axons are present, we examined the distribution of laminin αA in *nid-1(ur41)* animals (Fig. 3). This mutation causes the dorsal sublateral axons to migrate along

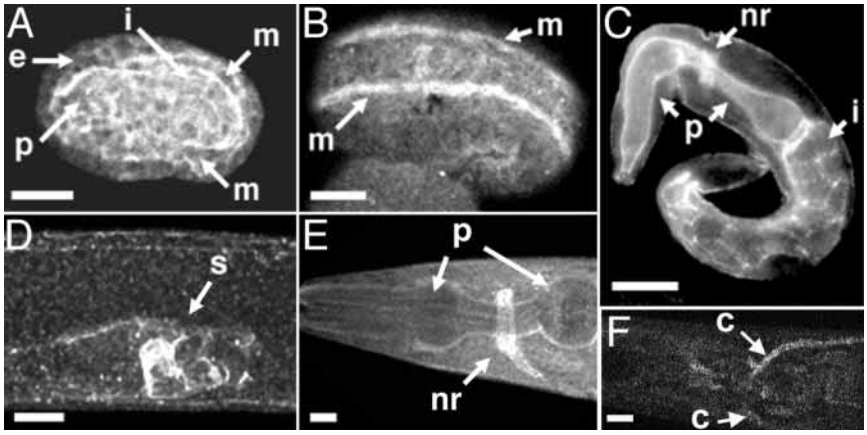

Fig. 2. Localization of laminin αA. Wild-type animals were stained using laminin αA antibodies. (A) The laminin αA protein is detected in the late gastrula between the rows of intestinal (i) and pharyngeal (p) precursor cells, the flanking myoblast cells (m), and the epidermoblast cells (e). Mid-plane optical section. (B) During embryo elongation, laminin αA is strongly detected between muscle and epidermal cells. Shown is staining associated with the two dorsal muscle quadrants. Dorsal lateral optical section. (C) In two- and threefold embryos, intense staining for laminin αA is observed at the basement membranes associated with the pharynx and intestine. Staining is also detected at the nerve ring (nr). (D) In larvae and adults, staining is detected at the spermatheca (s) where the protein localizes between individual endothelial cells. (E) In larvae and adults, staining is weak at pharyngeal and intestinal basement membranes but is stronger at the nerve ring. (F) Laminin αA is also detected in the basement membranes associated with the excretory canal (c). Anterior is towards the left, dorsal towards the top. Scale bars: 10 μm.

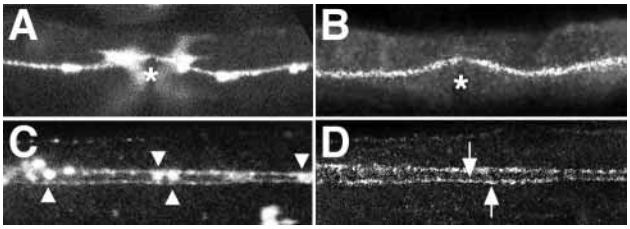

Fig. 3. Laminin αA is associated with neurons. Immunofluorescence micrograph of *nid-1(+)* (A,B) and *nid-1(ur41)* (C,D) animals that express GFP in right fascicle axons of the ventral nerve cord were co-stained with anti-GFP (A,C) and anti-laminin αA (B,D) antiserum. In wild type, there is an average of 54 axons in the right fascicle and six in the left (Hedgecock et al., 1990). (C) In *nid-1(ur41)* mutants, several axons from the right fascicle are mispositioned to the left fascicle (Kim and Wadsworth, 2000). (D) Laminin αA associates with the mispositioned axons in the left fascicle (arrows). Ventral aspect. The midline is defined by the vulva (*) and midline motor neuron cell bodies (arrowheads in C). Anterior is towards the left, dorsal towards the top.

the dorsal midline and axons of the right ventral nerve cord fascicle to migrate in the left fascicle (Kim and Wadsworth, 2000). We observe that Laminin αA is localized with the mispositioned axons rather than along the normal nerve pathways (Fig. 3D). This suggests that laminin αA is localized to neuronal cell surfaces by specific laminin αA receptors.

Staining with the laminin αB antiserum shows that, like laminin αA, the αB subunit accumulates between the primary tissue layers near the completion of gastrulation (~250 minutes) (Fig. 4A). Laminin αB antiserum stains all the major basement membranes during the remainder of embryogenesis and throughout larval development and in the adult (Fig. 4B-E). Although laminin αB staining is weak in the basement membranes surrounding pharynx, intestine, body wall muscle and epidermis, the staining is strong in the basement membranes surrounding gonad, distal tip cell, vulval muscle, intestinal muscle, anal depressor muscle and coelomocytes (Fig. 4F-H). These basement membranes are notable in that they are thick and appear to have mainly the αB-containing laminin isoform. Although the staining is diffuse within most basement membranes, at the muscle/epidermis membrane a distinct pattern is observed (Fig. 4B). On the muscle surface, a grid-like network that comprises regularly spaced bands running circumferentially and longitudinally is observed (Fig. 5). The longitudinal bands correspond to the thin-filament-containing (I-band) region of the muscle myofilament lattice. In this region, thin filaments are anchored by dense body structures that are in turn linked by transmembrane complexes to the basement membrane (Moerman and Fire, 1997). Laminin αB is also strongly detected at muscle-muscle cell boundaries.

In order to compare directly the distribution of the two laminin α subunits, we co-stained for both subunits using species-specific secondary antibody conjugates. As the germ layers develop, both α subunits are deposited between the layers. However, the staining for laminin αA is most intense around the pharyngeal and intestinal precursor cells, whereas staining for laminin αB is most intense around the myoblast cells and along epidermal cells (Fig. 6A). By the onset of elongation, distinct layers of laminin αA and laminin αB staining can be distinguished, particularly anteriorly between the developing pharynx and the body wall (Fig. 6B). This indicates that the segregation of the laminin isoforms begins early, before or as organogenesis proceeds.

To distinguish whether the two laminin α subunits retain an association with different basement membranes as elongation proceeds, we used mutants homozygous for the deficiency *mnDf90*. In these animals, the pharynx differentiates, but it does not attach to the buccal cavity, causing the pharynx to become displaced from the anterior body wall (M. Portereiko and S. E. Mango, personal communication). This allows the two basement membranes, which in mature wild-type animals are juxtaposed, to be visualized separately. We observe that

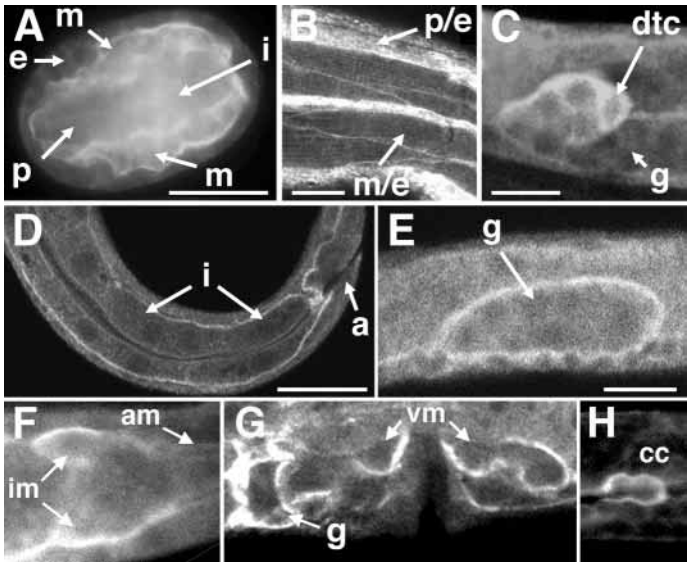

Fig. 4. Localization of Laminin αB. Wild-type animals were stained using Laminin αB antibodies. (A) The Laminin αB protein is detected in the late gastrula between the rows of intestinal (i) and pharyngeal (p) precursor cells, the flanking myoblast cells (m), and the epidermal cells (e). Mid-plane optical section. (B) Laminin αB is localized to the muscle and epidermis basement membranes in late stage embryos, larvae and adults. The protein has different distribution patterns in the pseudocoelom/epidermis (p/e) and muscle/epidermis (m/e) basement membranes. Lateral view showing two muscle quadrants, each two cells wide. The muscle cells are anchored to the epidermis through connections involving the muscle/epidermis basement membrane. The lateral epidermal ridge separates the quadrants. (C) In L3 and L4 larvae, Laminin αB is localized to the gonad basement membrane and is associated with the distal tip cell (dtc), a migratory cell that helps form the gonad (g). (D) In late stage embryos, larvae and adults, Laminin αB is localized to the basement membranes that separate the intestine (i), rectal epithelium (a; anus), epidermis and pseudocoelom. (E) Laminin αB is localized to the basement membrane that encloses the gonad (g) primordium cells in the L1 and L2 larval stages. (F) Laminin αB is detected in the basement membranes associated with intestinal muscle (im) and anal depressor muscle (am). (G) Laminin αB is detected in vulval muscle (vm) basement membrane and somatic gonad (g; uterine epithelium and/or uterine muscle). Staining of all specialized muscles is asymmetric: stronger near their anchorage to bodywall or other epithelia. (H) Laminin αB is associated with coelomocytes (cc). Scale bars: 20 μm for A-E.

the two α subunits remain differentially localized after elongation. Laminin αA is localized to the pharyngeal basement membrane, whereas laminin αB is associated mainly with the body wall basement membranes and only weakly with the pharyngeal basement membrane (Fig. 6C). These results indicate that each laminin α subunit is segregated in the embryo to different adjacent basement membranes and that each membrane retains its unique α subunit composition.

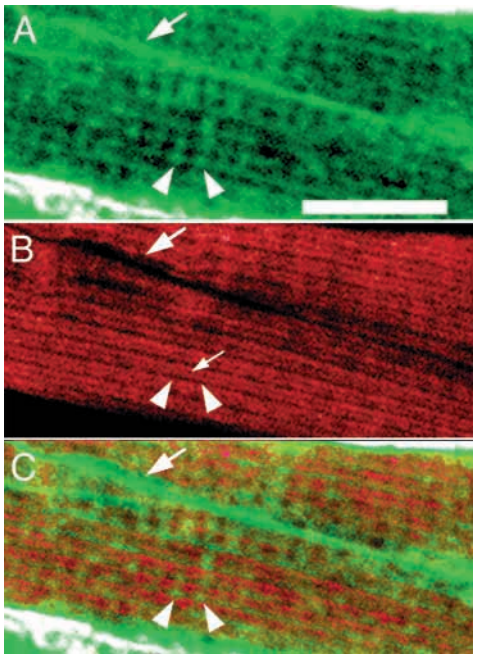

Fig. 5. Laminin αB forms a repeating network in the muscle/epidermis basement membrane. Immunofluorescence micrograph of the same wild-type animal co-stained with Laminin αB (green) and myosin heavy chain B (red) antibodies. (A) Laminin αB is distributed in a grid-like pattern on the muscle surface. The signal appears as periodic dots (arrowheads) and as regularly spaced bands running circumferentially and longitudinally between the dots. The signal is also enhanced at muscle-muscle cell boundaries (arrow). (B) Staining showing myosin thick filament structure in the muscle cell. Thick filaments emanate from the M-line (small arrow) and interact with thin filaments that are in turn anchored to dense bodies, which are functionally analogous to vertebrate Z-lines. The arrowheads indicate the region where dense bodies are located; large arrow indicates muscle-muscle cell boundaries. (C) Superimposition of the two images above showing the relationship of the extracellular laminin network to the myofilament lattice. The longitudinal laminin band and dots corresponds to the thin-filament-containing regions of the muscle.

Expression of laminin α subunit genes

Using antisense cDNA probes and the expression of laminin promoter-GFP transgenes, we examined the expression of the α subunit genes during embryogenesis and larval development (Table 1). In situ hybridization was used to examine early *epi-1* gene expression. Expression of *epi-1*, the gene encoding laminin αB, is first detected in the nucleus of cells entering the gastrula (Fig. 7A). As the cells arrange into the endodermal and mesodermal layers, *epi-1* mRNA is detected within cytoplasm (Fig. 7B). Expression continues as the intestinal cells and the precursors of the pharynx form a central cylinder with the myoblasts filling in between this cylinder and the outer layer of cells. Strong expression by the myoblasts is detected (Fig. 7C). Besides the cell movements, this period of development is characterized by rapid cell divisions as the number of cells increase from 28 to ~350. These results indicate that the regulation of *epi-1* expression is coordinated with the events of gastrulation. In larvae, *epi-1* is expressed in the body wall, vulva and anal depressor muscles, as well as intestinal cells and in somatic cells of the gonad (not shown).

In situ hybridization for *lam-3*, the gene encoding laminin αA, was not successful. However, promoter-GFP transgenes indicated that *lam-3* is expressed during gastrulation and through embryogenesis in pharyngeal, intestinal and epidermal cells (Fig. 7D). In the larvae, *lam-3* gene expression is maintained in the spermatheca (Fig. 7E) and in the pharyngeal m3-m8 and mc cells (Fig. 7F). These results show that the laminin α subunit genes have different expression patterns and, together with the protein localization studies, indicate that each α subunit can localize to basement membranes not associated with the cells that express the gene.

To determine whether the localization of each laminin isoform depends on the other isoform, the distribution of each laminin α subunit in the loss-of-function mutant of the other laminin α subunit was examined. The results showed that the localization of laminin αA does not depend on laminin αB, nor does the localization of laminin αB depend on laminin αA (see Fig. S1 at http://dev.biologists.org/supplemental/).

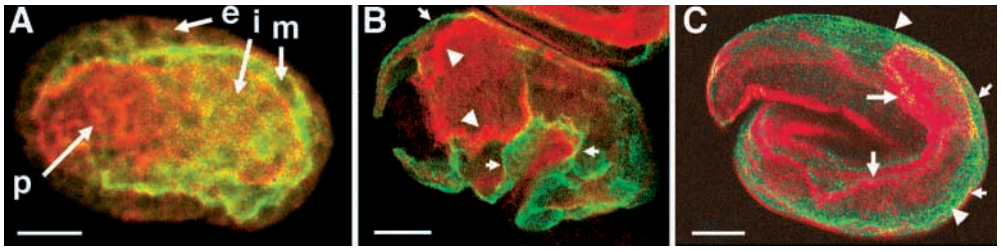

Fig. 6. Laminin αA and Laminin αB segregate to different basement membranes. Immunofluorescence micrograph of embryos co-stained with Laminin αA (red) and Laminin αB (green) antibodies. (A) By late gastrulation, both α subunits are deposited between the germ layers. While staining for Laminin αA is intense around the pharyngeal precursors (p), both subunits are detected between the muscle (m), intestinal (i) and epidermal (e) precursor cells. (B) As the embryo elongates, areas of distinct Laminin αA and Laminin αB composition can be distinguished between the developing pharynx/intestine (arrowheads) and body wall muscle (arrows). (C) Co-staining of a late stage *mnDf90* embryo, in which the pharynx and intestine has detached from the body wall muscle. Laminin αA associates with the pharynx and intestine (large arrows) and Laminin αB associates with the body wall muscles (arrowheads), suggesting that these basement membranes, which are juxtaposed in wild-type animals, each retain a unique α subunit composition. Juxtaposed muscle/epidermis associated membranes appear yellow (small arrows). Anterior is towards the left, dorsal towards the top. Scale bars: 10 μm.

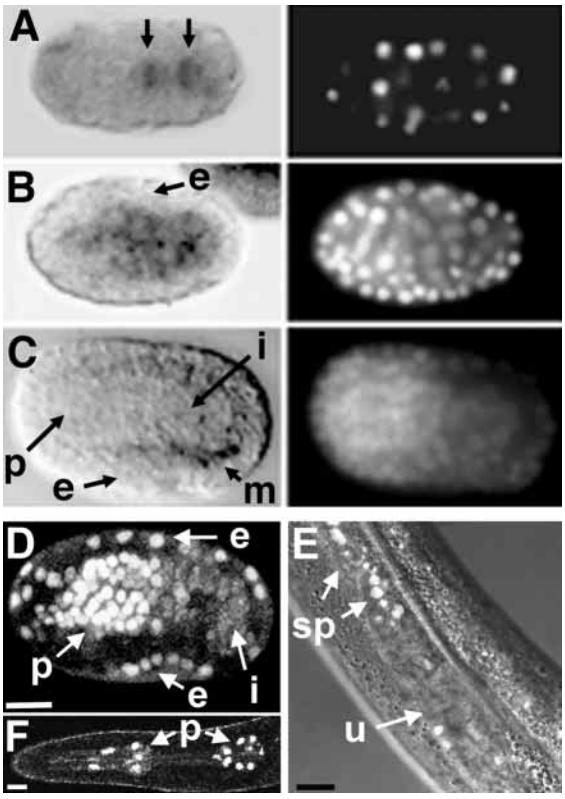

Fig. 7. Expression of the Laminin α genes, *lam-3* and *epi-1*. To observe *epi-1* RNA expression, embryos were hybridized with antisense *epi-1* probes (left panels in A-C) and stained for DNA with DAPI (right panels in A-C). To observe the expression pattern of *lam-3*, the *lam-3* promoter was used to drive GFP expression (D-F). Anterior is leftwards. (A) The endodermal and mesodermal precursor cells sink inwards from the ventral surface as gastrulation begins. epi-1 RNA is detected in the nuclei of the ingressing cells (arrows). (B) At midgastrulation, *epi-1* RNA is detected in the cytoplasm of intestinal, pharyngeal and myoblast cells, but not epidermal (e) precursor cells. (C) At the beginning of elongation, expression is detected primarily in the body wall myoblast cells (m). Expression is weak or is not detected in intestinal (i), pharyngeal (p) and epidermal cells (e). (D) By the onset of elongation, *lam-3::gfp* is expressed in pharyngeal (p), intestinal (i) and epidermal cells (e), but not in myoblast cells. As the embryo elongates, expression in all these cells declines. (E) In larvae and adults, *lam-3::gfp* expression is detected in spermatheca (sp) cells. The fluorescence image is superimposed on top of the DIC image of the same animal to show the uterus (u). (F) In larvae and adults, *lam-3::gfp* expression is also detected in pharyngeal muscle cells (p). Scale bars: 10 μm in D-F.

Collagen type IV is not required to localize the laminin α subunits

Basement membranes comprise networks of laminin and collagen type IV. These networks interact with other extracellular matrix proteins and cell-surface receptors, such as integrins. Collagen type IV is first detected intracellularly as the embryo begins to elongate and is detected extracellularly after the animals have elongated by 1.5-fold (Graham et al., 1997). This expression is later than the expression of laminin, suggesting that laminin is localized to cells before collagen type IV and that laminin does not require collagen type IV to associate with cell surfaces. To test this experimentally, the distribution of the laminin α subunits in collagen type IV mutant animals was observed. We used the mutation *emb-9 (g23)*, which is semidominant and causes both the collagen type IV chains to accumulate intracellularly, apparently by blocking assembly or secretion of the type IV collagen heterotrimers (Graham et al., 1997). Both laminin αA and laminin αB show normal distribution patterns until after early elongation, when collagen type IV is secreted (see Fig. S3 at http://dev.biologists.org/supplemental/). These results indicate that the correct distribution of the laminin α subunits does not initially require collagen type IV and is consistent with the notion that laminin is localized to cell surfaces before a prototypical basement membrane assembles.

epi-1 and *lam-3* loss-of-function mutations cause lethality

Mutations in the *epi-1* gene were isolated in a screen devised to isolate mutants defective in gonad conversion from mesenchyme to epithelium. The female somatic gonad of *C. elegans* is a cylindrical myoepithelium that surrounds the germ cells and sustains their maturation (Buechner et al., 1999; Hirsh et al., 1976; Kimble and Hirsh, 1979). Like epidermis, pharynx and intestine, the gonad will epithelialize during its morphogenesis. However, unlike other tissues in which the cell polarization occurs during gastrulation, the gonad polarizes late in larval development and is larger. As a result, its morphogenesis is more easily observed. Mutants were isolated at the L3 and L4 stage, in which the uterine precursors failed to exclude germ cells from the center of the gonad (the uterus), spermathecae failed to form a closed lumen, or the ovarian sheath cells failed to spread over the adjacent germ cells. One of the genes identified in this screen, was designated *epi-1* (epithelialization).

Mutations in the *lam-3* gene were isolated after the possible phenotypes of *lam-3* mutants were identified by examining animals made deficient for laminin αA using RNA-mediated interference, or RNAi (Fire et al., 1998). The *lam-3(RNAi)* animals arrest during early elongation or at the L1 larval stage. The L1 animals have abnormal pharyngeal development and have shorter bodies (posterior to the pharynx) at the time of arrest. From a screen for mutations that cause pharyngeal defects, mutants with phenotypes similar to the *lam-3(RNAi)* phenotype were isolated. Four non-complementing alleles, *n2488*, *n2493*, *n2561* and *n2563*, were mapped to the region of linkage group I where the physical sequence data suggested the *lam-3* gene should reside. A 20 kb laminin αA DNA sequence was amplified by PCR and was found to rescue the mutant phenotypes when expressed in *n2561* animals.

The strongest alleles of *epi-1* and *lam-3* and *RNAi* animals cause embryonic and larval lethality (Table 2). The *epi-1(rh199)* mutant embryos and *epi-1(RNAi)* embryos lack detectable Laminin αB antiserum staining and *lam-3(n2561)* and *lam-3(RNAi)* larvae lack detectable Laminin αA antiserum staining. Animals deficient for both α subunits were made by double RNAi as the genetic construction of *epi-1* and *lam-3* double mutants was problematic. Compared with single *epi-1*- or *lam-3*-null mutants or RNAi animals made deficient for a single subunit, the double RNAi animals were more likely to arrest development during embryogenesis. This suggests that each laminin has separate functions required for viability during embryogenesis. Although a pharyngeal development is not required for embryonic viability, the observation that only

Table 2. *epi-1* and *lam-3* are both essential for viability

	Developmental arrest (%)		Sterile	
	Embryo	Larvae	adult (%)	*n*
epi-1(rh199)lf	37	36	27	324
epi-1 RNAi	46	26	28	420
lam-3(n2561)lf	51	49 (L1 stage)	0	227
lam-3 RNAi	34	66 (L1 stage)	0	464
epi-1 RNAi + lam-3 RNAi	85	15 (L1 stage)	0	802

lam-3 larvae always arrest at the L1 stage with abnormal pharynxes is also consistent with the idea of distinct developmental roles for the laminins during embryogenesis.

Distinctive basement membranes separate tissues

The differences between the distribution and phenotypes of the two laminin subunits suggests that they function within different extracellular environments. The basement membranes of *C. elegans* have been examined in different studies (Albertson and Thomson, 1976; White et al., 1986; White et al., 1976); however, a methodical comparison of membranes has not been carried out. We compare here, using transmission electron microscopy (TEM) of thinly sectioned wild-type adults, the distinctive basement membrane that separate each major tissue (see Figs S4, S5 at http://dev.biologists.org/supplemental/). For example, the bodywall consists of two distinct parts, the somatic musculature and the epidermal tissue. All muscle cells within a given quadrant are surrounded by a single basement membrane, which covers their surfaces without entering the spaces between neighboring cells. Similarly, the cells of the epidermis, including all hypodermal cells, neurons, glial cells and the excretory and gland cells are covered by a single layer of basement membrane, which covers the entire epidermis without invading between cells. It is very often possible to see a periodic series of dark dots, or 'lollypops' (terminology of J. White), which lie on the outer surface of the epidermal basement membrane in transverse sections (facing away from the tissue of origin), that seem to comprise a second layer to this very thin membrane. In favorable oblique sections, these dots comprise thin parallel rods (about 2 nm diameter) encircling the basement membrane (not shown). Their spacing is often 20 nm or 40 nm between dots or rods. Endodermal tissues are similarly covered by a single basement membrane. Thus, the pharynx displays a very thick basement membrane that covers basal surfaces of muscles, support cells, glands and neurons without separating any of them. This thicker basement membrane seems to consist of a single layer. Where the pharynx meets the intestine, the thick basement membrane stretches to cover the pharyngeal/intestinal valve cells on their basal surfaces, before merging into the thinner basement membrane that covers the basal side of the intestine. Only a few other cells display similar thick basement membranes, including the mature oocyte before fertilization (Hall et al., 1999), the uterine epithelium, and many specialized alimentary and sex muscles where they oppose the cuticle (see below). The extracellular material surrounding the oocytes in some other organisms is referred to as a glycocalyx; this distinction might also be appropriate in *C. elegans* once molecular compositions are known.

Body wall muscles appear to support different basement membranes on each face, with a thickened membrane facing the outer epidermis and cuticle (featuring laminin αB expression; see below), and a very thin membrane facing lateral and inward-facing surfaces (pseudocoelom). In many places the basement membranes of the muscle and neighboring epidermis are together; however, in various places where the tissues are further separated, the muscle basement membrane appears separately very thick, whereas the neighboring epidermal membrane is thin, just as in other parts of the anatomy. This suggests that the basement membrane associated with the muscle mostly contributes to the thick basement membrane between the cells. A single layer of basement membrane similarly covers the gonadal cells, both over the sheath cells of the proximal arm and over the bare germ cells of the distal arm (Hall et al., 1999). A rather thick basement membrane lies over the distal tip cell (DTC) of the gonad that merges at the trailing edge of the DTC with the thin layer covering the bare germ cells. These descriptions of the basement membrane come from TEM of immersion-fixed specimens. Preliminary studies of fast-frozen worms suggest the basement membranes are more lacey or flocculent and seem to extend farther into the space between tissues (D.H.H., unpublished).

One striking feature revealed is the degree of asymmetry of basement membranes associated with some cells (see Fig. S5 at http://dev.biologists.org/supplemental/). Besides the bodywall muscles, this is a feature of many classes of alimentary and sex muscles, often to a more dramatic extent. Thus, the muscles of the alimentary tract [intestinal, sphincter and anal depressor (DA)] show very thick basement membranes in limited regions where they are anchored to the bodywall or rectal cuticle via thin epidermal interfaces (an example for the muDA anchorage is shown on SW-Worm Tiler, Slice 726, at *wormatlas.org/SW/SW.htm/WormTiler.htm*). Similarly the sex muscles of the hermaphrodite (vulval and uterine muscles) and of the male tail (spicule, gubernacular and bursal muscles) show very robust basement membranes at their anchorages to various specialized cuticle regions, but thin membranes elsewhere. The uterine epithelium also shows this asymmetry, with a very thick basement membrane where it anchors to the seam and alar cuticle, and a thin basement membrane elsewhere (shown in SEAM FIG5 at *wormatlas.org/handbook/hypodermis/hypsupportseam.htm*).

Laminin α subunits are required for intact basement membranes

We examined the laminin α subunit requirements for basement membrane structures by comparing basement membranes in wild-type and laminin α subunit mutants (Table 3). Basement membranes where laminin αB is primarily localized are disrupted in *epi-1* mutants. For example, in severe alleles broken pieces of thick membrane are occasionally found in the body cavity at midbody, perhaps derived from the spermatheca. The final TEM appearance of these broken membranes, as described below, are subject to secondary tissue displacement after initial weakness of the basement membranes. Thus, when a tissue breaks through its covering, the membrane pieces may snap back, fold, clump or become distorted as viewed by EM. The thinner basement membranes surrounding the epidermis, intestine and gonad show a wide variety of defects in all *epi-1* alleles. Multiple layers, large whorls and clumping of material are very common in adult *epi-1* animals (Fig. 8). More

Table 3. Summary of the principal defects caused by viable loss-of-function and partial loss-of-function mutations

Gene	Tissue/cells	Phenotypes
epi-1	Body wall muscles	Disrupted basement membranes; failure to adhere to epidermis; ectopic dense bodies; improperly oriented myofilaments.
	Epidermis	Disrupted basement membranes; adherence to the intestine; loss of compactness near muscle attachment sites.
	Gonad	Disrupted basement membranes; germ cells escape and proliferate in body cavity and adjacent tissues.
	Intestine	Disrupted basement membranes; adherence to the epidermis.
	Nerves	Disorganized and mispositioned; axon outgrowth defects; extracellular matrix accumulation around individual axons.
lam-3	Pharynx	Disrupted basement membranes; cells adhere and protrude into body wall muscle and epidermis; ectopic adherens junctions; improperly oriented filaments; extracellular matrix accumulation between adjacent pharyngeal cells.

rarely, the membrane material forms a diffuse granular lumpy substance filling the pseudocoelom. In *epi-1* mutants, the thicker basement membrane covering the pharynx does not show many disruptions. There are only rare ruptures of pharyngeal basement membranes in embryos and none in adults, although the entire pharynx is sometimes grossly twisted in adults. This latter phenotype could be a secondary consequence of the disruption of basement membranes elsewhere.

In contrast to the *epi-1* laminin αB mutants, the body wall structure and most other tissues in the *lam-3* laminin αA mutants appear normal by electron microscopy. However, in *lam-3* mutants, the thick pharyngeal basement membrane has distinct gaps that permit pharyngeal cells to adhere to surrounding tissues (Fig. 9B). These results are in agreement with the unique distribution patterns of each subunits, the morphological distinctions between membranes, and the idea that each laminin isoform contributes specific properties.

Laminin α subunits are required to prevent cells from adhering to and invading neighboring tissues

Using light microscopy, the phenotypes of *epi-1* and *lam-3* mutants suggest gross distortions of the normal separation of tissues. The *epi-1* mutants that do not die early often adopt a distinctive appearance, they have a swollen midbody and become practically immobile owing to apparent friction between adjacent tissues. At some points, the epidermis and intestine appear to adhere together. Adults are immobile and sterile, having distended midsections with germ cells

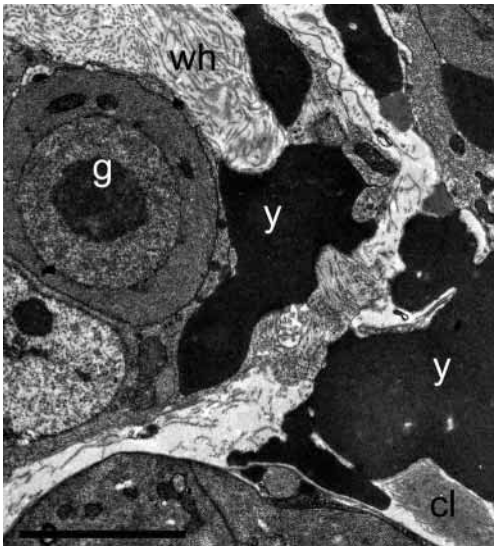

Fig. 8. Basement membranes are disrupted in laminin αB mutants. Mutations in the laminin *epi-1* gene disrupt the integrity of basement membranes. In the body cavity, multiple layers, large whorls (wh) and clumping (cl) of material are common in adult animals. This electron micrograph shows a region between the gonad and intestine, a germ cell (g) and yolk (y) are indicated. Scale bar: 5 μm.

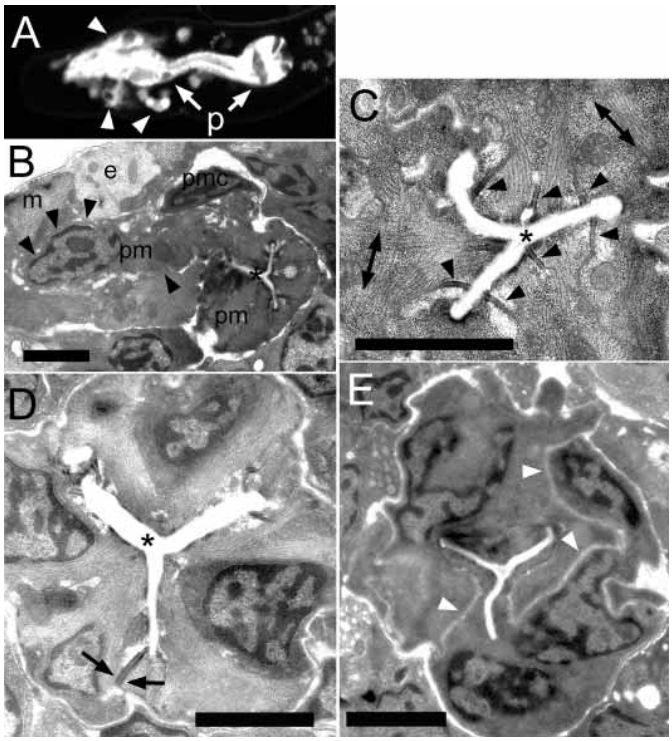

Fig. 9. Laminin αA-deficient animals. (A) The pharynx (p) of predicted *lam-3* null mutants do not properly form; cell bodies (arrowheads) are mispositioned into the surrounding tissues. In this animal the pharyngeal muscle cells (p) were visualized by expression of a *myo-2::gfp* transgene. (B) Electron micrograph of a *lam-3* mutant reveals that the pharynx, which is normally cylindrical, is distorted because of the displacement of pharyngeal muscle (pm) and marginal (pmc) cells. The pharyngeal basement membrane is discontinuous and pharyngeal cells directly adhere to the body wall muscle (m) and epidermis (e) cells of the surrounding tissues (arrowheads). Asterisk indicates the lumen. (C,D) In the pharyngeal cells of *lam-3* mutants, the apical membrane domain appears to develop normally as judged by the adherens junctions (C, arrowheads) that form by the lumen (asterisk). In addition, ectopic adherens junctions (D, arrow) also form at what should be the basolateral side of cells. Myofilaments in muscles and intermediate filaments in marginal cells may not assume their normal radial orientation (C, double-headed arrows). (E) In some cases, the lateral cell membrane appears greatly reduced in *lam-3* mutants. Increased space between cells with what appears to be excess basement membrane forms between adjacent pharyngeal cells (arrowheads). Scale bars: 2 μm in B-E.

proliferating in the body cavity. At the L1 stage, *lam-3* mutants have deformed pharynxes, suggesting that larvae die because they are unable to eat. The development of the pharynx was also followed by expression a *myo-2::gfp* transgene, which marks the pharyngeal muscle cells. Epifluorescence microscopy reveals that during gastrulation the pharyngeal muscle cells are arranged properly but as the embryo elongates and organs begin to form, many of the cell bodies move into the surrounding tissue, leaving a process behind that remains attached to the pharynx (Fig. 9A).

Using electron microscopy we observe that embryonic lethality caused by mutations in laminin α subunit genes is primarily caused by improper separation of tissues and/or detachment of cells (Fig. 10). In addition, we observe in larvae and adult *epi-1* mutants areas where basement membrane is ruptured and adjoining tissues are adherent, indicating places where tissues can not slide past each other. In adults, failure of the sheath cells to cover the developing gonad leads to germ cells breaking through the lamina and invading neighboring tissues (Fig. 11B), and producing germ cells free in the pseudocoelom (Fig. 8). In *lam-3* mutants, cell bodies of both muscle cells and marginal cells from the pharynx are sometimes displaced into the surrounding tissue. Despite this dramatic displacement of cell bodies, all the pharyngeal cells remain connected to the lumen of the pharynx. On their lumenal surface, as in wild type, apical adherens junctions are found connecting adjacent cells (Fig. 9C).

Laminin α subunits are required throughout development for cell polarity, differentiation, proliferation and migration

The examination of an allelic series of *epi-1* mutations, *rh199*, *rh200* > *rh165* > *rh191* > *rh92* and *rh152* > *rh27* > *rh233*, reveals the role that laminin plays in controlling cell polarity, differentiation, proliferation and migration throughout development. Although the strongest alleles, *rh199* and *rh200*, are often embryonic lethal, we find that the rare slowly developing homozygotes have gross mistakes in polarization, such that apical and basal features are poorly segregated. In mutants, muscle cells, which normally have two non-equivalent 'faces' with different basement membranes, can show misallocation of dense bodies to the inward-facing cell membrane (Fig. 11A). Myofilaments are disorganized and the individual muscle sarcomeres or whole muscle cells are not attached to the epidermis (Fig. 11C). The epidermis overlying muscle is thick, suggesting that the organization of the epidermal cytosketelon is abnormal. Normally the epidermis overlying muscle is compressed; filaments extend across the epidermis and attach to the cuticle in order to transmit the contractile force from the adjacent muscle.

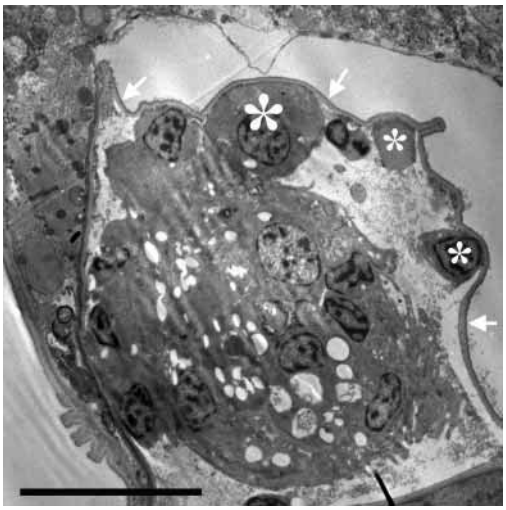

Fig. 10. Early embryonic lethality in *epi-1* mutants. A homozygous *epi-1(rh199)* embryo inside of the heterozygous parent shows cells (asterisks) that are separated and detached from the tissues. The detachment of cells during embryonic development and the failure of organogenesis cause embryonic lethality in predicted *epi-1* null mutants. The eggshell (arrows) is slightly shrunk owing to the fixation. Scale bar: 5 μm.

Germ cell invasion of neighboring tissues is a characteristic phenotype in mutants that manage to develop to later stages (Fig. 11B). These cells inappropriately remain in mitosis and proliferate within the neighboring tissues. This proliferation defect causes a gross enlargement of the midbody in adult animals. This is a predominant phenotype observed in the *rh165* strain.

In the mutants, striking defects in cell and axon outgrowth are observed, apparently owing to misguidance along broken or misassembled basement membrane. This phenotype is easily observed in the more active and more fertile mutants, particularly with the temperature sensitive allele *rh191*. All longitudinal nerves show occasional defects in final positions, and the ventral nerve cord often wanders from its normal position at the ventral midline (Fig. 11D). This phenotype was also observed by Forrester and Garriga (Forrester and Garriga, 1997). Interestingly, individual axons can become surrounded by separate sheets of basement membrane and leave the fascicle. Axons may also defasciculate in regions where the basement membrane forms clumps rather than sheets. Such errors probably cause defects in synaptic connectivity, although we have not tried to reconstruct the nerve circuits. The *rh191* allele frequently retains cuticle on the tail, perhaps owing to difficulties in molting.

All of the weak or moderate alleles may show 'strong phenotypes' at low frequencies. Alleles such as *rh92* and *rh152* are almost normal in fertility, but still show many tissue defects, including some disorganization of the gonad, extracellular accumulation of yolk granules and whorls of material (presumed to be basement membranes), milder muscle defects and occasional guidance errors by touch dendrites and excretory canals. Although the gonad sheath cells usually cover the gonad successfully in these alleles, their processes show irregular folds where they oppose the basement membrane, and sheath cell somata fail to flatten normally and may contain whorls of membranous material (data not shown). Excess yolk accumulates in the pseudocoelom, possibly owing to poor development of the oocytes, which normally take up yolk only at maturity, or possibly owing to failure to form sheath pores that admit yolk from the pseudocoelom to the oocytes (Hall et al., 1999; Grant and Hirsh, 1999). Weak *epi-1* alleles such as *rh233* and *rh27* show milder versions of these same defects.

Finally, the allele *rh233* is unique in that it has specific effects on the migrations of the canals of the excretory cell. The excretory cell is the largest mononucleate cell in the animal (Buechner et al., 1999; Nelson et al., 1983). Its cell

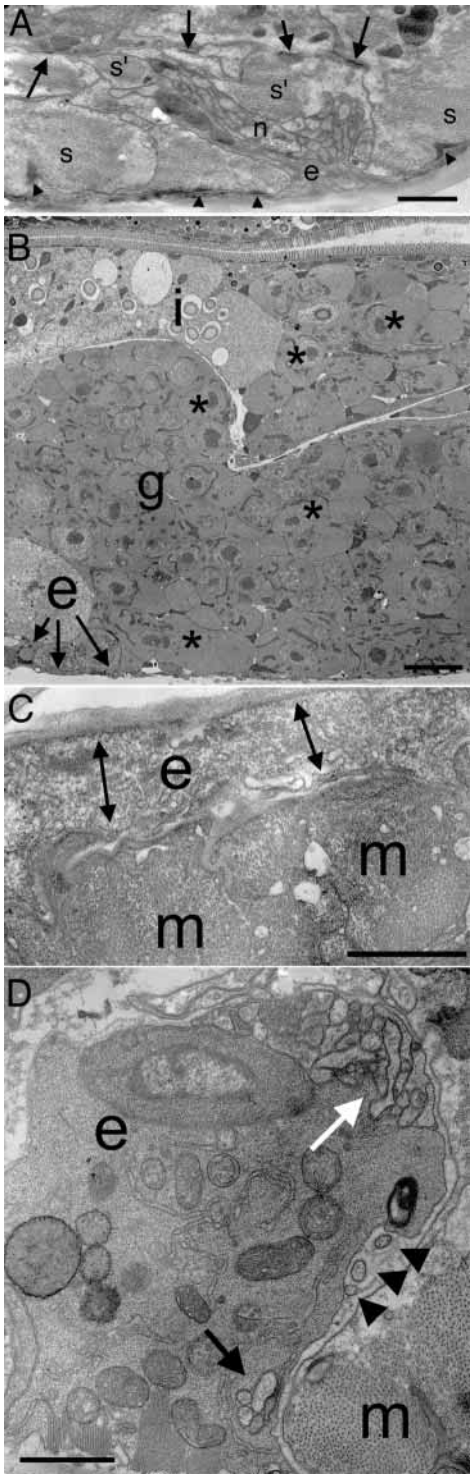

Fig. 11. Cell polarization, cell proliferation, cell differentiation and migration defects in *epi-1* mutants. (A) In this predicted *epi-1* null mutant, *epi-1(rh199)*, cell polarity is disrupted such that, in the muscles, additional dense bodies (arrows) form ectopically on the pseudocoelomic side of the cell, and sarcomeres (s′) organize in an unusual position away from the epidermis. Normally positioned dense bodies (arrowheads) and sarcomeres (s) are observed on the epidermal (e) side of the muscle cells. Dense bodies are analogous to vertebrate Z-lines and function to maintain the alignment of the thin filaments. A displaced muscle cell distorts the shape of a nerve (n). (B) Strong *epi-1* alleles cause sterility owing to failure of gonadogenesis during the third larval stage. The gonadal basement membrane is weakened or missing, and the gonadal sheath fails to enclose the germline, which permits germ cells to escape into the body cavity and to invade neighboring tissues. In this *epi-1(rh165)* mutant, germ cells (asterisks) have invaded the intestine (i). A basement membrane separates the intestine from the gonad arm below it, however no basement membrane separates the gonad (g) and the thin layer of epidermis (e) and here the tissues adhere to one another (arrows). (C) In *epi-1* mutants, the development of the body wall muscles is compromised. In this *epi-1(rh165)* mutant, the muscle cells (m) of a quadrant show incomplete differentiation. The organization of the sarcomere is primitive, with poor segregation of thick and thin filaments and little evidence of dense bodies to anchor them. The entire muscle has failed to settle closely onto the bodywall and the intervening epidermis (e), normally a thin layer, is abnormally wide (double-headed arrows). (D) Axon migration and nerve positioning defects are often observed in *epi-1* mutants. In this *epi-1(rh191)* mutant, the right bundle of the ventral cord (white arrow) is mispositioned to the dorsal side of the ventral epidermal ridge (e). At the normal position of the right bundle, four or five axons are seen (black arrow). In addition, two or three axons are mispositioned to the lateral side of the ridge (arrowheads). Interestingly, a basement membrane appears to be associated with each of these individual axons, a phenotype never observed in wild type. Scale bars: 5 μm in A; 10 μm in B; 2.5 μm in C; 1 μm in D.

body is positioned ventrally near the terminal bulb of the pharynx. Two arms of the cell, the canals, extend laterally along the length of the animal. Frequently, in the mutants, these canals are ventrally mispositioned. Sometimes the canals are shortened or both canals travel along the same side. Similar excretory guidance defects are observed in other alleles, but less frequently.

The reconstructions of *lam-3* mutants also demonstrate the importance of laminin in regulating cell polarity. Although wild-type pharynx cells show radial organization, with myofilaments or intermediate filaments oriented between apical and basal membranes, in *lam-3* animals these filaments become disordered, with some running to the lateral membranes in the pharyngeal muscle cells and marginal cells, respectively (Fig. 9C). We also observe adherens junctions between pharyngeal cells at abnormal locations (Fig. 9D) and greatly increased basal cell membrane surrounded by extracellular matrix, resulting in very little lateral membrane and little cell-cell contact (Fig. 9E). These results suggest that in *lam-3* mutants the apicobasal polarity of the pharyngeal cells is compromised, as well as the ability to maintain or establish lateral identity.

DISCUSSION

We have investigated the role that laminin α subunits play in the development of *C. elegans*. Each α subunit is distributed to specific cell surfaces that are exposed between tissue layers near the end of gastrulation. Based on *epi-1* and *lam-3* phenotypes, laminin is necessary to assemble a stable basement membrane and for organizing receptor complexes and cytoskeletal components to the proper cell surfaces. We consider that these results are consistent with an idea that laminin can organize extracellular matrix, receptor and

cytoskeletal elements into a supramolecular configuration that is crucial for regulating interactions between adjacent tissues.

The *C. elegans* α subunits are members of phylogenetically conserved protein families

The *C. elegans* Sequencing Consortium has revealed only two α subunits and a β and a γ subunit predicting that only αAβγ and αBβγ laminin heterotrimers are present. Alternatively spliced forms of laminin β or γ subunit have not been detected by RT-PCR (C.-c.H., D.H.H., E.M.H., G.K., V.K., B.E.V., H.H., A.D.C., P.D.Y. and W.G.W., unpublished). Overall, the size and primary structure of laminin α subunits are conserved between phyla. We find that the *C. elegans* laminin αA chain is typical with two exceptions: there are only four LG domains instead of the usual five and there are an additional two LE modules (10 instead of eight). In *Drosophila*, two α subunits and a β and γ subunit have been described (Chi and Hui, 1988; Chi and Hui, 1989; Chi et al., 1991; Garcia-Alonso et al., 1996; Haag et al., 1999; Henchcliffe et al., 1993; Kusche-Gullberg et al., 1992; Martin et al., 1999; Montell and Goodman, 1988; Montell and Goodman, 1989; Yarnitzky and Volk, 1995). One of the *Drosophila* α subunits is similar to the *C. elegans* αA subunit and the vertebrate α1 and α2 subunits, whereas the other is similar to the *C. elegans* αB subunit and to the vertebrate α3, and α5 subunits (Martin et al., 1999). In general, the αB-like laminins are the most widely expressed of the laminin α subunits, whereas the αA-like laminins appear to have more restricted expression patterns (Martin et al., 1999; Miner et al., 1995; Miner et al., 1997). The laminin α subunits of *C. elegans* and *Drosophila* appear during gastrulation, suggesting a common requirement for having different laminin α subunits during early development.

Each laminin α subunit is segregated to different cell surfaces

Our study reveals early events that lead to the assembly of basement membranes in vivo. Both laminin α subunit genes are apparently expressed under the control of signals that initiate and regulate gastrulation. Gene expression is first detected in the nuclei of cells that are ingressing through a furrow along the ventral midline and, as the tissue layers begin to be organized, cytoplasmic RNA is detected. At this time, the gene encoding laminin αA, *lam-3*, is expressed in pharyngeal and epidermal cells, and weakly in intestinal cells, whereas the gene encoding laminin αB, *epi-1*, is expressed in intestinal, pharyngeal and myoblast cells. Both laminin α subunit proteins are then deposited between the tissue layers. Near the end of embryogenesis, laminin α subunit gene expression changes, the laminin αA gene being expressed most notably in the pharynx and the laminin αB gene in the muscle cells.

The distribution of the different laminin subunits is probably a cell-surface receptor-mediated process. Although both laminin proteins are secreted between tissue layers during gastrulation, they do not indiscriminately assemble. Rather, each subunit is distributed in a different pattern to cell surfaces and, furthermore, they are not necessarily associated with the cells that express the subunit (Table 1). The staining pattern of laminin αA along the nerve tracts is revealing because the basement membrane associated with the nerve tracts is not morphologically distinguished from other regions of the epidermal basement membrane. We hypothesize that laminin αA is concentrated at neuronal cell surfaces by specific cell-surface receptor(s) and that the laminin αA containing trimer mixes with the αB trimer in the basement membrane at these locations. The association of laminin αA even when the axons are mispositioned supports this conclusion. Also revealing is the finding that even when the two laminins might appear to be able to intermingle, such as where the pharynx and body wall basement membranes are juxtaposed, they in fact remain separated, indicating that each is anchored to a particular architecture.

The laminin α subunits associate with cell surfaces before the reported expression of other basement membrane components and they are required to assemble stable basement membranes. Evidence from other systems also suggests that early laminin expression is essential for further basement membrane assembly. For example, antisense experiments that disrupt laminin α subunit expression in Caco2 epithelial cells blocks laminin secretion and prevents the subepithelial accumulation of entactin/nidogen and type IV collagen, and the formation of a basement membrane (De Arcangelis et al., 1996). In addition, the laminin γ1 knockout arrests at peri-implantation and neither the embryos nor derived embryoid bodies form basement membrane (Aurelio et al., 2002; Smyth et al., 1999). In both these cases other basement membrane proteins are detected but only as disorganized extracellular deposits.

Like the laminin α subunits, other extracellular matrix proteins in *C. elegans* also localize to different basement membranes and are not necessarily associated with the cells that expressed the protein (Graham et al., 1997; Kang and Kramer, 2000; Kim and Wadsworth, 2000). This suggests that cell surface-associated molecules are required for the assembly of the extracellular matrix proteins into basement membranes (Graham et al., 1997). Collagen IV localizes to all basement membranes except those between the pseudocoelomic cavity and the body wall muscles or the epidermis (Graham et al., 1997). Nidogen (entactin), which can bind both collagen IV and laminin with high affinity (Fox et al., 1991), is associated with muscle cells as the embryo begins to elongate and subsequently is detected at the pharynx, intestine and gonad primordia (Kang and Kramer, 2000; Kim and Wadsworth, 2000). In larvae and adults, nidogen is detected in most basement membranes, but is most strongly detected around the nerve ring and developing gonad. It becomes concentrated at the edges of muscle quadrants and on the sublateral nerves, which run longitudinally along the center margin of the muscle quadrants. This staining pattern is different from either laminin α subunit, although there are striking similarities to the laminin αA pattern in regards to nervous system staining. Nidogen is found at all locations where collagen IV localizes. The *C. elegans* homolog of mammalian perlecan, a heparan sulfate proteoglycan that binds nidogen/entactin and laminin, plays an essential role in muscle development has been shown by antibody staining to localize to basement membranes at the bodywall and anal muscles, and at the pharynx and gonad (Rogalski et al., 1993). Perlecan may be produced by epidermal cells and recruited to the body wall muscles cells (Spike et al., 2002).

Our results suggest that although laminin may associate with many different surfaces of cells, it is normally excluded from doing so. In *lam-3* mutants, an inappropriate matrix can

accumulate between the pharyngeal cells, presumably because defective adhesions between the pharyngeal cells allow laminin inappropriate access to lateral surfaces. Where body wall basement membrane is defective in *epi-1* mutants, growth cones, which normally migrate between the body wall basement membrane and the basal surface of the epidermis, may become inadvertently exposed to secreted non-polymerized laminin. As a result, laminin is inappropriately assembled at exposed surfaces all around the axons.

The laminin α subunits are required to control cell-cell adhesions between tissues

Basement membranes are observed to separate different tissues. The basement membrane appears to distinguish discrete (basal) borders between specialized tissues, but is generally excluded from any spaces formed by the lateral surfaces of adjacent cells within a tissue, perhaps permitting these cells to stick tightly to their neighbors. In a few locations, two neighboring tissues may make close approaches and need to stick together. In these locales, their basement membranes appear to be able to fuse end-to-end (as at the joining of the pharynx to its valve or at the joining of the DTC to the germline) or to fuse face-to-face (as at neuromuscular junctions of the nerve ring and the longitudinal nerve cords, where the parallel membranes of the nerves and of the somatic muscles seem to become one layer at the point of neuromuscular contacts). In all other locales, it is notable that each tissue is bounded by its own basal lamina, perhaps to permit easy sliding of one tissue past another, as during the flexion of the nematode body. Preventing cell-cell adhesions between tissues is critical. For example, where body wall muscles face the cuticle, the basement membranes form an integral link from muscle to epidermis and to cuticle. During normal body flexions when the nematode moves through the environment, most other internal adhesions must be minimized or, as in the case of *epi-1* mutants, the animal will be paralyzed.

Laminin forms a network between the body wall muscle cell surface and the basal surface of the epidermis. Our results suggest that this is important for forming the apparatus that physically link the muscle, basement membrane and the overlying epidermis and cuticle. This apparatus is necessary for transferring contractile force. In the muscle, the myofilament lattice is just below the cell surface and is anchored to the muscle cell membrane and adjacent basement membrane by integrin-containing dense bodies and M-line complexes. In turn, the complexes are linked through the basement membrane to the intermediate filament arrays that extend across the compacted epidermis and attach to the cuticle (Francis and Waterston, 1991; Hresko et al., 1999; Moerman and Fire, 1997).

In *epi-1* mutants, dense bodies are missing and can ectopically assemble, the myofilament lattice is disrupted, and the overlying epidermis may not compact. These observations suggest that laminin αB is required for properly organizing the receptor complexes and filament arrays of muscle and epidermis. During early body wall muscle development, myofibrillar components accumulate at membranes adjacent to the epidermis and other muscle cells, whereas in the epidermis hemidesmosome components become restricted to regions adjacent to the muscle cells (Hresko et al., 1994). After this polarization, integrin-containing cell-matrix adhesion complexes appear, followed by the assembly of the highly ordered dense body and M-line structures. Thick filaments associate with the M-line and thin filaments with the dense bodies (Barstead and Waterston, 1991; Gettner et al., 1995; Hresko et al., 1994; Rogalski et al., 1993; Waterston, 1989). The myofilaments run longitudinally in the animal, while the intermediate filaments in the epidermis become organized in circumferentially oriented bands. The assembly of the dense body and M-line components requires the extracellular matrix protein UNC-52/perlecan and UNC-112, an intracellular protein that colocalizes with integrin at the cell-matrix adhesion complexes (Francis and Waterston, 1991; Rogalski et al., 2000; Rogalski et al., 1993).

The ectopic assembly of dense bodies in *epi-1* mutants suggest that laminin is required during the early step of muscle cell polarization. This is consistent with observations that laminin αB is present before the adhesion complexes are assembled and before UNC-52/perlecan and the muscle integrins are functionally required for muscle development. Defective polarization may not preclude assembly of adhesion complexes if the signals responsible for driving the assembly are still present.

The *lam-3* phenotypes suggest laminin is required to polarize cells and to organize adhesion complexes. In the mutants, adherens junctions are positioned at inappropriate cell surfaces of the pharyngeal cells. Current models predict that epithelial cell polarity is initiated by contact between cells or between a cell and the extracellular matrix (Yeaman et al., 1999). These contacts trigger the assembly of cytoskeletal and signaling networks at the contact sites and the establishment of an apical membrane domain at the non-contacting surface. In a process that is not well understood, global changes in the organization of the cytoskeleton are induced. Microtubules are redistributed into long bundles along the apicobasal axis of the cell. The mechanism regulating the orientation of the microtubule assembly is not known, although linkages to the cytoskeletal networks at the lateral and basal membranes or to the actin cytoskeleton assembly at the apical membrane domain have been suggested. There is evidence that suggests cell adhesion to the extracellular matrix is important for organizing the apicobasal axis. In Madin-Darby canine kidney (MDCK) culture, tight junctions become localized to the apicolateral membrane boundary only after extracellular matrix accumulates on one side of the cells (Wang et al., 1990). The tight junctions and the axis of polarity can be reoriented by exposing a different surface to extracellular matrix (Wang et al., 1990). In the *lam-3* mutant, all pharyngeal cells form mature apical adherens junctions and remain attached to the lumen, indicating that apical membrane domains are established and maintained. However, the adherens junctions forming at abnormal locations suggests that the loss of contact sites because of the absence of laminin αA might establish other ‘non-contacting surfaces’ and, in turn, induced the formation of apical-like membrane domains. As a result, some cytoplasmic filaments orient relative to these domains, instead of perpendicular to the lumenal surface.

We observe other cases as well where epithelial cells fail to form the specialized structures of the apical and basolateral domains. For example, the spermatheca will not form a closed lumen. Furthermore, the accumulation of yolk and the molting

defects of some *epi-1* mutants are associated with the improper uptake or secretion of proteins from mature epithelial cells. In the most severe events, cells may fail to assemble into tissues at all. In mutant embryos with a predicted laminin α null allele, some cells were seen to lose contact with all neighbors and round up into isolated spherical units. The inability of cells to polarize and correctly adhere to their neighbors probably accounts for the embryonic lethality of *epi-1* null animals.

The laminin α subunits are required for inductive signaling

A dramatic case where cellular development is altered occurs when the gonad sheath cells fail to enclose the germline. The normal proliferation and differentiation of the germ cells requires cell-cell signaling from elements of the somatic gonad (Austin and Kimble, 1987; Buechner et al., 1999). Once the developing germline is unsheathed, the germ cells can remain in mitosis and create a vigorous pathological phenotype, in which immature germ cells invade and multiply within other tissues. In strong *epi-1* alleles, dividing germ cells are observed in all regions of the body from head to tail.

Cell and axon migrations also require laminin. In *C. elegans*, axons migrate between the basement membranes and the epidermis, whereas mesodermal cells migrate on the opposite side of the basement membrane (Hedgecock et al., 1990; Hedgecock et al., 1987). Extracellular cues, such as the laminin-related guidance molecule, netrin UNC-6 are thought to be associated with the basement membrane (Wadsworth et al., 1996). In the *epi-1* mutants, both cell and axon migrations are disrupted (see also Forrester and Garriga, 1997). In most cases this may be the result of the physical disruption of the basement membrane; however, it is also possible that some alleles of *epi-1* interfere with the ability of guidance cues or guidance receptor molecules to interact with basement membrane components. This may be the case for *epi-1(rh233)*, which specifically affects the migrations of the excretory cell canal processes.

A model for laminin function

It is proposed that receptor-facilitated laminin self-assembly sets up a supramolecular architecture that is necessary for organizing adhesion and cell signaling between adjacent tissues (Colognato et al., 1999; Colognato and Yurchenco, 2000). This model predicts dynamic reorganization involving cell receptors, intracellular proteins and the extracellular matrix. We show that laminin associates with cell surfaces in a process that is probably receptor mediated and occurs before the assembly of a prototypical basement membrane. These observations, coupled with the mutant phenotypes that show disorganized basement membranes, receptor complexes and cytoskeletal components, provide in vivo evidence for this model. A particularly attractive system for further study is the role of laminin in the development of the body wall muscle attachments. We are struck by the similarity between the polygonal arrays observed on the body wall muscles and myotube surfaces in culture. In *C. elegans*, laminin polymerization between the surfaces of the epidermis and muscle cells might be a key event for the organization of muscle integrin-containing receptor complexes, extracellular matrix components and epidermal hemidesmosomes into a supramolecular configuration that spans the tissues.

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