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Genetic analysis of laminin A reveals diverse functions during morphogenesis in *Drosophila*

Claire Henchcliffe, Luis García-Alonso, Joyce Tang and Corey S. Goodman*

Howard Hughes Medical Institute, Department of Molecular and Cell Biology, 519 Life Science Addition, University of California, Berkeley, Berkeley, CA 94720 USA

*Author for correspondence

SUMMARY

In order to dissect the functions of laminin A in vivo, we have undertaken a molecular and genetic characterization of the laminin A subunit (*lamA*) gene in *Drosophila*. Sequence analysis predicts a multidomain structure similar to mammalian homologs. We generated a series of complete and partial loss-of-function mutant alleles of the *lamA* gene; complete loss-of-function mutations lead to late embryonic lethality. Certain combinations of partial loss-of-function *lamA* alleles give rise to escaper adults, which have rough eyes associated with changes in cell fate and pattern, misshapen legs and defects in wing structure. These phenotypes suggest that laminin A has diverse functions during morphogenesis in *Drosophila*.

Key words: laminin A, *Drosophila* genetics, cell fate, limb structure, wing structure

INTRODUCTION

Laminin is a large glycoprotein complex, which forms a major component of basement membranes in a wide variety of organisms. Although variant forms have been described, the most studied form of laminin in vertebrates exists as a heterotrimer of subunits A (400×10^3 M_r), B1 (220×10^3 M_r) and B2 (200×10^3 M_r). Tissue culture and biochemical studies using purified laminin have made considerable progress in dissecting its different activities. Laminin has complex biochemical properties, binding both to cell surfaces through multiple receptors (including six identified integrins) and to other components of the extracellular matrix, such as collagen type IV, nidogen/entactin and heparan sulfate proteoglycan. Laminin affects cell behavior in vitro in many ways, including, for example, morphology, differentiation, mitosis and migration (reviewed by Martin and Timpl, 1987; Beck et al., 1990) and promotes substratum adhesion for many cell types, including epithelial, myoblast and Schwann cells (Kleinman et al., 1981, 1985; Terranova et al., 1980).

Studies using proteolytic fragments of laminin and synthetic peptides have allowed mapping of function domains. Within the laminin A chain, an activity located in fragment E8 (comprising the end of the long arm including the G domain) is necessary for development of polarity in mouse embryonic mesenchymal cells during formation of a polarized, differentiated kidney epithelium (Klein et al., 1988). This same fragment promotes neurite outgrowth in tissue culture (Edgar et al., 1984) and the peptide IKVAV within E8 promotes cell attachment and neurite outgrowth (Tashiro et al., 1989).

Many of the in vitro activities of laminin vary according to the cell type studied and the conditions of the experiment. For example, although laminin promotes substratum adhesion for many cell types, it appears to interfere with substratum adhesion for neurons of the embryonic olfactory epithelium (Calof and Lander, 1991; also see Chiquet-Ehrismann, 1991). Attachment of cultured neural retinal cells to a laminin substratum may be dependent on the conformation or distribution of binding sites of laminin (Adler et al., 1985). Although laminin promotes cell migration in many experiments, at high concentration it inhibits migration of avian neural crest cells (Perris et al., 1989).

Given this diverse and at times opposing array of activities of laminin in tissue culture, it is not surprising that laminin has been predicted to have many different functions in the developing organism. As a step towards unravelling these roles in vivo and dissecting its structure and function, we have used a combined genetic and molecular genetic approach in *Drosophila*. Laminin is one of a number of cell and substratum adhesion molecules that are well conserved between vertebrates and arthropods (reviewed by Hortsch and Goodman, 1991). Just as in vertebrates, so too in *Drosophila*, the laminin complex consists of three subunits, A (400×10^3 M_r), B1 (220×10^3 M_r) and B2 (180×10^3 M_r) (Fessler et al., 1987; Montell and Goodman, 1988). The genes encoding all three subunits have been cloned (Montell and Goodman, 1988) and the complete open reading frames for the B1 and B2 subunits have been sequenced

(Montell and Goodman, 1988, 1989; Chi and Hui, 1989). The B1 and B2 subunits show similarity to their vertebrate homologs in both the arrangement and sequence of their multidomain structures. Furthermore, in tissue culture, preparations of *Drosophila* laminin are able to promote attachment and spreading of cells cultured from late *Drosophila* gastrulae (Volk et al., 1990). Although the receptors for laminin in *Drosophila* are not yet known, a number of integrins have been cloned in this organism (Bogaert et al., 1987; Leptin et al., 1987, 1989). Thus, laminin provides the opportunity to begin to use a genetic approach to understand the roles of individual components of the extracellular matrix during development, and to elucidate further their interactions with cell receptors in a pathway in which extrinsic cues are translated into changes in such events as signal transduction, cytoskeletal reorganization and cell behavior.

As in vertebrates, *Drosophila* laminin has been localized to basement membranes such as those covering the inside of the epidermis, developing muscles, and internal glands and organs of the embryo (Fessler et al., 1987; Montell and Goodman, 1989; Kusche-Gullberg et al., 1992). Laminin is also an abundant component of the basement membrane surrounding the larval imaginal discs (Fessler et al., 1987). These are unique to holometabolous insects and arise during embryonic development. At the end of larval development, the discs consist of an epithelial monolayer surrounded by a basement membrane. During metamorphosis, adult structures develop from these discs by complex morphogenetic changes and cell differentiation. The final cuticular adult structures are easily scored and in particular cases such as the compound eye, many steps in the differentiation of cell fate and pattern have been well described. Thus, the derivatives of imaginal discs, and in particular the compound eye, are ideal systems to begin a genetic analysis of the role of laminin during development.

In this paper, we present the open reading frame from the *Drosophila* laminin A subunit (*lamA*), which predicts a multidomain protein similar to vertebrate laminin A. We have generated mutations in the *lamA* gene, some of which correspond to a complete loss of function leading to lethality at a late embryonic stage and others of which are hypomorphic alleles which in certain combinations give rise to escaper adults with reduced viability and defects in a number of tissues. The pleiotrophy of these defects indicates that laminin A has diverse functions during morphogenesis in *Drosophila*.

MATERIALS AND METHODS

Isolation of cDNA and genomic clones

A short cDNA clone corresponding to part of the laminin A subunit of *Drosophila* was previously isolated by screening the Crews 10-13 hour *Drosophila* embryo CNS gt11 cDNA library with anti-laminin antisera (Montell and Goodman, 1988). The corresponding gene was localized by in situ hybridization to polytene chromosomes to 65A10-11. This cDNA was used to isolate a 3.8 kb cDNA clone and genomic clones of 13.5 kb and 13.9 kb by screening the Zinn 9-12 hour *Drosophila* cDNA library (Zinn et al., 1988) and a *Drosophila* genomic library (Maniatis et al., 1978). Fragments of these genomic clones were then used to isolate overlapping cDNA clones. A genomic clone encompassing the 5 end of the laminin A gene was isolated by screening the genomic library with a DNA fragment isolated from the P element insert line $l(3)neo^{11}$ by following the plasmid rescue method (Hanahan et al., 1980). This was in turn used to screen a cDNA library resulting in a 3 kb cDNA clone being obtained which covered the 5 end of the translated sequence of the laminin A subunit. Thus we were able to obtain overlapping cDNA clones covering the entire translated region of the laminin A subunit, as determined by sequencing.

Sequencing of cDNA clones

Subclones of cDNA clones were sonicated and cloned into M13 following standard procedures (Maniatis et al., 1982). Single-stranded templates obtained from the M13 transfectants were sequenced according to the dideoxy method (Sanger et al., 1977), using Sequenase (US Biochemical Corp.). Five stretches of sequence information were obtained to fill gaps by sequencing single-strand templates with specific 20-mer oligonucleotides. The cDNAs were sequenced on both strands. Sequence data were assembled and analyzed using the Intelligenetics software package, and alignments to determine the level of identity between given sequences were performed with the FASTDB program.

Genetic methods and mutations

The P insertion line AS249 or *l(3)PneO65A* (formerly called $l(3)neo^{11}$) was obtained from Allan Spradling. It contains a P element, pUChsneo, encoding neomycin resistance (Steller and Pirrotta, 1985) inserted at 65A10-11. A recessive lethal phenotype was separable from the P element insert by recombination and no phenotype found in this stock could be attributed to presence of the P element insertion. The transposon was mobilized by crossing to flies carrying a stable source of transposase activity (Robertson et al.,1988). From the progeny of each of 40 single F_1 males, 30 single males were used to generate a total of 939 fertile lines. These were screened for: recessive lethal mutations and viable visible mutations. Loss of the P-element insert, which contains a gene conferring resistance to neomycin as a marker, was examined by testing for survival and development of eggs laid on yeast-glucose food containing neomycin (Genetecin, from GIBCO, at 1 mg/ml in yeast-glucose food). Of 939 total lines generated after P element mobilization, 71 recessive lethal mutations have been obtained, of which 20 (neomycin sensitive) fall into a single complementation group. Members of this complementation group fail to complement a deficiency, *Df(3L)Vn-2*, which deletes the region between 64E and 65C-D (Budnik and White, 1987). Southern blot analysis of the genomic DNA of these lines showed that all had rearrangements in the sequences upstream and/or including the 5 part of the laminin A gene. Some of the restriction patterns were identical, indicating that 11 out of the 20 of these were independent alleles. Identical alleles can arise by excision of the P element during pre-meiotic stages of sperm development. One of these excision alleles, $lamA^{7\text{-}5}$, was used to isolate all further mutations. Canton S flies were mutagenized with 15 mM EMS and crossed to *TM1/TM2* females. 2400 F_1 males were successfully crossed to $lamA^{7\text{-}5}$/*TM3* females and 9 alleles were recovered, all of which were lethal in combination with $lamA^{7\text{-}5}$ and also *Df(3L)Vn-2*.

For analysis of interactions between *lamA* with the subunit of the integrin encoded by the *lethal myospheroid* gene, we used the viable mys^8 (= *nj42*) allele of *l(1)mys*. For analysis of interactions with the subunit of integrin encoded by the *inflated* gene, we used the viable if^1 allele.

Characterization of mutant phenotypes

Mutant adults and pupae from crosses of different combinations of alleles were identified by absence of the dominant markers *Sb*

or *Tb* present on the *TM3* and *TM6* balancer chromosomes, respectively. Embryos were identified by the absence of -gal expression from a TM3, -gal balancer. Scanning electron microscopy of adult eyes was performed as described by Kimmel et al. (1990). For light microscopy, eyes were prepared for sectioning as in Ready et al. (1976). 1 µm or 2 µm sections were stained with hot toluidine blue (1% in a 1% borax solution). Cobalt chloride staining, used to visualize the apical surface of the pupal retina (taken at 40 hours at 25°C after the white prepupal stage), was performed as described in Cagan and Ready (1989). Adult cuticles were prepared for light microscopy by incubating flies in a solution of three parts 70% ethanol to 1 part glycerol for several days. Following this, flies were dissected, heated at 90°C for 10 minutes in a 10% KOH solution, dehydrated in an ethanol series, cleared in xylene and were then mounted in Permount (Fisher Scientific) for viewing.

Production of *lamA* clones

Mitotic recombination clones were induced using the Minute technique (Morata and Ripoll, 1975). 1st and 2nd instar larvae of the genotype: w^{1118}; *mwh* $lamA^{7-5}$/*M(3)67C* $P[w^{+}]B122$, were irradiated with X rays to a total dose of 1000 rads. The mutation *M(3)67C* maps at 67 C1-10 and the insertion $P[w^{+}]B122$ at 68 C1-4 on polytene chromosomes. Clones of cells homozygous for $lamA^{7-5}$ were recognized by the presence of the *w* marker in the eye and *mwh* in other cuticular regions. From several dozen clones induced in the eye, 22 were sectioned and stained with toluidine blue for study. We also analyzed seven large $Minute^{+}$ $lamA^{7-5}$ clones in the wing that were induced in 1st instar larvae (32-48 hours AEL); these seven clones were found in 263 wings examined.

RESULTS

Cloning and characterization of the laminin A subunit in *Drosophila*

A short cDNA corresponding to part of the laminin A subunit was previously isolated (Montell and Goodman, 1988) and was used in this study as the starting point to isolate cDNA clones covering the entire *lamA* gene. We have determined the sequence of the entire open reading frame and part of the flanking non-translated region from a series of overlapping cDNA clones isolated from cDNA libraries constructed from 9-12 hours *Drosophila* embryos (Zinn et al., 1988). The deduced protein sequence is shown in Fig. 1. The putative translational start site is followed by a stretch of hydrophobic amino acid residues which together with flanking sequences code for an apparent signal sequence (von Heijne, 1986), likely to be cleaved from the mature protein. Following the signal sequence, there is more than one potential site for cleavage by peptidases; as a result we have numbered the amino acids starting with the initiation methionine. The total open reading frame is 3712 amino acids; this is 628 amino acids longer than the mouse A chain due to differences in domains V, IVb, IIIb, IIIa and G. Shortly before we finished the complete sequence, as reported here, the nucleotide sequence of a 1951 amino acid C-terminal portion of the laminin A sequence was published (Garrison et al., 1991). While this paper was in review, the same group published the remaining 1761 amino acid N-terminal portion of the laminin A sequence (Kusche-Gullberg et al., 1992).

The deduced amino acid sequence was compared with that for the mouse laminin A subunit (Sasaki et al., 1988) and predicts a protein with a multidomain structure similar to that proposed for the mouse. The overall level of amino acid similarity is 29% between *Drosophila* and mouse, compared with 78% between mouse and human laminin A subunits (Haaparanta et al., 1991): this level of similarity differs considerably between domains as shown in Fig. 2. In vertebrates, a laminin A variant called merosin has been characterized and a partial sequence published (Ehrig et al., 1990). We compared domain G of *Drosophila*, mouse and human laminin A to the available human merosin sequence comprising the carboxyl-terminal fragment (Fig. 2). *Drosophila* laminin A is 26% identical at the amino acid level to both mouse and human laminin A and human merosin. This compares with 41% identity between human merosin and both human and mouse laminin A.

Domains IIIa, IIIb and V are cysteine-rich and in both *Drosophila* and mouse may be divided into repeats of about 50 amino acids which have homology with motifs found in a number of epidermal growth factor (EGF)-related proteins [first noted by Sasaki et al. (1987) for mouse laminin] and are proposed to fold into a similar conformation to EGF (Engel, 1989), although the repeats of laminin are unusual in that they contain eight cysteine residues per repeat rather than the normal six. The level of amino acid similarity between mouse and *Drosophila* in these three domains is high (see Fig. 2). However, in contrast to the laminin B1 and B2 subunits in *Drosophila* and vertebrates in which the number of these EGF-like repeats has been conserved, in the laminin A subunit the number of these EGF-like repeats differs between mouse and *Drosophila* (see Fig. 2).

A number of activities have been attributed to specific peptide sequences in laminin. In mouse laminin A at amino acids 1123-1125 (domain IIIb) an RGD sequence is present: this is a cell recognition sequence which binds to integrin receptors (Ruoslahti and Piersbacher, 1987). This sequence is not present at any position in the *Drosophila* sequence. Thus *Drosophila* laminin has no RGD sequence in any known chain and must adhere to cell surfaces through other active sites. A candidate for such a site is the peptide IKVGV (amino acids 2671-2675) in domain I/II. This is similar to the sequence IKVAV (amino acids 2100-2104 at the C-terminal end of domain I/II) in mouse which as a peptide interferes with cell attachment and spreading, migration, and neurite outgrowth in tissue culture (Tashiro et al., 1989). The sequence LRE has been defined as a component of an attachment site for motoneurons to vertebrate S-laminin, a B1 laminin-like molecule (Hunter et al., 1989). In S-laminin, the LRE motif is present in domain I, in mouse laminin A, it is found in domains I/II and, in human laminin A, the sequence is found twice in domains I/II and once in the G domain. In *Drosophila* laminin A, LRE is found in domain IVa (position 1627). Since the position is not conserved it is unclear whether this represents an active site in *Drosophila* laminin A.

Generation of mutations in the laminin A gene

The *lamA* gene maps cytologically to 65A10-11 on the left arm of the third chromosome (Montell and Goodman, 1988). There is a P element insert line, AS249, with a

Fig. 1. Open reading frame encoded by the laminin A gene. Overlapping cDNA clones were isolated from the Zinn gt11 library constructed from 9-12 hour *Drosophila* embryos (Zinn et al., 1988) and sequenced, giving a 11,136 bp open reading frame, which encodes the 3712 amino acids shown here. The complete nucleotide sequence can be found in the GenBank database. The predicted initiation codon is 48 nt from the 5 end of our analyzed nucleotide sequence. Preceding this codon is the nucleotide sequence CACA, which is in agreement with the consensus sequence (C/A A A A/C) at translation initiation sites in *Drosophila* genes (Cavener, 1987). At the end of the open reading frame are two stop codons and we find multiple potential sequences in agreement with the consensus sequence for poly(A) addition (Proudfoot and Brownlee, 1976) within the 347 nt of 3 non-translated sequence that follow (data not shown). The putative signal sequence is double-underlined. The first amino acid for each domain (VI, V, IVb, IVa, IIIb, IIIa, I/II and G) is delimited by vertical arrows and named in bold type. EGF-like repeats within domains V, IIIb and IIIa are similarly marked and named (eg V-1, V-2, etc.) in plain type. Potential sites for N-linked glycosylation are underlined. Five differences in nucleotide sequence, presumed to be polymorphic, were found between our complete sequence and the partial sequence published by Garrison et al. (1991). Four of these changes are silent (at nucleotides 5940, 6008, 9722 and 10906). One difference results in a substitution: amino acid number 1912, E (ours) to Q (theirs). While our paper was in review, Kusche-Gullberg et al. (1992) published the rest of the sequence. The N-terminal portion of their sequence differs from ours with four additional amino acid substitutions: a.a. 45, Q (ours) to P (theirs); a.a. 1032, R to L; a.a. 1407, A to R; and a.a. 1598, Q to H.

pUChsneo P element conferring neomycin resistance (Steller and Pirrotta, 1985) inserted at 65A10-11. Southern blot analysis was used to show that this P element is inserted 2.5 kb upstream of the translational start site of the laminin A subunit (Fig. 3). The presence of the P element in this chromosome does not alter laminin A expression

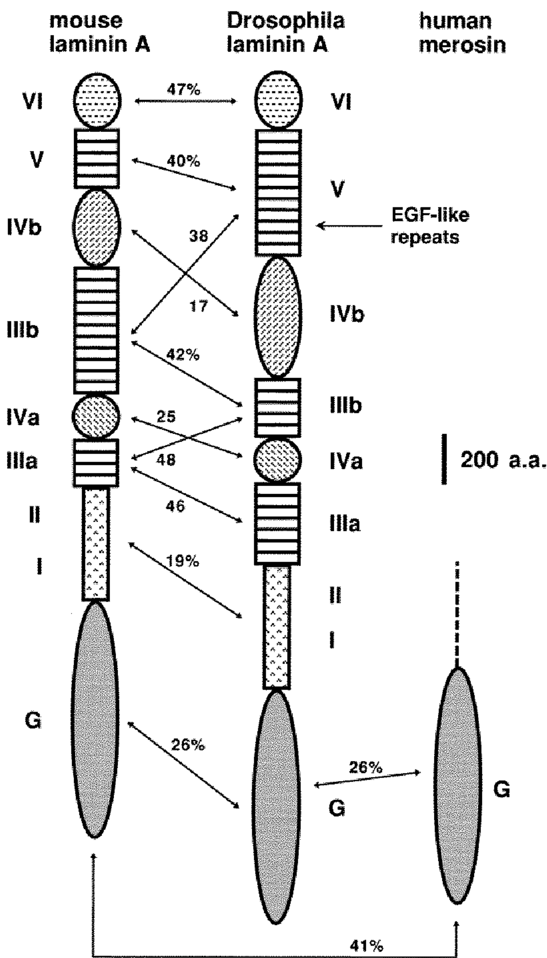

Fig. 2. Schematic diagram showing the domain structure (G, I-VI) of the 3712 amino acid *Drosophila* laminin A subunit as compared to the complete structure of mouse laminin A and the partial published sequence of human merosin (only the G domain). Whereas the *Drosophila* B1 and B2 subunits have the same domain structure and number of EGF-like repeats as compared to their mouse counterparts (Montell and Goodman, 1988, 1989), the *Drosophila* A subunit has different numbers of EGF-like repeats in domains V, IIIb and IIIa as compared to its mouse counterpart. The numbers indicate the percentage amino acid identity between the *Drosophila* and mouse domains. Note that the *Drosophila* A subunit has 26% amino acid identity with the G domains of both the mouse A and human merosin subunits, whereas the G domains of these two vertebrate subunits have 41% amino acid identity with each other, suggesting that they may have evolved by a gene duplication after the evolutionary split leading to chordates and arthropods.

throughout embryogenesis as determined by antibody staining. Moreover, we have observed no phenotype that can be attributed to the presence of this P element insert.

To generate mutations in the *lamA* gene, we mobilized the pUChsneo element in the AS249 stock by genetically introducing a source of transposase. In such a cross, the majority of P element excision events are precise, but a small proportion of such excisions are imprecise, removing flanking genomic sequences and leading to small deletions ranging from several hundred to several thousand nucleotides. Thus, deletions spanning part or all of a neighboring gene can be isolated. Of 939 total lines generated after P element mobilization, 11 independent recessive lethal mutations have been obtained which fall into a single complementation group. These alleles fail to complement a deficiency, *Df(3L)Vn-2* (Budnik and White, 1987), which deletes the *lamA* gene. Southern blot analysis of the genomic DNA of each lethal allele was undertaken using fragments within a 6 kb *Eco*RI genomic fragment (comprising 370 bp translated sequence plus upstream flanking DNA, Fig. 3) as probes. All 11 alleles show rearrangements in genomic DNA upstream of the *lamA* gene.

One of the lines with the largest deletion, $lamA^{9-32}$, has been mapped in more detail and shows that the deletion has one breakpoint 2.5 kb upstream of the translational start site and the second breakpoint between an *Eco*RI site at nt 370 and a *Pvu*I site at nt 806 (Fig. 3). This deletion therefore removes at least 370 bp of translated sequence at the N terminus of the protein. By probing northern blots of embryonic poly(A)+ RNA with probes within the 6 kb *Eco*RI restriction fragment, no transcription unit other than that for laminin A could be detected (data not shown), suggesting that the lethal phenotype is due to lack of laminin A function.

In order to isolate a range of different alleles at this locus, we undertook a screen for EMS-induced mutations in the *lamA* gene. From 2400 chromosomes screened in F2, we isolated nine new alleles, all of which were lethal in the initial screen in combination with one of the excision alleles, $lamA^{7-5}$. These EMS-induced alleles also fail to complement the deficiency *Df(3L)Vn-2.* Embryos of all alleles were examined by immunohistochemical techniques, using antisera specific to laminin A to test for expression of this subunit in each mutant (data not shown). No laminin A protein could be detected above background in any allele obtained by P element excision. Of the nine EMS-induced alleles, five showed wild-type levels of laminin A expression and four had reduced levels. These data are summarized in Table 1 for the alleles used in phenotypic analysis.

Abnormal adult phenotype of *lamA* alleles

To examine the effect that altering or removing laminin A

Table 1. *lamA* mutant alleles used in phenotypic analysis

Mutant abbreviation	Origin	Expression of laminin A protein‡
$lamA^{9-32}$	P element excision*	Not detected
$lamA^{6-36}$	P element excision*	Not detected
$lamA^{216}$	EMS†	Wild-type
$lamA^{160}$	EMS†	Wild-type
$lamA^{81L}$	EMS†	Reduced
$lamA^{25}$	EMS†	Reduced

*Isolated as imprecise excisions of AS249 with recessive lethal phenotype.

†Isolated as failing to complement *lamA* P element imprecise excision alleles.

‡As detected during embryogenesis of mutants with rat polyclonal antisera specific to laminin A isolated from S2 cell conditioned medium (C. H., C. S. G., unpublished results).

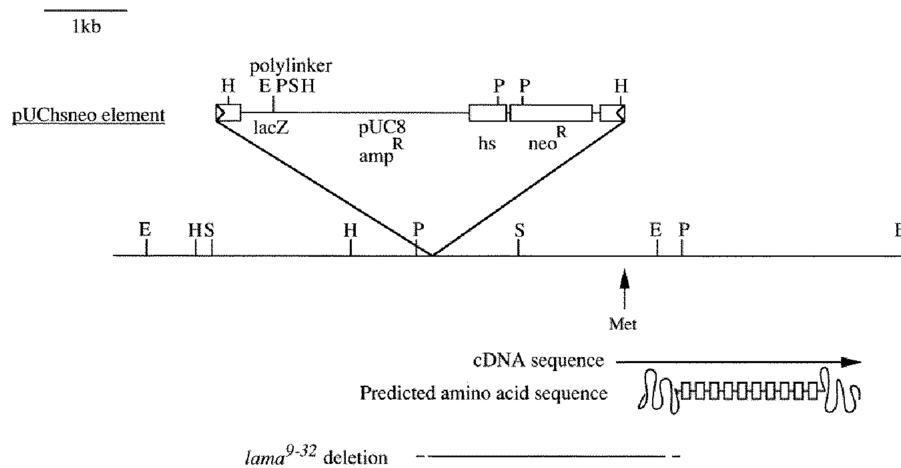

Fig. 3. Schematic representation of the P element insert and genomic region deleted in *lamA*$^{9\text{-}32}$. A restriction map is shown for the region upstream and including the 5 part of the *Drosophila* laminin A gene. Restriction sites are marked, E, *Eco*RI; H, *Hin*dIII; S, *Sal*I; P, *Pst*I; Pv, *Pvu*I (not all restriction sites are shown). The initiation methionine at the translational start site is marked by a vertical arrow labeled Met. Below this, the deduced protein structure is shown where it aligns with the genomic sequence with EGF-like repeats represented as open boxes between the two globular domains VI and IVb. The extent of genomic sequences deleted in DNA of *lamA*$^{9\text{-}32}$ is shown as a solid line; the dashed lines show the limits of uncertainty from mapping. The pUChsneo element in line AS249 is shown above its insertion position in the genomic restriction map. P element sequences are represented by the end open boxes and inverted repeats within these are marked as open arrows in each box. Two more open boxes are labeled hs, heat shock promoter element; and neo^{R}, encoding resistance to neomycin. Plasmid-derived sequences are labeled pUC8, containing a gene for ampicillin resistance (amp^{R}), and the galactosidase gene (lacZ).

function has on the development of the fly, we examined progeny from crosses of almost all possible combinations of the *lamA* alleles obtained. All combinations of excision alleles with the deficiency *Df(3L)Vn-2* or each other produced embryos that developed until stage 17 of embryogenesis but failed to hatch (these embryos can, however, move within the egg case). At the light microscopic level, we have been unable to detect any dramatic morphological abnormality in these mutant embryos. This analysis involved the use of the following monoclonal antibodies, which recognize different subsets of PNS and CNS neurons: mAb BP102, BP104, 22C10 and 1D4. In addition, we examined muscle development in dissected preparations and the cuticle including the head, mouth parts and denticle bands. In summary, the CNS, PNS, motoneurons and muscles, and the head, mouth parts and denticle bands appear normal in these mutant embryos. We do not detect the presence of laminin A protein in these embryos using immunohistochemical methods (Table 1).

All heteroallelic combinations of EMS-induced alleles tested with other EMS-induced alleles or with excision alleles were lethal, except in the case of four alleles: *lamA*^{25}, *lamA*^{81L}, *lamA*^{160} and *lamA*^{216} which in the crosses shown in Table 2 resulted in a number of escaper adults. We have characterized the phenotypes of these adults with respect to frequencies of survival as compared to balancer carrying sibs. All combinations of *lamA* alleles giving rise to escaper adults do so at a frequency that differs between heteroallelic combinations (Table 2). The reduced viability measured for these mutants is due to death during several phases of development, varying between genotypes. For example, 20% of *lamA*^{216}/*lamA*$^{6\text{-}36}$ pupae compared to 80% of *lamA*^{25}/*lamA*^{160} pupae fail to eclose. Of the escaper adults that eclose, many are lethargic and unable to jump or fly, and a large number die within one or two days after hatching.

Examination of the escaper *lamA* adults revealed the presence of morphological malformations of their eyes, legs

Table 2. Dependence of *lamA* mutant adult escapers on the specific allelic combinations

lamA mutant alleles	Number of adult mutant animals/ expected number* ×100(%)				
	lamA$^{6\text{-}36}$	*lamA*^{216}	*lamA*^{160}	*lamA*^{81L}	*lamA*^{25}
lamA$^{6\text{-}36}$	0	32	30	0	0
lamA^{216}	–	0	23	0	0
lamA^{160}	–	–	0	9	1
lamA^{81L}	–	–	–	0	0
lamA^{25}	–	–	–	–	0

*Expected number calculated from number of sibling progeny. Results are based on 1000-2000 adults scored from crosses at 25°C.

and wings (Table 3) (described in more detail below). Defects have also been seen in other parts of the cuticle (the antennae, ocelli, post-vertical and ocellar bristles) at a lower penetrance. A striking feature of the phenotypes of the adult escapers in these different combinations of *lamA* alleles is their variability: progeny from any one cross range from wild type to strong phenotype. Moreover, within a single structure, for example the compound eye of a particular individual, variability is seen between different regions of that eye. This variability is not surprising, since these escapers represent hypomorphic conditions of the gene. Moreover, presumably, those few individuals that survive to adulthood represent the least severe phenotypes within a given genotype.

Phenotypes were scored in comparison to balancer carrying sibs and to flies heterozygous for each *lamA* allele in combination with *AS249*$^{1\text{-}30}$, a wild-type stock derived from the P element excision screen which had undergone precise P element excision (as judged by Southern blot analysis of the genomic DNA). Of 100 flies scored for each control, none of the mutant phenotypes scored in Table 3 were observed. The differences in viability and penetrance of particular phenotypes seen between allelic combinations indicates that these EMS alleles reduce or alter laminin A func-

Table 3. Dependence of *lamA* mutant phenotypes on the specific allelic combinations

		Penetrance of phenotypes† Number of animals showing defect/total number mutant × 100 (%)			
lamA allelic combinations	Number of mutant animals/ expected no.* ×100(%)	Rough and/or misshapen eyes	Malformed legs	Blistered wings	Wing venation defects
160/6-30	30	82	77	29	35
216/6-36	32	82	91	<5	15
216/160	23	90	33	5	30
81L/160	9	91	38	20	38
21/160	1	82	95	24	10

*Expected number calculated from number of sibling progeny. Results are based on 1000-2000 adults scored from crosses at 25°C.
†Mutant phenotypes were scored for 70-100 animals. A mutant phenotype was counted where present in at least one single structure (for example an individual with a defect in one leg out of six is counted as showing the mutant defect).

tion in different ways or to different extents. This agrees with results of immunohistochemical localization of laminin A in embryos of the EMS-induced alleles (Table 1), which demonstrates that, although each expresses laminin A protein in a spatial and temporal patterns that are similar to wild type, $lamA^{160}$ and $lamA^{216}$ have apparently wild-type levels and $lamA^{25}$ and $lamA^{81L}$ have reduced levels.

Laminin A functions during development of the compound eye

The compound eye of *Drosophila* develops from the eye imaginal disc and is made up of a precise array of several hundred ommatidia, each comprising a stereotyped array of photoreceptor cells, pigment cells, cone cells and mechanosensory bristles (reviewed by Ready et al., 1976; Tomlinson, 1988; Cagan and Ready, 1989). The scanning electron micrograph in Fig. 4A shows the surface of the wild-type eye with straight rows of hexagonal lenses, each of which has bristles positioned at alternate vertices. Fig. 4B-F show examples of *lamA* mutant eyes which have abnormal overall shape and a rough appearance at the surface of the eye resulting from changes in the array of hexagonal facets and bristles. Changes in eye shape are most often associated with a reduction in the number of lens facets leading to a narrowing of the eye (Fig. 4C), but often a local indentation is seen where a number of facets appear to be missing (typically at the equatorial region; Fig. 4B). The roughness observed is associated with several factors (Fig. 4D-F): disruption of the normal array of lenses, misplaced and extra bristles, differences in lens shape and occasional fusion of lenses. The phenotypes shown are not specific to any one genotype, but rather are found in *lamA* escapers of all genotypes.

To examine the arrangement of cells in the retina, we cut plastic sections (transverse relative to the ommatidia of adult eyes) and stained them with toluidine blue. Wild-type ommatidia are precisely arrayed in rows. In each ommatidium, seven of the eight photoreceptors are visible and each project a darkly stained rhabdomere towards the center of the cell cluster (Fig. 5A). Shared between ommatidia are the secondary and tertiary pigment cells and bristles. Fig. 5B and C show examples of mutant eyes with strong phenotypes as found in adult escapers within each mutant genotype. Within each mutant genotype, the phenotypes range from wild type to strongly disrupted, with the most common being phenotypes that do not affect the entire eye. The most frequent phenotype is one of disruption of the array of ommatidia: the trapezoidal patterns of cells are oriented in different directions, unlike the regular lattice in wild-type eyes, and individual rows of ommatidia may run together. The majority of ommatidia have the wild-type complement of photoreceptor cells. However, two or more ommatidia may be fused (Fig. 5B). Ommatidia may develop with fewer than normal photoreceptors; in most *lamA* mutant eyes, this is seen infrequently but Fig. 5C shows an extreme example. Sections have also been examined that were cut perpendicular to the plane of the retina. These show normal organization of cells present in the retina: stained rhabdomeres spanned the entire thickness of the retina (data not shown).

The arrangement of cells on the apical surface of the 40 hours pupal retina of laminin A mutants was examined by cobalt sulfide staining (Fig. 5D-F). In the wild-type pupal retina (Fig. 5D), each cluster of cells visualized by this method comprises four central cone cells, surrounded by two primary pigment cells. Between these arrangements are a stereotyped array of secondary and tertiary pigment cells and bristles. In the mutants examined, the phenotypes observed range from wild type to severe. Fig. 5E and 5F show phenotypes in which the arrangement of cell clusters within the array is disorganized; bristles are frequently misplaced and fusion of ommatidia can be inferred from cell clusters possessing close to twice the normal complement of cone cells. This agrees with sections cut through the adult retina. Interestingly, although the majority of cell clusters possess the wild-type complement of cells, ommatidia can possess three or five cone cells, rather than four as in wild type. As mentioned earlier, given that these mutants are hypomorphs, the penetrance of this phenotype should represent a fraction of the requirement for laminin A. We do not know the penetrance of the complete loss-of-function condition, because null alleles are embryonic lethal. Were this gene to function in a cell-autonomous fashion, this issue could be easily resolved by examining mitotic recombination clones of null mutants. However, as described below, and as expected for an extracellular matrix protein, this gene does not function in a cell-autonomous fashion.

To study the phenotype of the null condition of *lamA* in the adult eye**,** we produced clones of cells homozygous for the P-element excision allele $lamA^{7-5}$. We induced somatic recombination in *w; mwh* $lamA^{7-5}$/$P[w^+]$ *M(3)67C* larvae. Thus, these clones were marked with the pigment-deleting mutation *white*. In addition, the presence of the heterozy-

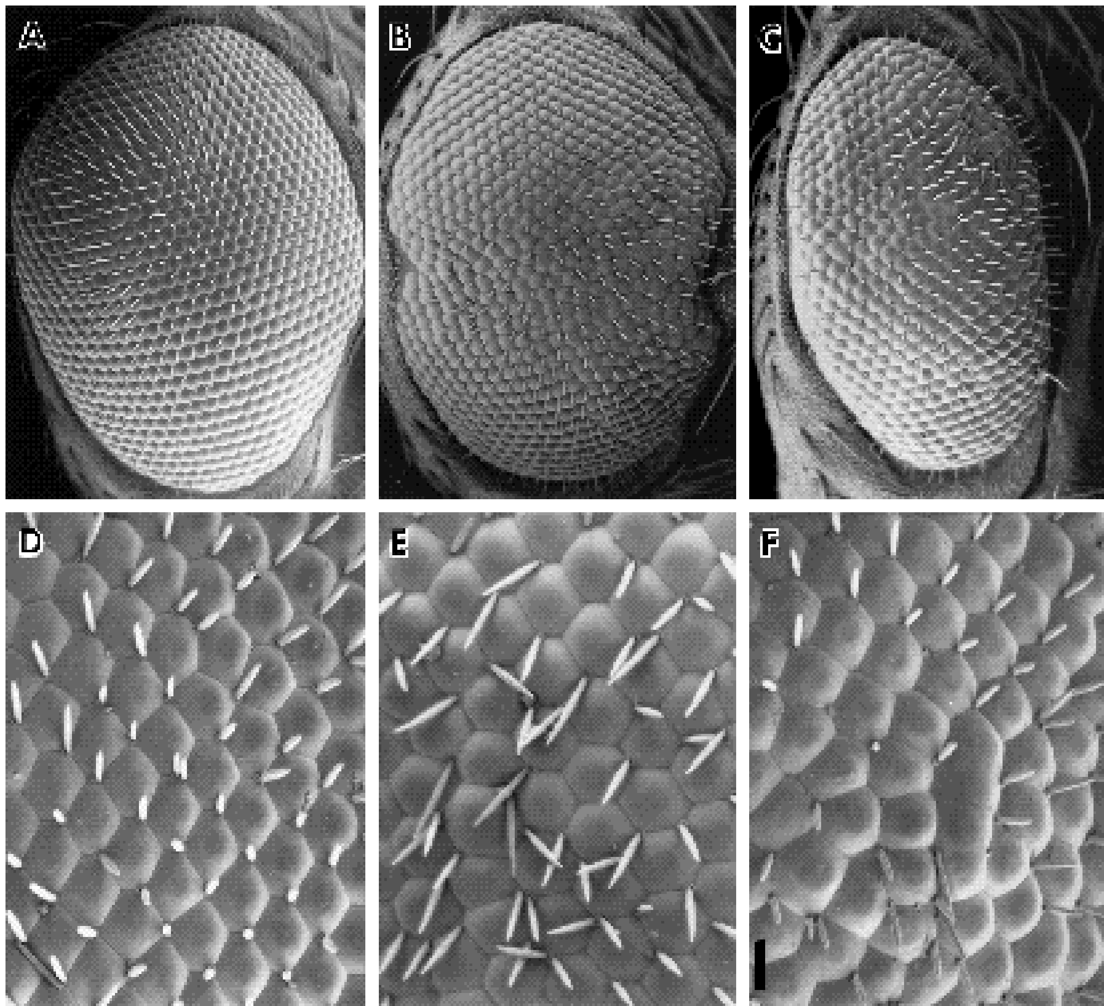

Fig. 4. Scanning electron micrographs comparing wild-type and *lamA* mutant adult eyes. (A) Wild type; hexagonal lenses are arrayed in straight rows with bristles evenly distributed. (B-F) *lamA* mutants; the eyes are misshapen with irregularly shaped edges (B) or are smaller than wild-type (C). (D-F) Rows of lenses poorly aligned, irregularly shaped and occasionally fused (F) and with mis-positioned bristles. The phenotypes shown are $lamA^{216}/lamA^{160}$ (B,C,F), $lamA^{160}/lamA^{9-32}$ (D,E). Phenotypes presented are common to all genotypes of escaper mutant adults. Bar, 50 μm in A-C, 12 μm in D-F. Anterior is to the right in A-C.

gous *Minute* mutation in the background enables the *lamA* mutant cells to span a large portion of the eye (Morata and Ripoll, 1975). Plastic sections stained with toluidine blue were analyzed for adult eyes containing clones homozygous for $lamA^{7-5}$. Within these clones, all ommatidia possess the wild-type complement of photoreceptor cells. The arrangement of ommatidia within the eye is usually normal, although some large clones show a loose packing (data not shown). A phenotype comparable to that described for escaper adults from different combinations of hypomorphic *lamA* mutant alleles is not seen. The *lamA* mutant phenotype is therefore non-cell autonomous as expected for a secreted molecule that is a component of the extracellular matrix.

Laminin A has diverse functions during adult development

In addition to its role during the development of the compound eye, laminin A also has a role in the development of other imaginal disc derivatives: *lamA* mutant adults have defects in structures of the legs and the wings (Table 3). In the wings, these defects are in the form of minor changes in wing venation and occasional separation (blistering) of the two surfaces of the wing. Malformations in *lamA* mutant adult legs occur at a high frequency. In *Drosophila,* the leg forms by evagination and elongation of a monolayer of undifferentiated epithelial cells which forms the imaginal disc (described in Fristrom and Rickoll, 1982), a process involving cell shape changes (Condic et al., 1991). Fig. 6A shows a wild-type leg with four tarsal segments, metatarsus, tibia and femur, separated by joints formed from infoldings of the epithelium. A set of thickened specialized bristles form the sex comb, present only in the male first leg. A wide range of structural defects are seen in *lamA* mutant adults and representative phenotypes are shown in Fig. 6B-D. The least severe phenotypes are bending within the normally straight segments (Fig. 6B). Individual segments, although present in the normal number, may be short

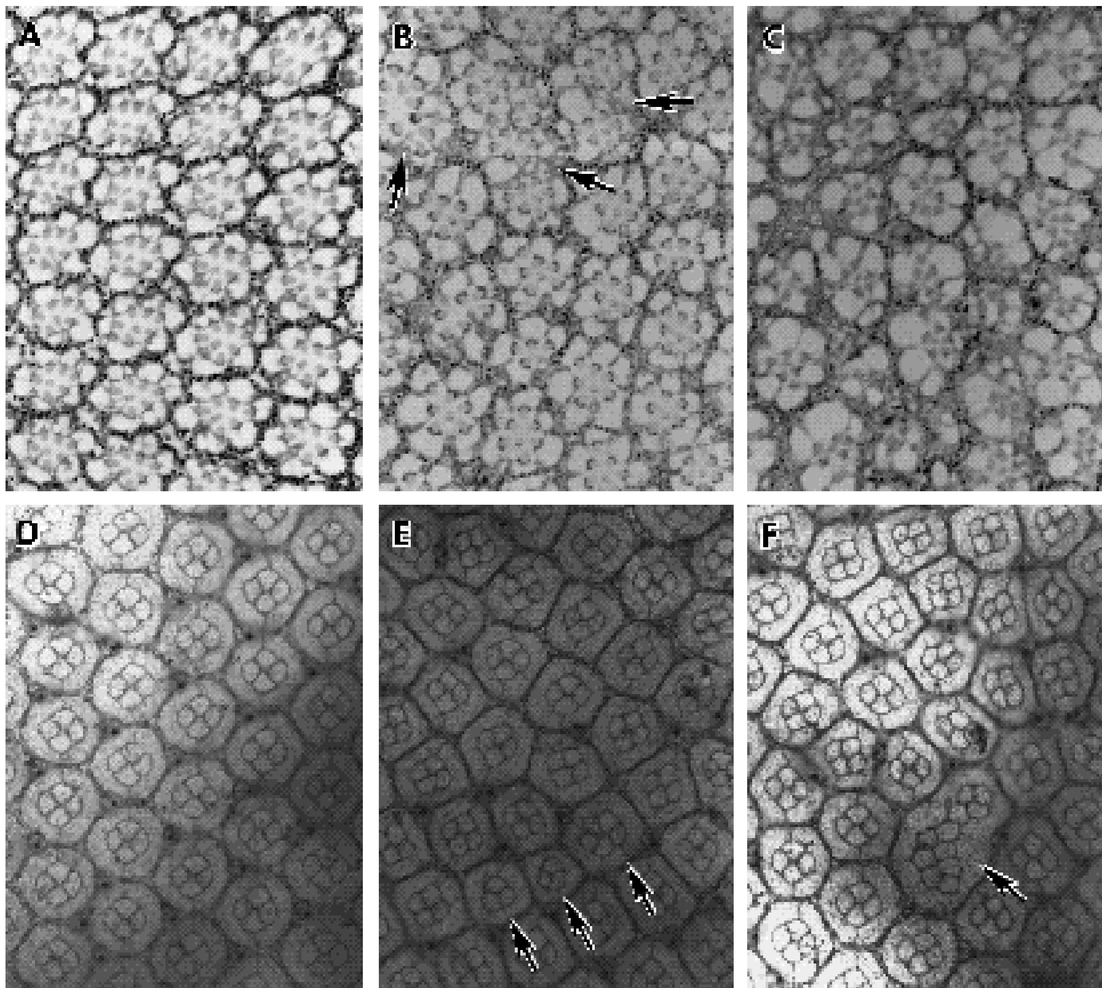

Fig. 5. Phenotypes in the adult and pupal retina of *lamA* mutants. (A) Photomicrograph of a sectioned wild-type eye. Each ommatidium comprises a trapezoidal cluster of cells separated from neighboring ommatidia by pigment cells. Ommatidia are precisely arrayed in rows. The stained structures in the middle of each cluster are the rhabdomeres of the photoreceptor cells. Central to each cluster is the R7 cell rhabdomere, surrounded by rhabdomeres of photoreceptor cells R1-R6. (B,C) Sections through eyes of flies with genotypes: $lamA^{25}/lamA^{160}$ (B), $lamA^{81L}/lamA^{160}$ (C). These show representative examples of defects found in all genotypes. (B) A region in which many cell clusters are fused (marked by arrows). Although the majority of normal sized ommatidia possess the wild-type complement of cells, a few have just 5 or 6 photoreceptor cells. (C) A region in which many ommatidia have fewer photoreceptor cells than wild-type and cell bodies are irregularly shaped. The array of ommatidia is disturbed, with occasional large spaces between individual ommatidia. (D) Photomicrograph of the apical surface of the 40 hours wild-type pupal retina, in which cells are viewed outlined by cobalt sulfide staining. At the center of each cluster are 4 cone cells, surrounded by 2 primary pigment cells. These clusters are separated by secondary and tertiary pigment cells and bristles (present at alternate apices). In *lamA* mutants (E and F), the organization of cell clusters is disrupted and bristles are mispositioned. Clusters may have an abnormal number of cone cells, marked by arrows in E, and occasionally fusion of cell clusters is seen where a number of pigment cells are absent (F). The genotypes of the mutants shown are $lamA^{25}/lamA^{160}$ (E), and $lamA^{81L}/lamA^{160}$ (F).

and bulbous: most clearly observed in the metatarsus and tarsal segments (Fig. 6C). Together, these account for the most commonly observed phenotypes. Although the majority of *lamA* mutant adult legs have the normal number of segments, a significant number of each heteroallelic combination have fused or deleted metatarsus and tarsi (Fig. 6D). Occasionally, *lamA* mutant legs are completely malformed and very short, with fusion of segments and ingrowths and outgrowths of the cuticle and duplication of recognizable structures such as the sex comb.

Clones of cells in the wing homozygous for a *lamA* null mutant ($lamA^{7-5}$) display weaker phenotypes than escaper wings entirely mutant for any one of a number of different hypomorphic *lamA* alleles, further supporting the conclusion from the eye clones that the gene functions in a non-cell autonomous fashion. In seven large $Minute^{+}$ clones examined in the wing, only one clone showed a weak phenotype in which the two surfaces of the wing were not together as normal.

To investigate potential interactions between *lamA* and the subunit of the integrin *lethal myospheroid*, we examined wings in adults that were homozygous for mys^{8} (same

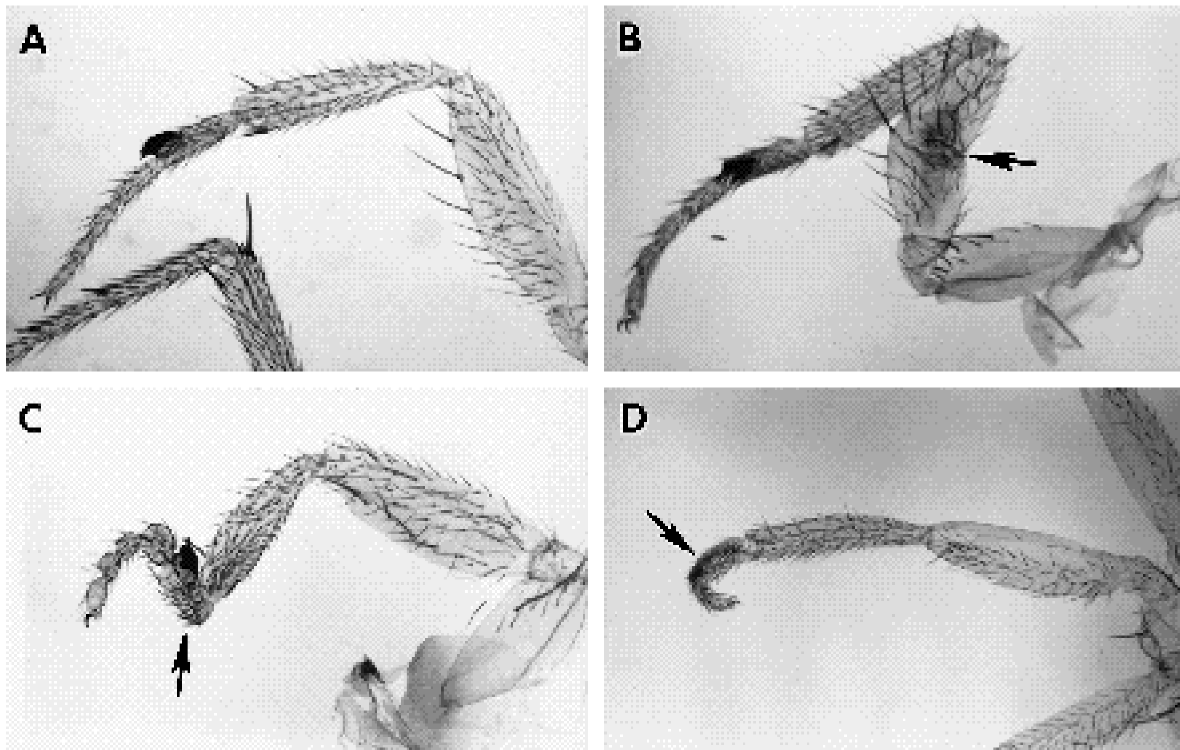

Fig. 6. Mutant leg phenotypes in *lamA* adult flies. Photomicrographs of cuticle of the male foreleg (A-C) and third leg (D) compare wild type (A) with a range of mutant phenotypes: (B) bend in femur (arrow); (C) shortened, bulbous tarsi and metatarsus, bent metatarsus (arrow); (D) fusion and shortening of metatarsal and tarsal segments (arrow). Genotypes of the adults are: $lamA^{216}/lamA^{9\text{-}32}$ (B,C), $lamA^{160}/lamA^{216}$ (D).

as *nj42*, a viable allele in which nonjumper adults show defects in certain muscles) and heterozygous for the *lamA* null allele $lamA^{7\text{-}5}$. Moreover, to investigate potential interactions of *lamA* with the subunit of the integrin *inflated*, we examined wings in adults homozygous for if^{1} and heterozygous for the same *lamA* null allele. We detected no significant enhancement of either integrin wing blistering phenotype.

DISCUSSION

To begin a genetic analysis of the roles of laminin during development, we have exploited the ability to manipulate *Drosophila* genetically and study the developmental consequences, particularly in light of the extensive homology between *Drosophila* laminin and vertebrate laminin (Montell and Goodman, 1988, 1989; Chi and Hui, 1989). Here we report on the molecular and genetic characterization of the laminin A subunit (*lamA*), which demonstrates diverse functions for laminin during morphogenesis.

The deduced amino acid sequence of *Drosophila* laminin A presented here predicts a multidomain structure similar to mouse and human homologs. Three of these domains, IIIa, IIIb and V, are cysteine-rich and are formed of EGF-like repeats. In contrast to the B1 and B2 subunits (Montell and Goodman, 1988, 1989; Chi and Hui, 1989), the number and arrangement of EGF-like repeats is not conserved between *Drosophila* and mouse laminin A subunits. It is known that different EGF-like repeats in the Notch protein have unique functions (see for example Rebay et al., 1991), and thus it is possible that these structural differences between species might reflect differences in function.

The structure of vertebrate laminin was initially determined to be composed of three subunits: A, B1 and B2. Recently, however, laminin complexes have been isolated that replace individual subunits in the heterotrimer (see Engvall et al., 1990, for a description of their distribution). These include S-laminin (Hunter et al., 1989), which is a laminin B1-like subunit found specifically enriched at synaptic junctions, and merosin (Ehrig et al., 1990), which is a laminin A-like subunit isolated from the trophoblast basement membrane of human placenta and present in striated muscle and peripheral nerve. The existence of such related genes in vertebrates leads to the question of whether alternative subunits exist in *Drosophila* and whether the laminin A subunit studied in this paper is a true homolog of the vertebrate laminin A chain or merosin. We compared the sequence of *Drosophila* laminin A with the available sequence for merosin (which comprises part of the domains I and II and the entire G domain). In terms of both amino acid identity and arrangement of subdomains within domain G, human laminin A and merosin are more similar to each other than either are to *Drosophila* laminin A. This suggests that laminin A and merosin may have arisen by a gene duplication after the evolutionary split leading to the arthropods and the chordates.

We have generated a series of mutant alleles in the *lamA* locus. Recessive lethal mutations were first obtained as imprecise P element excisions. We then used one of these P element excision alleles (*lamA*$^{7-5}$) to conduct an EMS mutagenesis (to generate *lamA* point mutations) in which we isolated nine additional *lamA* alleles. Although all *lamA* alleles were isolated due to their recessive lethal phenotype, certain heteroallelic combinations gave rise to adult progeny with reduced viability.

The phenotypes observed for the *lamA* mutant adults demonstrate pleiotropic effects of laminin A on differentiation and morphogenetic events occurring during the development of the imaginal discs to form adult structures. The compound eye of *Drosophila*, with its stereotyped array of defined cell types, presents an ideal system for analysis of laminin A requirements during cell interactions and differentiation. Laminin A is present in the basement membrane surrounding the eye disc in the larva, and cells of the retinal epithelial monolayer are in direct contact with this basement membrane during a wave of differentiation passing through the disc associated with the morphogenetic furrow (described in Cagan and Ready 1989).

Examination of the neurons of the retina of escaper adults (from combinations of hypomorphic mutant alleles) shows that, although most individual ommatidia contain the wild-type complement of photoreceptors, the arrangement of the cell clusters often does not form a precise array as in wild type. In eyes with a severe phenotype, smaller ommatidia are occasionally seen, possessing fewer photoreceptor cells. In some instances, cell clusters are found in which twice or almost twice the number of photoreceptor cells are present. These are probably due to fusion of ommatidia in which the secondary and tertiary pigment cells normally separating the ommatidia are absent (or misplaced). We examined the pupal retina for defects in development of cone cells and pigment cells. As in the adult eyes examined, the arrangement of cell clusters is frequently disturbed and changes in both the number and arrangement of each cell type are seen. Interestingly, often ommatidia are found with five cone cells, rather than the normal four, suggesting that a change in cell fate has occurred. Defects are also seen as a lack of cells, but in contrast to a gain of cells, such abnormalities could result from either a change in cell fate or a failure of a cell to differentiate along a particular pathway. These effects on cell fate seen in *lamA* mutants (e.g. extra cone cells) may arise as a consequence of inappropriate cell-cell contacts during the sequential series of inductive interactions (Tomlinson, 1988).

To examine the effect of total lack of function on development of the compound eye, we produced clones of cells homozygous for the null allele *lamA*$^{7-5}$ in a wild-type background. However, even in clones covering almost the entire retina, the external appearance of the eye and the arrangement of neurons appears wild type. The *lamA* mutation is therefore non-cell autonomous (this conclusions is also supported by the results of clones in the wing). Since many of the clones observed covered the majority of the retina, laminin A function in these regions of the developing eye must derive either from laminin A already synthesized which persists in the tissue, or from laminin A synthesized by other tissues or cell types (e.g., hemocytes or fat cells; Kusche-Gullberg et al., 1992).

The leg disc also undergoes major structural changes during metamorphosis and it appears that at least some of these processes require laminin function. Phenotypes seen at high penetrance in *lamA* mutants are bent and malformed (often bulbous) segments. At a lower frequency, segments are shortened and fused. This suggests that reduced laminin A function affects changes in cell morphology and rearrangements required to produce the final conformation of the epithelium. Adult *lamA* mutants also have defects in other cuticle structures, including wings, ocelli, antennae and bristles (notably ocellar and post-vertical bristles).

All of the defects studied in the adult are visible in the external cuticle. However, it is likely that the defects extend to the internal tissues since laminin A appears to affect a wide range of developmental processes. This is supported by the observation that many of the mutants die as pharate adults or as young adults unable to jump or fly. This lethargy may be due to structural defects in the muscles or inappropriate or absent motor innervation or sensory projections into the CNS.

Although the cellular receptors for laminin in *Drosophila* have not yet been defined, integrins have been isolated in this organism which are similar in structure to vertebrate integrins (of which a subclass bind laminin). Genetic loci have been identified corresponding to two *Drosophila* integrins: *inflated (if)*, an subunit and *l(1)myospheroid (l(1)mys)*, a subunit (Leptin et al., 1989). However, comparison of the phenotypes of mutations in these integrin genes (with the phenotypes of the mutations in *lamA* described here) suggests that these integrins and laminin A might be involved in different mechanisms. For example, *l(1)mys* embryos suffer defects in dorsal closure, disruption of muscle attachment to the body wall and herniation of brain tissue (Newman and Wright, 1981), none of which are seen in *lamA* null alleles. Although mosaic analysis of *l(mys)* has demonstrated a requirement for this integrin in development of the adult eye and legs (Zusman et al., 1990), details of the observed phenotypes are quite different to those described here for *lamA*. In *l(1)mys* clones in the eye, the surface of the lens is normal, in contrast to *lamA* mutants in which the array of lenses is disrupted and extra and mispositioned bristles arise. Within the retina, although mutant *lamA* eyes show the array of ommatidia to be disrupted, each ommatidium has a normal structure and attachment to the fenestrated membrane when viewed in sections cut perpendicular to the eye surface. This too is in contrast with *l(1)mys* clones where disorganization of rhabdomeres is evident, particularly at basal positions, i.e. close to the fenestrated membrane (Zusman et al., 1990).

In support of this conclusion that *lamA* does not appear to interact with either *l(1)mys* or *inflated*, we have observed no significant enhancement of wing blistering phenotypes in adult wings homozygous for either the viable *mys*^{8} allele or the viable *if*^{1} allele and heterozygous for a null *lamA* allele.

It is difficult to reconcile the observations of diverse effects of laminin A upon adult development with the lack of obvious morphological abnormalities in mutant embryos. One potential explanation for such a difference would be a

maternal contribution of the gene product (Kusche-Gullberg et al., 1992), although other Northern and in situ hybridization studies have seen no evidence for this (Montell and Goodman, 1988, 1989). Alternatively, it is possible that a number of different extracellular matrix components (either other molecules, or laminin complexes without the A subunit) can fulfil some of the normal functions of laminin in the embryo and thus that removal of the laminin A subunit may be compensated by others present.

The mutant *lamA* alleles described here provide an opportunity to use the approaches of *Drosophila* genetics to elucidate further the developmental and cellular mechanisms of action of laminin A. Furthermore, it may be possible to look for interacting loci and thus to study genes whose products are involved in these events. In this way, genetics could be used to begin to dissect the signalling pathways that transmit information from the extracellular matrix, across the cell surface, to ultimately effect changes in cell behavior. For example, although several integrins have been identified thus far in *Drosophila*, for the reasons outlined above, it is likely that none of them function as a laminin A receptor. It should now be possible to use genetic approaches to identify laminin receptors and other downstream genes that mediate the diverse range of laminin functions controlling differentiation and morphogenesis.

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