Zebrafish mutants identify an essential role for laminins in notochord formation

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SUMMARY

Basement membranes are thought to be essential for organ formation, providing the scaffold on which individual cells organize to form complex tissues. Laminins are integral components of basement membranes. To understand the development of a simple vertebrate organ, we have used positional cloning to characterize grumpy and sleepy, two zebrafish loci known to control notochord formation, and find that they encode laminin β1 and laminin γ1, respectively. Removal of either chain results in the dramatic loss of laminin 1 staining throughout the embryo and prevents formation of the basement membrane surrounding the notochord. Notochord cells fail to differentiate and many die by apoptosis. By transplantation, we demonstrate that, for both grumpy and sleepy, notochord differentiation can be rescued by exogenous sources of the missing laminin chain, although notochordal sources are also sufficient for rescue. These results demonstrate a clear in vivo requirement for laminin β1 and laminin γ1 in the formation of a specific vertebrate organ and show that laminin or the laminin-dependent basement membrane is essential for the differentiation of chordamesoderm to notochord.

Key words: Laminin, Notochord, Basement membrane, Differentiation, Zebrafish

INTRODUCTION

Organogenesis requires the precise coordination of a number of distinct cellular processes. Among these, establishment of an appropriate extracellular environment is crucial for the proper exchange of information between tissues and for the ultimate shape and function of the organ. The vertebrate notochord is an early embryonic structure ideally suited to study organ formation, as it comprises a single cell type that is specified early in development. The notochord is essential for normal development of vertebrate embryos, providing both mechanical and signalling functions. Systematic screens for mutations affecting zebrafish embryogenesis have identified several genes that control notochord differentiation and morphogenesis (Odenthal et al., 1996; Stemple et al., 1996).

As the notochord differentiates, expression of a group of early genes is extinguished, a large vacuole within each cell becomes inflated and a fibrous extracellular sheath is formed. The sheath, in combination with the turgor pressure exerted by the vacuolated cells, provides the notochord with the rigidity required for its function as the major skeletal element of lower-vertebrate embryos. The notochord also provides essential signals for midline patterning (Cleaver and Krieg, 2001; Placzek et al., 1993; van Straaten et al., 1989; Yamada et al., 1993). For example, the notochord is a source of Hedgehog proteins that instruct the formation of ventral-specific fates in the neural tube (Ericson et al., 1995; Tanabe et al., 1995) and, in zebrafish, induce somitic mesoderm to form slow-twitch muscle (Barresi et al., 2000; Currie and Ingham, 1996; Krauss et al., 1993; Lewis et al., 1999).

The zebrafish notochord arises from the embryonic shield, which is the teleost equivalent of the amphibian Spemann organizer (Oppenheimer, 1936; Saudê et al., 2000; Shih and Fraser, 1995; Spemann and Mangold, 1924). Embryonic shield cells involute during gastrulation, giving rise to chordamesoderm, which then differentiates to form mature notochord (Gritsman et al., 2000; Melby et al., 1996; Talbot et al., 1995). Two genes, bozozok and floating head, have been identified as essential for specification of shield cells to chordamesoderm (Fekany et al., 1999; Talbot et al., 1995). Another nine loci are essential for the differentiation of chordamesoderm into mature notochord. Three loci of this latter class, bashful (bal), grumpy (gup) and sleepy (sly), also control guidance of axons in the developing brain (Karlstrom et al., 1996; Odenthal et al., 1996; Stemple et al., 1996).

We have characterized gup and sly, and show here that they encode the laminin β1 and γ1 chains, respectively. Laminins are heterotrimeric proteins consisting of α, β and γ chains. There are 12 known isoforms of laminin generated by distinct combinations of the five α, four β and three γ chains (for reviews, see Colognato and Yurchenco, 2000; Timpl and Brown, 1996). Laminins are extracellular basement membrane proteins that have a variety of roles during development and in the mature animal. For example, they have been found to control cellular differentiation of mammary epithelia, to
participate in axis specification in Drosophila and to control directed growth of axons (Deng and Ruohola-Baker, 2000; Hopker et al., 1999; Serafini et al., 1994; Streuli et al., 1995). Laminins have been shown to play an important role in neuromuscular junction formation and maintenance (Patton, 2000). Laminins have also been implicated in disease. For example, the disruption of laminin α2 or laminin-interacting proteins can lead to muscular dystrophy (Cote et al., 1999; Miyagoe et al., 1997) (M. J. P., unpublished).

As laminins are a major constituent of basement membranes, their role in the organization of basement membrane structure has also been an area of active investigation. In addition to laminins, most basement membranes contain type IV collagen, nidogen/entactin and a variety of heparin sulfate proteoglycans, such as perlecan (Durkin et al., 1988; Gupta et al., 1997; Hassell et al., 1980; Mann et al., 1989; Yasothornsrikul et al., 1997; Yurchenco and Ruben, 1987). Cell culture and genetic studies in both vertebrate and invertebrate species have provided a detailed picture of basement membrane organization (Colognato and Yurchenco, 2000; Timpl and Brown, 1996). Cell receptors bind the C-terminal globular domain of the laminin α chain, while the short arms protruding laterally from the laminin molecule interact to form a hexagonal lattice covering the cell surface (Colognato et al., 1999). Nidogens crosslink laminins to a collagen IV matrix (Fox et al., 1991; Mayer et al., 1993; Poschel et al., 1994). Proteoglycans can interact simultaneously with laminins and growth factors, such as FGF, TGFβ or Wnt family members (Costell et al., 1999; Perrimon and Bernfield, 2000).

When specific laminin or collagen IV isoforms, or cell-surface laminin receptors are missing, the basement membrane fails to form (Aumailley et al., 2000; Cote et al., 1999; Henry et al., 2001b; Miyagoe et al., 1997; Smyth et al., 1999). This loss of basement membrane can produce specific defects in the function of organs and tissues. For example, loss of collagen IV is implicated in the failure of kidney glomerular function in Alport syndrome (Hudson et al., 1993). Similarly, loss of laminin α5 leads to disruption of glomerular function in the mouse mutant (Miner and Li, 2000). Loss of specific laminin isoforms in both vertebrates and invertebrates has been shown to cause specific tissue defects; however, the study of the late roles of one key vertebrate laminin, laminin 1 (α1, β1, γ1), has been difficult because of the requirement of one of its components, γ1, for implantation and formation of extra-embryonic endoderm in mice (Smyth et al., 1999; Timpl et al., 1979).

We find that laminin 1 in zebrafish is produced from both maternal and zygotic sources, and is expressed at all stages of embryogenesis. When the zygotic supply of the β1 or γ1 chain is disrupted, laminin 1 protein is lost throughout the embryo. These disruptions also cause a loss of basement membrane around the notochord and notochord cells fail to differentiate. By transplantation, we find that providing the missing laminin chain from exogenous sources can restore notochord differentiation. We also find that wild-type chordamesoderm in a grumpy or sleepy mutant host environment is able to provide sufficient wild-type laminin to rescue notochord differentiation.

MATERIALS AND METHODS

Embryo collection

General maintenance, collection and staging of zebrafish were carried out according to The Zebrafish Book (Westerfield, 1994). The approximate stages are given in hours post-fertilization at 28.5°C (hpf), according to morphological criteria (Kimmel et al., 1995).

Embryo labelling and microsurgery

Donor embryos were labelled with biotin dextran and tetramethylrhodamine dextran and shield transplants were performed as previously described (Saude et al., 2000). In order to trace cell lineage following fixation, embryos were incubated overnight in Vectastain ABC-AP reagents A and B in phosphate-buffered saline, 0.1% Tween-20 (PBT) (Vector Labs). After washes in PBT, embryos were further washed in 0.1 M Tris pH 8.2, 0.1% Tween-20. The embryos were placed in 4 ml of 0.1 M Tris pH 8.2, 0.1% Tween-20 into which one Fast Red tablet had been dissolved (Roche). The color reaction was monitored constantly and the reaction stopped with PBS and 2 mM EDTA.

Preparation of genomic DNA from mutant embryos

The digestion of each single embryo was done by incubating in 100 µl of lysis buffer (0.5% SDS, 0.1 M EDTA pH 8, 10 mM Tris pH 8, 100 µg/ml Proteinase K) for 5 hours at 55°C. DNA was purified using Sephacryl S-400 (sigma) in Multiscreen-GV, column plates (0.22 µm hydrophilic, low protein binding, Durapore Membranes). DNA was diluted 1:10 and 5 µl used in a 20 µl PCR reaction.

Oligonucleotide labelling

For SSLP analysis, both primers were radiolabelled. Each labelling reaction consisted of 0.5 µl 1×T4 polynucleotide kinase buffer, 2.9 µl of 10 µM oligonucleotide, 0.1 µl T4 polynucleotide kinase (10 U/µl) and 1.5 µl γ32P dATP (6000 Ci/mmmole, 10 mCi/µl), and was incubated for 30 minutes at 37°C.

Polymerase chain reaction (PCR)

All PCR reactions were carried out in 96-well plates with a final reaction volume of 20 µl. For a 20 µl reaction, we used 14.3 µl PCR mix, 0.15 µl of each labelled oligonucleotide, 0.2 µl of Taq polymerase (5 U/µl) and 5 µl of DNA template. The PCR mix was made by adding 122 µl of 10× Taq buffer, 9.8 µl of 25 µM dNTPs and 740.8 µl of distilled water to a final volume of 872.6 µl. PCR conditions used consisted of 35 cycles of 92°C for 1 minute, 58°C for 1 minute and 72°C for 1.5 minutes. PCR products were separated on a 6% polyacrylamide denaturing gel and exposed directly to film at ~70°C.

PAC, BAC and YAC library screening and YAC end rescue

The zebrafish yeast artificial chromosome DNA pools were purchased from Research Genetics and the zebrafish BAC library was obtained from Genome Systems. Both libraries were used according to the manufacturers’ instructions. YAC end rescue was performed to obtain genomic sequence (Talbot and Schier, 1999).

Antibody staining and whole mount in situ hybridization

For immunostaining with the laminin 1 antibody (Sigma L-9393) (1:400), embryos were fixed in 4% paraformaldehyde (PFA) overnight at 4°C and then stored in methanol, followed by several washes in PBT and digestion in proteinase K (10 µg/ml) for 5-10 minutes. No further fixation was used. Secondary antibodies conjugated to HRP were used and detected using DAB supplemented with NiCl2.

Whole-mount in situ hybridization reactions were carried out according to published protocols (Thiss et al., 1993). Riboprobe for lamc1 was transcribed from a template comprising 950 bp of 3’UTR. This clone was linearized with XhoI and transcribed with T7. Riboprobe for lamb1 was transcribed directly from a cDNA clone, MPMGp669C1220Q (EST Accession Number, AI584363) obtained from the RZPD (Vente et al., 1999), linearized with EcoRI and transcribed with SP6. Other riboprobes used detected fltl (Brown et al., 2000), ptc1 (Concordet et al., 1996), ehh (Currie and Ingham, 1996) and twbb (Ekker et al., 1995).
**Antisense morpholino oligonucleotide injections**

Antisense morpholino oligonucleotides (MO) (Gene Tools, LLC) were designed complementary to the 5' sequence near the start of translation (Nasevicius and Ekker, 2000). The MOs used were: lamb1, 5'-TAATTCGATTTCTTTCTACACCGG-3'; laml, 5'-TGGTCCTTTTGCTATGGCAGACCTC-3' and 5'-GAATGCTACGGTACGTCACGTTCTGTGAT-3'; and lamb4, 5'-CTAGACGGAGACACATAGCTG-3'.

A volume of 1.4 nl was injected through the chorion of one-cell stage embryos to deliver 3.5 ng of MO. The lamb4 MO produced no discernible phenotype and was used as an injection control.

**TUNEL assay**

Embryos were fixed in 4% PFA overnight at 4°C and then stored in methanol. Following re-hydration and digestion with proteinase K (10 μg/ml), embryos were fixed with 4% PFA for 20 minutes, washed, then fixed further in ethanol:acetic acid for 10 minutes at −20°C. After further washes with PBT, embryos were incubated with equilibration buffer for 1 hour at room temperature then overnight in 37°C in TdT enzyme in reaction buffer (1:2) with Triton X-100 (0.3%). The reaction was stopped by a 3-hour incubation in Stop/Wash Buffer at 37°C. All reagents were from the Apoptag In Situ Apoptosis Detection Kit-Peroxidase (Oncor). The embryos were blocked and immunostained according to our whole-mount in situ hybridization procedure.

**Electron microscopy**

Whole zebrafish embryos were dechorionated manually and fixed overnight with 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.2 (SCB). They were washed for 10 minutes in SCB and postfixed for 1 hour in 1% osmium tetroxide and SCB. They were washed again with SCB and stained en bloc with 1% aqueous uranyl acetate for 1 hour. The samples were then dehydrated through a graded ethanol series, followed by two changes of propylene oxide over 20 minutes and embedded in Epon resin (Agar Scientific). Ultra thin sections (50 nm) were cut and mounted on pioloform coated slot grids and stained with 1% aqueous uranyl acetate for 15 minutes followed by Reynold’s lead citrate for 7 minutes. Sections were visualized on a Jeol 1200 EX electron microscope.

**Accession numbers**

Sequences have been submitted to GenBank and the Accession Numbers are lamb1 (AF468049); lamb4 (AF468050) and laml (AF468048).

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**RESULTS**

**gup and sly mutant embryos display identical phenotypes**

In previous descriptions of gup and sly, it was observed that their phenotypes are essentially indistinguishable (Odenthal et al., 1996; Stemple et al., 1996). A more extensive examination of molecular and morphological markers confirms this observation. Both sly and gup mutants have shortened body axes, which are correlated with a failure of notochord differentiation (Fig. 1A-C). This failure is manifest in at least two ways. First, mutant notochord cells fail to become properly vacuolated. Secondly, mutant notochord cells fail to extinguish the expression of several marker genes, including echidna hedgehog (ehh) (Fig. 1D,E, gup data not shown), while mutant floor plate shows a normal pattern of tiggy-winkle hedgehog (tvhh) expression (Fig. 1F,G). gup and sly mutants share at least three other phenotypes. Both mutants show abnormally high levels of apoptosis in the notochord (Fig. 1H-J). Both show undersized retinas and displacement of the lens (Fig. 1K-M). Finally, both mutants fail to form intersomitic blood vessels as judged by expression of fli1, a marker of vascular progenitor cells (Fig. 1N,O and data not shown) (Brown et al., 2000). At later stages, we observed no blood flow between the somites of mutant embryos (not shown).

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**Fig. 1.** Morphological and molecular analysis of sly and gup mutants. Lateral views show morphological phenotypes of wild-type (WT) (A), gup (B) and sly (C) mutant embryos at 22 hpf. (D) Whole-mount in situ hybridization shows that by 28 hpf ehh expression is no longer observed in the wild-type vacuolated notochord (arrow). (E) In sly mutants the notochord remains undifferentiated and expression of ehh is maintained (arrow). (F,G) In WT and sly mutants, the expression of tvhh is identical and restricted to the floor plate (arrows). (H) TUNEL analysis at 28 hpf shows that apoptosis is restricted to the dorsal neural tube in WT embryos. (I,J) In both gup (I) and sly (J) mutant embryos, more extensive apoptosis is observed along the embryonic midline. (K-M) Close-up images of WT (K), gup (L) and sly (M) mutant embryos at 22 hpf show the small retina and protruding lens of the mutants. (N,O) Whole-mount in situ hybridization to detect expression of fli1, a marker of vascular progenitor cells, shows that the inter somitic vessels seen forming in 22 hpf WT embryos (N) fail to form in sly mutants (O).
**sly encodes the laminin γ1 chain**

To understand the roles of *sly* and *gup* in notochord formation, both genes were isolated by positional cloning. Half-tetrad analysis was initially used to place *sly* onto linkage group 2. PCR-based markers that spanned CA repeats were used in recombination analysis of over 3000 meioses (Shimoda et al., 1999). We found the *sly* locus to lie within an interval of 3.4 cm spanned by the markers Z11023 and Z7632 (Fig. 2A). A YAC-based chromosome walk was carried out from the *elra* locus, which has been mapped to the same interval and found on the PAC clone 196M22 (Horne-Badovinac et al., 2001). We found that one end of YAC clone 148F11 contained an exon that displayed significant homology to exon 3 of the human laminin C1 gene, which encodes the γ1 chain that is found in 10 out of the 12 known laminin isoforms. We used degenerate PCR primers designed against exon 28 of the human gene to obtain a fragment from the 3' end of the zebrafish gene. Polymorphic markers generated from the 5' and 3' ends of the gene showed no recombination in more than 1000 meioses, indicating close linkage between *sly* and *lamc1*. We found the full-length cDNA sequence to span 6055 bp, predicting a protein of 1593 amino acids (~200 kDa predicted mass) (Fig. 2B). To identify the mutations associated with *sly* alleles we cloned and sequenced *sly* and *m466* embryos. We found both mutant alleles to contain premature stop codons (*m86*: Y131T3. Sequence of *lamininC1* exon 3 was found at the end of YAC 148F11. (B) Zebrafish laminin γ1 protein. The positions of the premature stop codons caused by mutation in the *sly* alleles are indicated. Protein domains are indicated by colored shapes: red, putative signal peptide; blue hexagon, laminin N-terminal globular domain; purple pentagons, laminin-type EGF-like repeats; orange octagon, laminin B globular domain; green rectangles, coiled-coil domains. (C) Meiotic map surrounding the *gup* locus compared with the physical map generated using RH analysis. A polymorphic marker, cat/gca, was identified by AFLP analysis and was found to map to LG25. Two candidate genes, *lamB1* and *lamB2*, were established to be closely linked. (D) Zebrafish laminin β1 protein. The position of the premature stop codon caused by the mutation in the *gupm189* allele is indicated. Protein domains are represented as in B above.

**Fig. 2.** Positional cloning of the *sly* and *gup* loci. (A) Meiotic map of the *sly* locus (including the nearest polymorphic CA repeat marker Z11023) compared with the chromosome walk undertaken. Starting from *elra* (PAC 196M22), the walk proceeded from left to right in a series of YACs, represented by labelled, coloured bars, e.g. Z7632. Polymorphisms were identified from YAC ends at various points and used to ascertain the distance and direction to the *sly* locus. These markers are indicated on the meiotic map, e.g. 177T3. Sequence of *lamininC1* exon 3 was found at the end of YAC 148F11. (B) Zebrafish laminin γ1 protein. The positions of the premature stop codons caused by mutation in the *sly* alleles are indicated. Protein domains are indicated by colored shapes: red, putative signal peptide; blue hexagon, laminin N-terminal globular domain; purple pentagons, laminin-type EGF-like repeats; orange octagon, laminin B globular domain; green rectangles, coiled-coil domains. (C) Meiotic map surrounding the *gup* locus compared with the physical map generated using RH analysis. A polymorphic marker, cat/gca, was identified by AFLP analysis and was found to map to LG25. Two candidate genes, *lamB1* and *lamB2*, were established to be closely linked. (D) Zebrafish laminin β1 protein. The position of the premature stop codon caused by the mutation in the *gupm189* allele is indicated. Protein domains are represented as in B above.
assignment of sly to the laminin c1 (lame1) gene. First we used a polyclonal antibody, generated against mouse laminin 1, which recognizes two bands of ~200 kDa and ~400 kDa on western blots of zebrafish extracellular matrix (not shown). We found that sly mutant embryos, at 24 hours post fertilization (hpf), exhibit very little laminin 1 immunoreactivity (Fig. 3B). By contrast, wild-type siblings showed extensive staining, especially surrounding the notochord and in intersomitic spaces (Fig. 3A,C). We then designed two distinct non-overlapping antisense morpholino oligonucleotides (MO) directed against sequences near the predicted start of translation for sly/lamC1. Whereas control MO-injected embryos appeared normal, sly/lamc1 MO-injected embryos displayed phenotypes identical to sly mutants. Laminin 1 immunoreactivity was severely disrupted in sly/lamc1 MO-injected embryos (Fig. 3F). Similar to sly mutants, the axes of sly/lamc1 MO-injected embryos were shorter than controls (Fig. 3I). Finally, notochords of sly/lamc1 MO-injected embryos fail to differentiate, as seen by the persistent expression of ehh and the failure to form properly vacuolated cells (Fig. 3L), and the lenses were abnormally displaced from undersized retinas (not shown). Taken together, these results show that sly encodes the zebrafish laminin γ1 chain.

**gup encodes zebrafish laminin β1 and is closely linked to laminin B4**

We used the amplified fragment length polymorphism (AFLP) method (Vos et al., 1995) to identify a genomic marker (cat/gca) 1.3 cM from the gupm189 (Fig. 2C). Using a radiation hybrid (RH) panel, we mapped this AFLP marker to within 21 cR of myod on chromosome 25 (Hukriede et al., 1999). Primers flanking a polymorphic region of the myod 3′UTR confirmed that the myod locus lies within 0.7 cM of gup (Postlethwait et al., 1998). By monitoring publicly accessible RH maps, we noted an expressed sequence tag (EST) marker that was both linked to laminin B4 and homologous to human laminin B genes. We sequenced the clone corresponding to this EST (Accession Number, AA495063) and found it to be homologous to human LAMB4 (Fig. 2C). Complete cDNA sequence for zebrafish lamb4 was obtained by screening additional cDNA libraries and by 5′ RACE. Database searches also yielded a zebrafish laminin B1 (lamb1) homologue (Accession Number, AI641157), which we mapped to within 20 cR of myod using the LN54 RH panel. Using this clone and 5′ RACE, we determined the complete lamb1 cDNA sequence. We obtained MOs targeted against lamb4 and lamb1 mRNA. Embryos injected with either the control MO or lamb4 MO, were phenotypically wild type (Fig. 3G and data not shown). By contrast, embryos injected with lamb1 MO displayed a morphological phenotype identical to that of gup mutants (Fig. 3H). More detailed analysis of the lamb1 MO phenotype revealed that, like gup mutants, LamB1 morphants display a loss of laminin 1 immunoreactivity (not shown), a persistence of ehh expression (Fig. 3K) and lenses abnormally displaced from retinas (not shown). As a further verification that gup corresponds to the lamb1 gene, we sequenced both cDNA and genomic DNA, taken from gupm189 mutant embryos, and identified a premature stop codon at amino acid 544 (Fig. 2D). Hence, gup encodes a laminin β1 chain and sly encodes a laminin γ1 chain. To elucidate their roles during embryogenesis further, we examined the expression of the mRNAs for these two genes.

![Fig. 3. gup and sly are phenocopied by antisense morpholino oligonucleotides (MO). (A-D) Laminin 1 staining in 32 hpf (A) and 24 hpf (C) WT, and 32 hpf sly (B) and 24 hpf gup (D) mutant embryos. (E,F) Control MO (E) and sly/lamC1 MO (F) -injected embryos at 24 hpf stained with anti-laminin 1 antibody shows the loss of laminin 1 in sly/lamc1 morphants. (G-L) Embryos injected with gup/lamb1 MO (H,K) or sly/lamc1 MO (L) possess a shortened body axis, and show maintenance of ehh expression in undifferentiated notochords (K,L) when compared with control MO injected embryos (G,J).](image)
consistent with a nonsense-mediated mRNA decay mechanism, and provides further evidence that the sly locus encodes laminin γ1 (Hentze and Kulozik, 1999).

We then compared the expression patterns of gup/lamb1 and sly/lamc1 mRNA to that of laminin 1 protein (a heterotrimer of α1, β1 and γ1 chains). Laminin 1 is expressed uniformly throughout the developing embryo at the blastula stage (Fig. 4C). By tailbud stage, however, laminin 1 immunoreactivity accumulates around the forming chordamesoderm (Fig. 4F). Laminin 1 immunoreactivity in sly mutants is similar to wild-type levels at these early stages of development (data not shown). As laminin 1 immunoreactivity is severely reduced in sly and gup mutants at later stages (Fig. 3B,D), it is possible that at this early stage the antibody detects unstable dimers or a different crossreactive laminin isoform.

Considering the overlapping expression of gup/lamb1 and sly/lamb1, we sought to test their epistatic relationship. We found that when gup/lamb1 MO and sly/lamc1 MO were co-injected, embryos expressed a phenotype that was indistinguishable from that caused by either MO alone (not shown). Similarly, when sly/lamc1 MO was injected into homozygous gup mutant embryos or gup/lamb1 MO was injected into homozygous sly mutant embryos there was no enhanced phenotype (not shown).

**Notochord differentiation and laminin 1 assembly can be directed either autonomously or non-autonomously**

The expression of both gup/lamb1 and sly/lamb1 in the chordamesoderm suggests the possibility that normal notochord differentiation depends on chordamesoderm-derived laminin. To test this idea, we conducted a series of transplantation studies. The zebrafish embryonic shield can induce the formation of a complete secondary axis when transplanted to lateral or ventral regions of an isochronic host embryo (Saude et al., 2000). The chordamesoderm (and therefore the notochord) of this secondary axis is entirely transplant derived, whereas surrounding tissue is host derived. Moreover, donor embryos fully recover from the removal of shield tissue, allowing the genotype of donor tissue to be scored according to the 24 hpf notochord phenotype (Saude et al., 2000). We took advantage of these features of the zebrafish embryo to generate twinned embryos in which the genotypes of the host and donor were different.

When gup or sly mutant shields were transplanted to wild-type hosts, the resulting secondary axes possessed phenotypically normal notochords (Fig. 5A,B). Therefore, laminin β1 and Laminin γ1 can non-autonomously rescue notochord differentiation. Wild-type shield cells transplanted into either gup or sly mutant hosts also form normal notochords (Fig. 5C-G). Hence, functional laminin chains can be supplied to the notochord from either autonomous or non-autonomous sources (Fig. 5B,D,E).

The results presented so far indicate that extracellular laminin is necessary for notochord cell differentiation. Wild-type notochords form a basement membrane and laminins are key components of basement membranes. Considering that immunostaining of laminin 1 is depleted in sly and gup mutants, we decided to examine the status of peri-notochordal basement membranes of sly and gup mutants using electron microscopy.

**Peri-notochordal basement membrane is disrupted in gup and sly mutants**

Basement membranes are complex structures formed by a variety of extracellular matrix proteins and proteoglycans. Laminins are thought to polymerize via the N-terminal domains of each subunit chain to interact with cell surfaces via the C-terminal globular domains of the laminin α chains (Colognato and Yurchenco, 2000; Yurchenco and Cheng, 1993). In addition, laminin matrices are thought to be cross-linked to collagen IV matrices via nidogen (Fox et al., 1991). The expected role of laminin as a linking protein suggests that, in the absence of Laminin, other components of the basement membrane will not be properly organized.

Electron micrographs of transverse sections from 28 hpf and 32 hpf wild-type zebrafish embryos reveal a number of features of the differentiated notochord. A thick basement membrane completely surrounds the notochord (Fig. 6A,C,E,G). In 28 hpf notochords, fibres running both parallel and perpendicular to the plane of section are seen within this basement membrane (Fig. 6E). By 32 hpf the basement membrane possesses many more parallel fibres in a more compact structure (Fig. 6G). Additionally, notochord cells possess large vacuoles that do not readily stain with osmium (Fig. 6A,C). Finally, the cytoplasm of wild-type notochord cells is filled with rough endoplasmic reticulum (RER; Fig. 6G).

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**Fig. 4.** Expression of lamb1 and lamc1. (A,B) Maternal expression of lamb1 (A) and lamc1 (B) is seen in 1000-cell embryos. (C) Maternal laminin 1 protein is also detected. (D-F) Posterior views reveal expression of lamb1 (D) and lamc1 (E) and laminin 1 protein (F) in tailbud stage embryos. (G,H) Flat mounts show lamb1 (G) and lamc1 (H) mRNA expression pattern in 15-somite stage embryos. (I,J) Lateral views of trunk show lamb1 (I) and lamc1 (J) mRNA expression at 22 hpf.
Role of laminin in notochord formation

Notochord cells of gup or sly mutants are dramatically different. As in wild-type embryos a thick layer surrounds the notochord at 28 hpf but no orderly arrangement of fibres is apparent (Fig. 6F); by 32 hpf this layer is largely undetectable (Fig. 6D). In the few places where this layer can still be seen, it remains severely disorganized (Fig. 6H). In addition, gup and sly mutant notochords contain fewer vacuolated cells, and those found have smaller vacuoles than do wild-type notochords (Fig. 6D). By 32 hpf, RER is difficult to spot and where detected, the lumen is much smaller than in wild-type notochord RER (not shown). Finally, by 32 hpf we observed darkly stained, apoptotic cells in and around the notochord (Fig. 6D). Thus, the absence of either gup/lamb1 or sly/lamc1 leads to disorganization and eventual loss of the basement membrane surrounding the notochord.

**DISCUSSION**

To understand how organs develop, it is essential to know how cellular differentiation is controlled. As part of the differentiation program, cells acquire a number of distinct mature properties, including a characteristic morphology, determined by both their intracellular cytoskeleton and the surrounding extracellular matrix. In our efforts to understand formation of the notochord, a simple essential vertebrate organ, we have found that extracellular matrix formation and cellular...
This differentiation event is affected by mutations in several genes. As chordamesoderm matures, it acquires a laminin-rich basement membrane. Subsequently, the transition from chordamesoderm to mature notochord is characterized by the formation of a thick extracellular sheath and the acquisition of large vacuoles. This differentiation event is affected by mutations in several genes, including gup and sly. The defects exhibited by gup and sly mutants can be divided into at least two classes: structural defects, exemplified by the loss of peri-notochordal basement membrane; and differentiation defects, as seen by the persistence of early marker genes. What then is the relationship between the failure to form an extracellular sheath and the failure to differentiate?

Our reciprocal transplantation experiments show that either autonomous or non-autonomous tissues can promote notochord differentiation. We propose that this is because laminin from either side of the mutant-tissue/host-tissue interface can rescue a peri-notochordal signalling apparatus that is lacking when all tissue is mutant. Consistent with this, we observe peri-notochordal laminin staining of wild-type tissue transplanted into mutant hosts. Whether Laminin is a direct component of the signalling apparatus is an unanswered question; however, our preliminary loss of function analysis of several candidate laminin receptors (dystroglycan, α3 integrin, α6 integrin and β1 integrin) has failed to reveal any notochord differentiation phenotype, suggesting that the laminin is not directly involved in the control of notochord differentiation (not shown) (Parsons et al., 2002).

Alternatively, the notochord differentiation signal may not directly involve laminin. In the mutants, loss of the basement membrane as a consequence of the loss of laminin, may lead to loss of a basement membrane associated signal. Electron microscopy of gup and sly mutants indicates that laminin 1 (or other laminin isoforms containing the β1 or γ1 chain) is crucial for the organization of the basement membrane surrounding the notochord. Thus, in the absence of laminin, other important basement membrane components will not be properly organized. These include proteoglycans, known to be mediators of growth factor activity (Costell et al., 1999; Perrimon and Bernfield, 2000). Indeed, both keratan sulfate proteoglycan and aggrekan, two proteoglycans normally associated with chondrogenesis, are localized to the peri-notochordal sheath (Domowicz et al., 1995; Smith and Watt, 1985). It is possible that in the absence of a peri-notochordal basement membrane growth factor signalling dependent on these proteoglycans would be disrupted. Additional insights into the mechanism of signalling from the basement membrane may be gained from studies of other notochord differentiation mutants.

As noted earlier, laminin α5 is important for murine glomerulogenesis (Miner and Li, 2000). Regarding a structural role for the basement membrane, a comparison between the notochord and the mammalian kidney glomerulus can be drawn. The mammalian kidney glomerular basement membrane (GBM) is an important part of the filtration machinery. In this role, it has been suggested that the GBM must withstand high hydrostatic pressures (Timpl, 1996). In lower vertebrates, for the notochord to possess the appropriate shape, stiffness and flexibility, its basement membrane must also withstand the turgor pressure exerted by the vacuolated notochord cells. It will therefore be of interest to determine if the notochord basement membrane shares any of the unique compositional qualities of the GBM.

Finally, we observed increased levels of apoptosis in the notochord and other tissues of the Laminin mutants. There are several possible explanations for this. On one hand, as discussed above, loss of basement membrane and its associated proteoglycans may lead to the loss of growth factors that provide trophic support. On the other hand, the loss of laminin and/or basement membrane may result in the general loss of attachment to the extracellular matrix. Many cell types are known to undergo cell death in response to loss of attachment via a mechanism known as anoikis, which is thought to involve integrin and focal adhesion kinase (FAK) signalling (Frisch and Francis, 1994; Frisch et al., 1996). Activated FAK has been observed in the differentiating notochord of zebrafish (Henry et al., 2001a). It will be of interest to know whether activation of FAK is perturbed in laminin mutants.

With the cloning and analysis of gup and sly we are beginning to understand how laminin is used to control notochord differentiation. The laminin chains encoded by gup and sly are constituents of several distinct laminin isoforms. Using these laminin chain mutants and antisense morpholino oligonucleotides, it should be possible to dissect the roles of various laminin isoforms in distinct developmental processes. For example, what are the roles of these laminins in axon guidance and how do they interact with other extracellular matrix components, such as netrin? And what are their roles during vasculogenesis? In the mouse, a basement membrane is required for implantation; therefore later developmental roles for molecules such as laminin 1 are difficult to study. As there is no requirement for zygotic laminin β1 or γ1 in early zebrafish embryogenesis, the zebrafish is an ideal animal in which to analyse the function of such basement membrane molecules in later processes.

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