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Primary structure, expression and function of laminin α4

by

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1 SUMMARY

Basement membranes (BMs) are thin extracellular sheet-like structures that compartmentalize tissues. They are found beneath epithelia and endothelia, and surrounding individual cells, such as muscle fibers, neurons and adipocytes. Laminins, type IV collagens, perlecan and nidogens are some of the main components of mature basement membranes. Laminins are composed of α, β and γ chains. They are not only major structural elements of basement membranes, but they also serve as cell-matrix linkers and signaling molecules through their interactions with cell surface receptors such as integrins. Laminins are multidomain glycoproteins with five α, four β and three γ chains identified in mammals so far. The chains combine to at least twelve heterotrimeric isoforms that have different tissue distributions and functions. Laminin α4 is recently described laminin chain, the role of which has been unknown. This study was part of a project aimed at characterizing laminin α4 and the laminin-8 isoform containing this polypeptide chain.

The complete primary structure of the mouse laminin α4 chain was derived from cDNA clones. Northern analyses of whole mouse embryo mRNAs revealed weak expression at day 7, but it later increased and peaked at day 15. In adult tissues the strongest expression was observed in lung and, in cardiac and skeletal muscles. Weak expression was also seen in other adult tissues, such as brain, spleen, liver, kidney, and testis. By *in situ* hybridization of fetal and newborn tissues, expression of the laminin α4 chain was mainly localized to mesenchymal cells. Strong expression was seen in the villi and submucosa of the developing intestine, the mesenchymal stroma surrounding the branching lung epithelia, and the external root sheath of vibrissae follicles, as well as in cardiac and skeletal muscle fibers. In the developing kidney, intense but transient expression was associated with the differentiation of epithelial kidney tubules from the nephrogenic mesenchyme. Immunohistological staining localized the laminin α4 chain primarily to lung septa, heart, capillaries, perineurium, and developing skeletal muscle.

To study the properties of laminin-8, recombinant laminin-8 heterotrimer (α4β1γ1) was produced in a mammalian expression system using triply transfected HEK-293 cells. The protein was purified using affinity chromatography aided by a fusion epitope tag followed by ion-exchange chromatography. The purified recombinant laminin-8 was shown to form the expected Y-shaped molecules in rotary shadowing electron microscopy. Recombinant laminin-8 supported cell adhesion, and integrins receptors mediating cell adhesion to laminin-8 were identified using function-blocking monoclonal antibodies. Integrin α6β1 was found to be a major mediator of adhesion of HT-1080 fibrosarcoma cells and cultured capillary endothelial cells to laminin-8. Integrin α6β4 was also able to mediate cell adhesion to

laminin. Antibodies to integrins α1, α2, α3 and α5 did not affect binding. Considering the expression patterns of laminin-8 and integrin α6β1 it is likely that the former is a ligand for the latter *in vivo* as well.

Platelets were found to contain laminin-8, and secrete some of it after stimulation with either thrombin or phorbol ester. Laminin-8 was purified from platelets using monoclonal anti-laminin β1 antibody column, and the identity of the three chains was determined with antibodies and amino-acid sequencing. Platelets adhered to purified platelet laminin-8 and recombinant laminin-8 using integrin α6β1.

To further explore the biological role of the laminin α4 chain, mice carrying inactivated *Lama4* alleles were generated. The laminin α4 null mice presented neonatal hemorrhages and a motorical dysfunction as adults. The hemorrhages were observed especially in the soft tissues of hind limbs, the head, lower back and neck regions, but also in the heart and meningies. As a consequence of the bleedings, the newborn mice suffered from anemia. There were also extensive bleedings and deterioration of microvessel growth in experimental angiogenesis in the cornea. Histological examination of newborn skeletal muscle revealed lack of other laminin chains and delayed deposition of type IV collagen and nidogen into capillary BMs. In contrast, perlecan deposition appeared normal. Electron microscopy showed discontinuities and absence of distinct capillary basement membranes, as compared to wild-type littermates, while other basement membranes appeared normal. In adult null mice, immunostaining for the BM components in muscle capillaries and muscle fibers were identical to those of controls, except for laminin α4. The fact that the vascular phenotype in null mice disappeared during the first weeks of life could be explained by initiation of expression and deposition of laminin-10 (α5β1γ1) into the capillary BMs, which probably then facilitates type IV collagen and nidogen deposition, restoring BM stability and structure. The results demonstrate a central role for the laminin α4 chain during microvessel growth and, in the absence of other laminin α chains, in the assembly of the type IV collagen network.

2 ABBREVIATIONS

α-DG	α-dystroglycan
BM	basement membrane
CMD	congenital muscular dystrophy
EGF	epidermal growth factor
EHS	Engelbreth-Holm-Swarm
ER	endoplasmic reticulum
GAG	glycosaminoglycan
N-linked	asparagine linked
VEGF	vascular endothelial growth factor

3 LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:

I Iivanainen, A., Kortesmaa, J., Sahlberg, C., Morita, T., Bergmann, U., Thesleff, I. and Tryggvason, K. (1997) Primary structure, developmental expression and immunolocalization of the murine laminin α4 chain. *J Biol Chem.* 272:27862-27868.

II Geberhiwot, T., Ingerpuu, S., Pedraza, C., Neira, M., Lehto, U., Virtanen, I., Kortesmaa, J., Tryggvason, K., Engvall, E. and Patarroyo, M. (1999) Blood platelets contain, secrete and adhere to laminin-8 (α4β1γ1) via α6β1 integrin. *Exp Cell Res.* 253:723-732

III Kortesmaa, J., Yurchenco, P. and Tryggvason, K. (2000) Recombinant laminin-8 (α4β1γ1): production, purification and interactions with integrins. *J Biol Chem.* 275:14853-14859.

IV Thyboll, J.*, Kortesmaa, J.*, Cao, R., Soininen, R., Wang, L., Iivanainen, A., Sorokin, L., Risling, M., Cao, Y. and Tryggvason, K. (2000) Absence of the laminin α4 chain disturbs endothelial cell basement membrane assembly leading to defective angiogenesis. Submitted.
*equal contribution

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*equal contribution

4 CONTENTS

5 INTRODUCTION

The first section is a review of the literature in the field of this study. Searching Medline with "laminin" produces over 10000 hits, and in almost 3000 articles the word "laminin" appears in the title, showing the enormous expansion of the field since the discovery of the first laminin in 1979 (Chung *et al.*, 1979; Timpl *et al.*, 1979).

Throughout this thesis, the established laminin nomenclature (Burgeson *et al.*, 1994) is used. A reader not already familiar with it should consult chapter 6.2.7 before continuing. The review section is divided into several chapters. First, a brief overview of what basement membranes are is presented to give context to the following chapters (6.1). Next, the overall laminin domain structure and different laminin isoforms are discussed (6.2.1). Laminin genes were not studied in this work, but are briefly discussed in chapter 6.2.2. Relevant for the present studies, laminin biosynthesis, interactions of laminins with other molecules and the role of laminin in basement membrane assembly are discussed (chapter 6.2.3). Finally, an overview of the complex patterns of laminin expression (6.2.4) and lessons learned on biological functions of laminin are presented (6.2.5).

I have chosen to refer to published parts of this work also in the review section, but the main discussion on the results is presented in a separate section (8).

6 REVIEW OF THE LITERATURE

6.1 BASEMENT MEMBRANES

Basement membranes (BMs, also known as basal laminae) are flexible, thin formations of extracellular matrix that lie underneath epithelial and endothelial cell layers and surround certain individual cells, e.g. muscle cells, fat cells and Schwann cells. In most cases, the basement membrane separates these cells from the underlying or surrounding connective tissue, but in some special locations, such as the lung alveolus and kidney glomerulus, it is located between two sheets of cells, where it acts as a size-selective filter. In addition to "simple" structural and filtering roles, basement membranes contribute to determination of cell polarity and induction of cell differentiation, influence cell metabolism, organize the proteins in adjacent plasma membranes and serve as substratum for cell migration. (Timpl, 1996b; Timpl and Brown, 1996)

Although the composition of basement membranes varies from tissue to tissue, certain proteins are usually present. Laminin is present in all known basement membranes, and it appears that laminin is required for the assembly of other basement membrane components (chapter 6.2.3). Most mature membranes contain a type IV collagen network, heparan sulfate core protein perlecan and nidogen (entactin), which is believed to serve as a bridge joining the independent networks of laminin and type IV collagen. A multitude of other, tissue-specific proteins and isoforms are also present in basement membranes as their many complex functions suggest (Timpl, 1996b; Timpl and Brown, 1996). A current model of a basement membrane is illustrated in figure 1.

6.2 INTRODUCTION TO LAMININS

Laminins are large (500-800 kDa) glycoproteins formed of three polypeptide chains of different types termed α, β and γ. The subunit chains associate by forming a triple-helical coiled-coil, producing a heterotrimeric molecule with up to three short arms and a long rod-like structure. Laminins are major structural components of basement membranes, but are also found in other locations (Colognato and Yurchenco, 2000; Ekblom, 1996). In recent years, laminins have been found in non-BM locations such as certain epithelia (Koch *et al.*, 1999) and intracellularly in monocytes, lymphocytes and platelets (Geberhiwot, 2000; Pedraza, 2000; II). In addition to structural roles, laminins function also as signaling molecules that regulate the cells that they contact. Cell proliferation, migration and

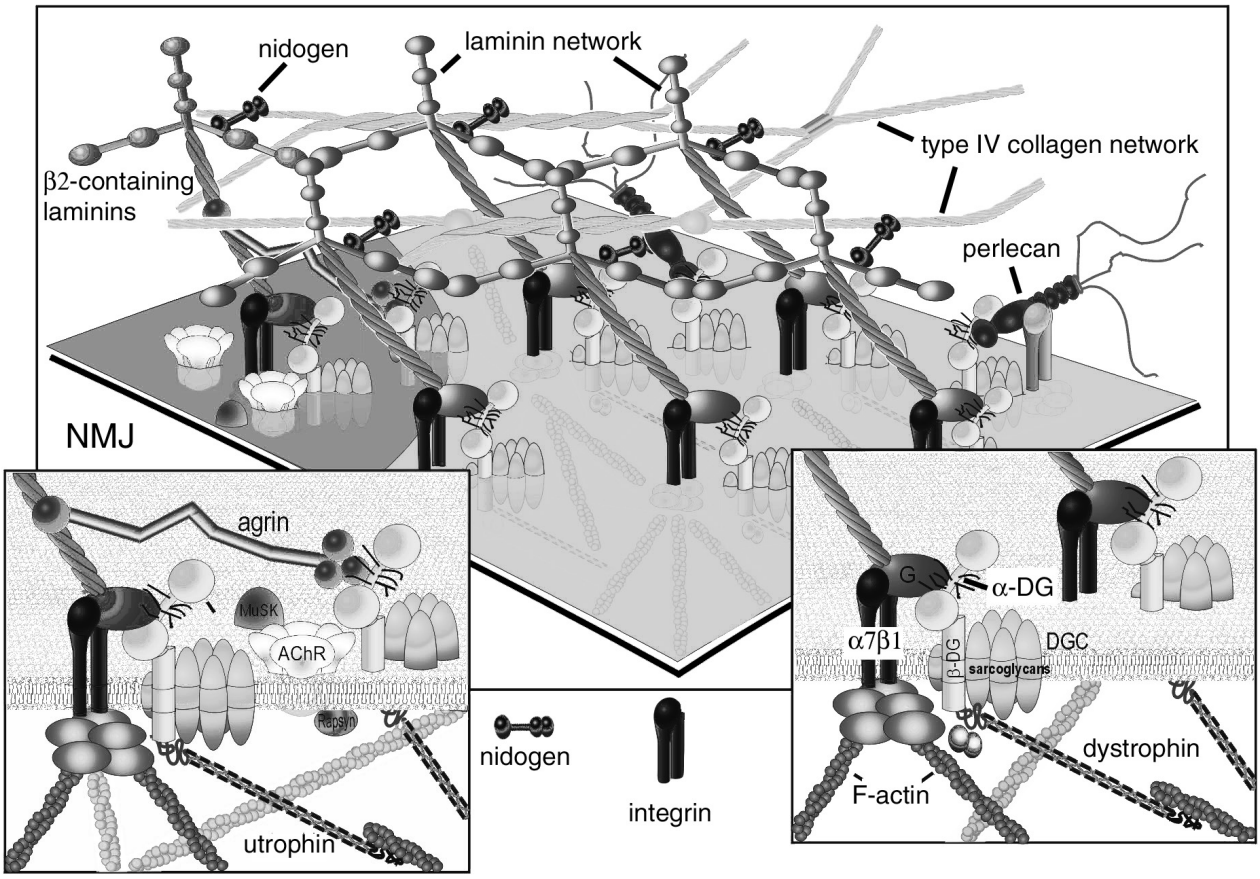

Figure 1. Current model of basement membrane structure. The laminin network is bound to the cell surface by α-dystroglycan and integrins, and nidogen links the type IV collagen network to laminin. This model represents muscle BM, but it is applicable to other BMs as well. **Left**, features specific for neuromusclular junctions (NMJ) are shown. **Right**, features of extrasynaptic muscle BM. Modified from image kindly provided by Peter Yurchenco.

differentiation are all modulated in part by laminins. As many other ECM proteins, laminins are composed of many structurally and often functionally autonomous domains. These domains often occur as modular units in several different proteins, including ones of non-ECM origin.

6.2.1 WHAT DO LAMININS LOOK LIKE: LAMININ STRUCTURE

6.2.1.1 GENERAL DOMAIN STRUCTURE

Laminins are heterotrimeric molecules, the chains of which are associated by an α-helical coiled-coil. All laminin chains share a degree of structural similarity, including globular domains, EGF-like laminin-repeats (LE-modules) and the domains forming the α-helical coiled-coil in the long arm. The α-chains are defined by a large globular domain (G-domain)

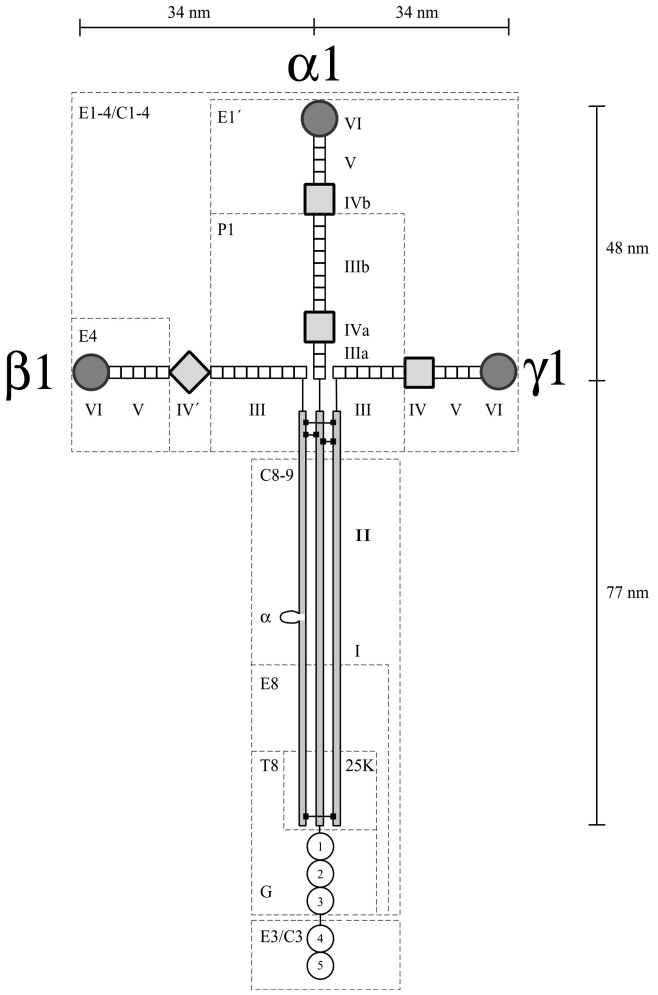

Figure 2. General domain structure and proteolytic fragments of laminin-1 (adapted from Maurer and Engel 1996 and Airenne, 2000). Domains are designated with Roman numbers according to Sasaki *et al.* 1988. Domains with internal homology or homology between chains are shown with identical symbols. The locations of proteolytic fragments E1-4/C1-4, E1', P1, E4, C8-9, E8, T8, 25K and E3/C3 are indicated. Disulfide bridges connecting the chains are shown as black squares connected by lines.

at their C-terminal. The β-chains have a characteristic small interruption in the coiled-coil domain, somewhat confusingly termed "α" (named prior to the current nomenclature). The general domain structure of laminins is presented in figure 2. Proteolytic fragments of laminin have been widely used to map functions to defined parts of the molecule, and the most common fragments are also indicated in figure 2. Domains can be designated by roman numerals according to (Sasaki *et al.*, 1988), or named by Swiss-Prot database convention (Bairoch, 1998)

The triple-helical coiled-coil long arm region is a defining feature of the laminins. It is formed by association of the α-helical domains (named I and II) of the three constituent

chains, which have heptad repeats $(abcdefg)_n$ typical to coiled coil structures. The repeats share little sequence homology, but have hydrophobic or non-polar amino acids preferentially located in positions a and d, charged residues in e and g and polar residues in b, c and f (Beck *et al.*, 1990). In essence, the hydrophobic residues are located on a strip along the α-helix coil, and when the coils associate, the hydrophobic strips form a hydrophobic core for the coiled-coil structure. Hydrophilic amino acids, in contrast, reside on the outside in a more aqueous environment. Ionic interactions or hydrogen bonds between residues on the outside stabilize the structure further. These ionic interactions, and those between residues embedded in the hydrophobic core, are likely to determine the specificity of chain assembly (Beck *et al.*, 1993).

The globular LN-domains located at the ends of the short arms are involved in polymerization (Yurchenco and Cheng, 1993). Little is known about the LF and L4-domains, which have no known function mapped to them so far. Major portions of the short arms are composed of small units known as EGF-like laminin repeats (LE), although their structure is only remotely similar to EGF. The LE-modules are small, compact units with a characteristic 8-cysteine pattern readily recognizable from the amino-acid sequence. A number of these modules are lined up in head-to-tail fashion in the short arm domains V and III. One of these modules in the γ1-chain hosts the nidogen binding site (Gerl *et al.*, 1991; Poschl *et al.*, 1994), but otherwise no specific functions have been mapped to the LE repeats. The crystal and solution structures of three consecutive γ1-chain LE-modules (III3-5) including the nidogen-binding site have been determined (Baumgartner *et al.*, 1996; Stetefeld *et al.*, 1996).

The G-domain present at the C-terminal part of the α-chains consists of five repeating modules called LG1-LG5. Similar motifs are also found in non-laminin proteins such as perlecan (Kallunki and Tryggvason, 1992; Noonan *et al.*, 1988), agrin (Rupp *et al.*, 1992) neurexins (Ushkaryov *et al.*, 1992), *Drosophila crumbs* protein and the sex-hormone binding globulin (Joseph and Baker, 1992). In the laminin α-chains, the modules LG1-LG3 are connected to the LG4-LG5 modules by a linker domain. Otherwise the LG-modules are separated only by a few residues. The linker could be sensitive to proteolysis, and this has been shown to be the case for at least the α2- and α4-chains (Talts *et al.*, 1998; Talts *et al.*, 2000). Crystal structures of LG-domain repeats have recently been determined (Hohenester *et al.*, 1999; Tisi *et al.*, 2000), and although the crystal structure of the whole G-domain has not been determined, a hypothetical model has been presented (Timpl *et al.*, 2000). Many binding sites for cell-surface receptors are located in the G-domain.

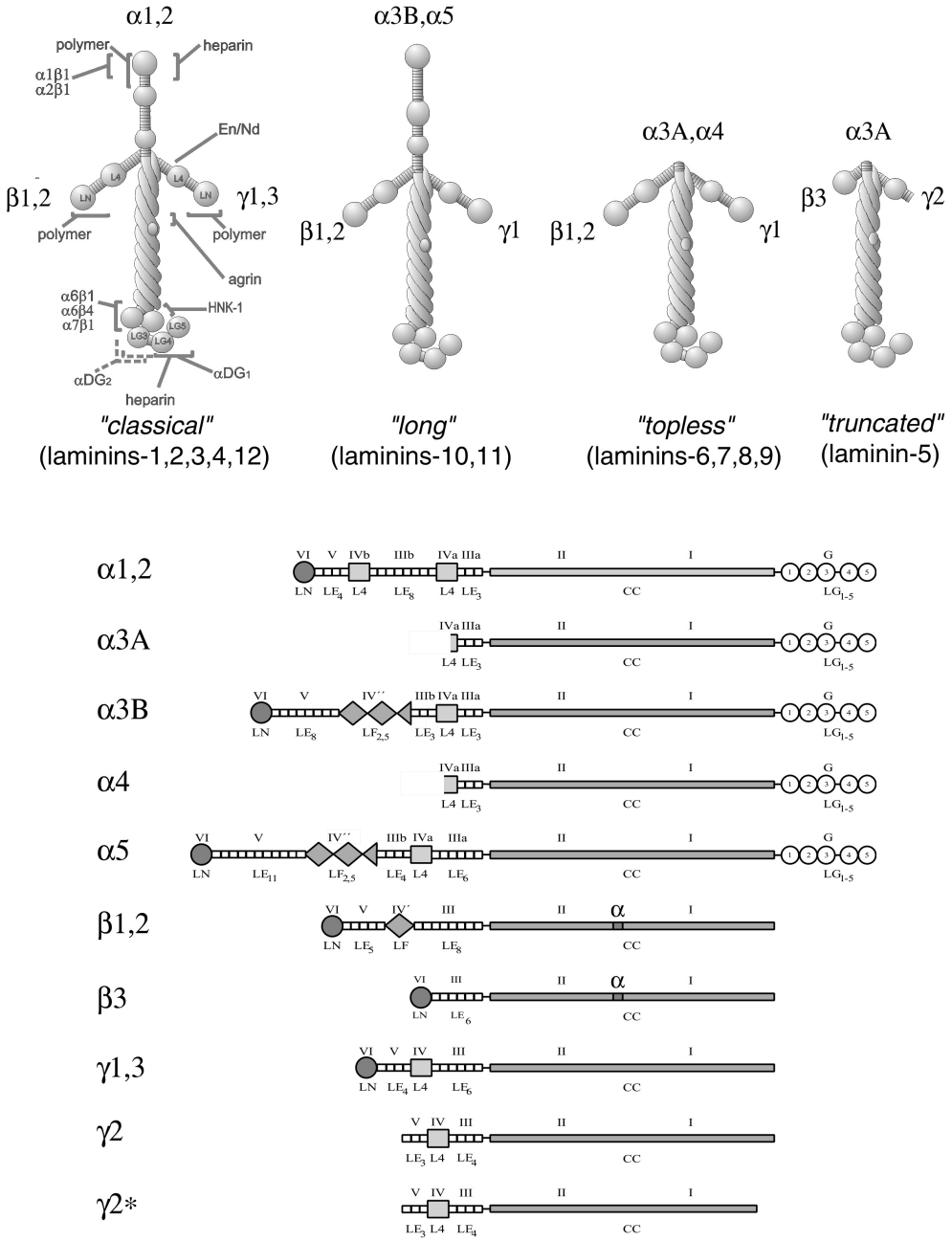

Figure 3. ***Upper,*** **Laminin heterotrimer structures.** Some binding sites that have been mapped to specific part of the molecule are shown. Adapted form Colognato and Yurchenco, 2000. (Data is mostly for laminin-1.) ***Lower,*** **Domain structures of laminin chains.** Structurally conserved or homologous domains are indicated by identical symbols. Numbering is as in figure 2, and letter codes (LN, LE, LF, L4, CC, LG) are according to Swiss-Prot database convention (Bairoch 1998). Modified from Airenne, 2000.

6.2.1.2 LAMININ ISOFORMS

As complex regulatory functions suggest, laminins exist in multiple isoforms with specific tissue- and developmental distributions. Thus far, the amino-acid sequences of twelve genetically distinct mammalian laminin chains (α1-5, β1-4, γ1-3) have been reported (for references, see chapter 6.2.7). A defining factor for the laminins is the presence of the coiled-coil region, which is common to all isoforms. The isoforms differ most strikingly with respect to their short arm domain structure (figure 3.) The first laminin to be discovered was laminin-1, which was isolated from a mouse sarcoma (Engelbreht-Holm-Swarm, EHS-tumor) (Timpl *et al.*, 1979). Due to its origin, it is also referred to as "EHS-laminin". For historical reasons, laminins with domain structure similar to that of laminin-1 are referred to as "classical" laminins, while the isoforms lacking short-arm domains are called "truncated". Laminins α3B and α5 are, in fact, larger than the original α1, and are thus "long" chains.

Laminins are also found in invertebrates, indicating that they arose early in evolution. Complete genomic sequences of the fly *Drosophila melanogaster* and the nematode *Caenorhabditis elegans* were recently determined, and both organisms have laminins with very similar domain structure to that of the vertebrate homologs. Both have two different α-chains, one "classical" (α1/2 like) and one "long" (α3B/α5-like). The β and γ-type chains are full-sized. Neither *C. elegans* nor *Drosophila* appear to possess any "truncated" laminins, which suggests that they have evolved later for a function specific for vertebrates (Hutter *et al.*, 2000). The presence of laminins in even more primitive lifeforms such as polyp *Hydra vulgaris* (Sarras *et al.*, 1994) and leech (Chiquet *et al.*, 1988) underlines the ancient origin of laminins.

6.2.1.3 HOW LAMININS ARE MADE: LAMININ BIOSYNTHESIS

Laminin chains are, as most other secreted proteins, transported into the endoplasmic reticulum (ER) as they are being synthesized. Laminin trimer assembly seems to occur exclusively intracellularly in the ER before secretion. It has been shown, using transfected cells, that simultaneous expression of all three chains of a heterotrimer is required for secretion of β1 and γ1-chains. The α1-chain can be secreted to a certain extent as a monomer but much more efficiently as part of a trimer. None of the various potential homodimers, trimers with two identical chains and other combinations, were secreted (Yurchenco *et al.*, 1997). Likewise, laminin γ1 null embryoid bodies failed to secrete laminin (Smyth *et al.*, 1999). It appears likely that the same scenario is true for other isoforms as well. Except for the removal of N-terminal signal peptides, no proteolytic processing prior to transport to the

extracellular space, has been shown to occur. Laminins are heavily glycosylated but the biological significance of the glycosylation is largely unknown. N-linked glycosylation occurs in the ER and the Golgi before laminin secretion (Cooper *et al.*, 1981; Peters *et al.*, 1985).

Fully assembled heterotrimers are furthermore stabilized by interchain disulfide bridges located in long arm at the N-terminal end, at least in most laminin isoforms (Beck *et al.*, 1990; Hunter *et al.*, 1990; Hunter *et al.*, 1992) (see Fig. 2). Platelets contain laminin-8, in which β1 and γ1 chains are disulfide bonded, but some of the α4-chain is non-covalently associated (II).

Laminins are large molecules that may contain many sites of interaction not exposed in the intact structure. Processing by proteases often results in fragments with properties radically different from the intact molecule. Various biological activities have been assigned to laminin fragments and peptides, that are not observed in the native molecule. Although the potential biological significance of the cryptic epitopes is controversial, they cannot be dismissed altogether. A parallel might be drawn to endostatin, a proteolytic fragment of type XVIII collagen, which is a highly potent inhibitor of angiogenesis (O'Reilly *et al.*, 1997). Some laminin chains are proteolytically cleaved *in vivo* after secretion. There is data indicating processing of most laminin isoforms, except laminin-1 (Leivo *et al.*, 1989). The G-domain of the α-chains contain a "linker" region between G-subdomains LG3 and LG4, which may be sensitive to proteolysis (discussed under "Laminin structure"), although the extent of proteolytic processing in various tissues and physiological states has not been studied conclusively. To date, proteolytic cleavage of laminins has been shown to have biological significance only in the case of laminin-5, so that cleavage of laminin-5 by MMP-2 reveals a cryptic site promoting keratinocyte migration (Giannelli *et al.*, 1997; Goldfinger *et al.*, 1998).

6.2.2 LAMININ GENES

Laminin genes are large and complex with many exons. Chromosomal locations of all known laminin genes in humans have been determined and are also known in some other organisms (see table in 6.2.7). The genes for laminins α2 and α4, and γ1 and γ2, respectively, are located close to each other both in mouse and man, but if there is a functional reason for this is unknown.

Complete gene structures have been determined for most chains, and the rest will probably soon follow, as the human genome sequencing is completed. At present, structures of LAMA2 (Zhang *et al.*, 1996), LAMA4 (Richards *et al.*, 1997), LAMB1 (Vuolteenaho *et al.*,

1990), LAMB2 (Durkin *et al.*, 1996), LAMB3 (Pulkkinen *et al.*, 1995), LAMC1 (Kallunki *et al.*, 1991) and LAMC2 (Airenne *et al.*, 1996) are known. It seems that the 5' ends of many laminin chain cDNAs have been unusually difficult to clone and sequence, which might depend on special features of mRNA or DNA secondary structure, or on the presence of sequences that are difficult to propagate in *E. Coli*. It will be interesting to see how the large-scale genomic sequencing efforts will be able to tackle the corresponding genomic sequences.

There are several documented splice variants of laminin chains. The two laminin α3 chain variants, α3A and α3B, arise from the use of different promoters (Ferrigno *et al.*, 1997), while the γ2* cDNA is formed by using only exon 22 and part of the following intron, leaving the last exon 23 unused (Airenne *et al.*, 1996). A very short (1 kb) variant was reported for laminin α4, named LAMA4* (Xiao *et al.*, 1997). This transcript consists of an incomplete LE-repeat and some additional unique sequence coding for a total of 129 aa. The splice variant was most highly expressed in the heart. The human genomic sequence covering the area has been published, and the exons corresponding to this transcript are present. We made an effort to clone the transcript from mouse but were unsuccessful (J. Jenner and J. Kortesmaa, unpublished). Its function, if any, is difficult to predict and is likely to be different from other laminin isoforms.

6.2.3 LAMININS IN THEIR SOCIAL CONTEXT: INTERACTIONS

Many different cell surface receptors bind laminins, performing both structural and signaling functions. In addition, laminins also interact with many other extracellular matrix molecules, often simultaneously and while being bound to receptors. The integrin and non-integrin receptors involved probably vary during tissue remodelling and development, as well as under pathological conditions. The multiple binding sites in laminins (see figure 3) might have one or more conformational modes which affect the interaction with the receptor. The spatial location and orientation of laminins in supramolecular assemblies could also affect the availability of certain sites for cellular binding *in vivo*. (Aumailley, 1996)

Due to the complexity of the system studied, it can be difficult to demonstrate the biological relevancy of an observed interaction. One good example is laminin interaction with glycosaminoglycans (GAGs). Laminin can bind heparin (Sakashita *et al.*, 1980), and also other GAGs such as heparan sulfate, dermatan sulfate and chondroitin sulfate (Del Rosso *et al.*, 1981; Fujiwara *et al.*, 1984). The major heparin binding activity is located in the G-domain, but the N-terminal domains of α1 and α2 also bind heparin (Colognato *et al.*, 1997). In practice most, if not all proteins with GAG chains, would interact with laminin in an *in vitro* binding assay. Whether all these interactions really occur *in vivo* is much harder to

show. Thus, in the following, only the most established interactions and those most relevant to this work are discussed.

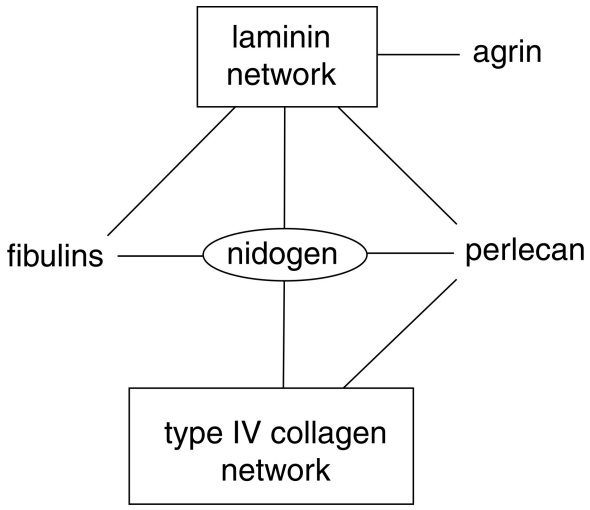

Figure 4. Interactions between BM components. The networks of type IV collagen and laminin are connected by nidogen. A central role for nidogen in BM assembly is apparent.

6.2.3.1 LAMININ RECEPTORS

6.2.3.1.1 INTEGRINS

Integrins are cell surface receptors that mediate many adhesive cell-matrix and cell-cell interactions. They are heterodimers of an α (19 variants in GenBank) and a β (8 variants in GenBank) subunit, forming more than 20 combinations. Integrins not only mediate cell adhesion, but they also transduce signals via activation of kinases such as focal adhesion kinase FAK and mitogen-activated protein (MAP) kinase (Cary and Guan, 1999; Giancotti and Ruoslahti, 1999). Some cases where intracellular signals induce a change in ligand-binding affinity ("inside-out signaling"), have been described (Hughes and Pfaff, 1998). Some integrins have only one or a few known ligands, whereas some appear to be very promiscuous. Binding is generally of low affinity, and both the adhesion and signaling functions depend on the binding of multiple receptors simultaneously.

A large variety of integrins has been implicated as receptors for different laminin isoforms (Aumailley, 1996). These include α1β1, α2β1, α3β1, α6β1, α6β4, α7β1, α9β1, αvβ3, albeit with varying affinities (see table I). The most laminin-specific integrin appears to be α6β1, which binds all laminin isoforms tested to date. The only other ligand described is sperm cell ADAM 2 (Almeida *et al.*, 1995; Chen *et al.*, 1999a; Chen *et al.*, 1999b). There seems to be

some difference between binding properties of different isoforms. Integrins α1β1 and α2β1 bind to the N-terminal end of laminin α2-chain, which the truncated isoforms lack (Colognato *et al.*, 1997), but it is too early to say if none of the truncated isoforms will bind to α1β1 or α2β1. In contrast, integrins α6β1, α6β4 and α7β1 bind to the C-terminal part which all the isoforms share (see figure 3.)

Table I: Laminin interactions with integrins.

Laminin isoform	*Integrin*	*References*
laminin-1	α1β1	(Ignatius and Reichardt, 1988; Tomaselli *et al.*, 1988)
	α2β1	(Languino *et al.*, 1989)
	α3β1	(Wayner and Carter, 1987)
	α6β1	(Sonnenberg *et al.*, 1988)
	α6β4	(Lotz *et al.*, 1990)
	α7β1	(Kramer *et al.*, 1989)
	α9β1	(Forsberg *et al.*, 1994)
	αvβ3	(Sonnenberg *et al.*, 1990)
laminin-2/4	α1β1, α2β1	(Colognato *et al.*, 1997)
	α3β1	(Delwel *et al.*, 1994)
	α6β1	(Delwel *et al.*, 1993)
	α7β1	(Yao *et al.*, 1996)
laminin-5	α3β1	(Carter *et al.*, 1991; Delwel *et al.*, 1994)
	α6β1, α6β4	(Delwel *et al.*, 1993; Niessen *et al.*, 1994)
	not α7β1	(Yao *et al.*, 1996)
laminin-8	α6β1, α6β4	(II, III)
laminin-10/11	α3β1, α6β1, α6β4, but not α2β1	(Kikkawa *et al.*, 2000; Kikkawa *et al.*, 1998).

Laminin-2/4 is a ligand for integrins α1β1, α2β1, α3β1, α6β1 and α7β1. Laminin-5 has been shown to be a ligand for integrins α3β1, α6β1 and α6β4, but not α7β1. The receptors binding to laminin-10/11 appear to vary from cell line to cell line. Laminin-10/11 was shown to be a ligand for integrins α3β1, α6β1 and α6β4, but not α2β1. (For references, see table I). In another study, function blocking antibodies against integrin α3, α6 and β1 subunits and α-dystroglycan had no effect on adhesion of epithelial cells to laminin-10/11 (Ferletta and Ekblom, 1999). In a yet another study, mouse multipotent hematopoietic FDCP-mix cells were shown to bind laminin-10/11 using integrin α6β1 (Gu *et al.*, 1999).

6.2.3.1.2 α-DYSTROGLYCAN

α-Dystroglycan (α-DG) is the best characterized of non-integrin laminin receptors. It was originally identified in skeletal muscle as the extracellular component of the dystrophin-dystroglycan transmembrane complex. In skeletal muscle, α-DG interaction with laminin α2 is thought to link the contractile cytoskeleton to the extracellular matrix (Hemler, 1999;

Henry and Campbell, 1996; Henry and Campbell, 1999). While α-DG was originally described in skeletal muscle, it is found in other tissues as well (Durbeej *et al.*, 1998; Ibraghimov-Beskrovnaya *et al.*, 1992; Ibraghimov-Beskrovnaya *et al.*, 1993). It was recently shown to be a receptor for laminin-1 in bovine aorta endothelial cells (Shimizu *et al.*, 1999).

α-DG is dumbbell-shaped with mucin-like carbohydrate moieties located in the central part. There is tissue specific variation in glycosylation. Deglycosylation abolishes laminin-binding activity in vitro, and laminin binding is subject to inhibition by various GAGs, such as heparin, with differences in sensitivity between laminin isoforms, suggesting a crucial role for the carbohydrates in the interaction. (Henry and Campbell, 1996) The binding site for α-DG is located in the G-domain. Apparently, the specific binding sites within the G-domain differ between different laminins; the α1LG4 is sufficient for binding (Andac *et al.*, 1999), while tandem repeats of α2-chain LG modules (α2LG1-3 and α2LG4-5) are required for strong binding (Talts *et al.*, 1999; Talts and Timpl, 1999). Corresponding modules from laminin α4-chain showed only marginal α-DG binding (Talts *et al.*, 2000). Site-directed mutagenesis experiments demonstrated that certain basic residues in α1LG4, some of which are also involved in heparin-binding, are required for α-DG binding (Andac *et al.*, 1999). Recent determination of G-domain structure has provided a molecular basis for α-DG binding. The binding involves coordination of a charged carbohydrate moiety of α-DG with a Ca^{2+}-ion bound to the LG-module (Hohenester *et al.*, 1999).

Indirect evidence suggests that laminin-8 might bind α-DG. Laminin purified from laminin α2-deficient dystrophic muscle binds α-DG in a manner that is sensitive to inhibition by heparin. In contrast, laminin from normal muscle binds α-DG even in the presence of heparin (McDearmon *et al.*, 1998). Since upregulation of laminin α4 has been observed in laminin α2-deficient muscular dystrophy (Patton *et al.*, 1997; Ringelmann *et al.*, 1999), laminin-8 is probably the main laminin in α2-deficient muscle. Thus, it would not seem unreasonable to assume that laminin α4 is involved in the interaction observed. On the other hand, (Talts *et al.*, 2000) recently reported that recombinant LG-module repeats from laminin α4 bound poorly to α-DG. Perhaps, even poor binding was strong enough to be detected in the study of (McDearmon *et al.*, 1998).

6.2.3.1.3 OTHER RECEPTORS

A glycolipid carbohydrate called HNK-1, found in several receptors in the nervous and immune systems, has been shown to bind the laminin-1 LG2 subdomain (Hall *et al.*, 1997a; Hall *et al.*, 1997b). Immunoglobulin-superfamily receptor B-CAM and its splice variant

Lutheran antigen bind laminin as well, and might function as laminin receptors especially in red blood cells and hematopoietic cells. B-CAM and Lutheran are also overexpessed in some tumor cells (El Nemer *et al.*, 1998; Udani *et al.*, 1998; Zen *et al.*, 1999). A transmembrane tyrosine phosphatase LAR (leukocyte-antigen-related) was recently shown to function as a receptor for laminin-1/nidogen complex (O'Grady *et al.*, 1998). Binding to laminin via LAR induced the formation of long cellular processes, suggesting a signaling function.

Other molecules proposed as laminin receptors include galactosyltransferase (Runyan *et al.*, 1986), galactosidases (Hinek *et al.*, 1993) and the 67kDa laminin receptor/elastin binding protein (Mecham *et al.*, 1989; Rao *et al.*, 1983).

6.2.3.2 TYPE IV COLLAGEN AND OTHER COLLAGENS

Type IV collagen forms a network, which is a major structural element of BMs. It is thought that the type IV collagen network is more rigid and mechanically stronger than the laminin network. Collagens are triple-helical and composed of three polypeptide chains, and type IV is no exception. There are six different type IV collagen chains numbered α1 to α6, coded by distinct genes. In most BMs, α1 and α2 are the predominant ones, while other components are found in more specialized locations such as the glomerular basement membrane (GBM) in the kidney (Hudson *et al.*, 1993; Kuhn, 1995).

Remarkably, laminins do not appear to bind directly to type IV collagens (Timpl, 1996a). Instead, several other proteins such as nidogen and perlecan (discussed below) that are able to bind both laminin and type IV collagen simultaneously, are thought to act as bridging mediators. Laminin-5, but apparently not other laminin isoforms, binds tightly to type VII collagen (Rousselle *et al.*, 1997).

Type XVIII collagen, the second most abundant basement membrane collagen, was recently described being a heparan sulfate proteoglycan (Halfter *et al.*, 1998). Interaction between the collagen XVIII fragment endostatin and laminin-1 has been reported (Sasaki *et al.*, 1998b).

6.2.3.3 NIDOGENS

Nidogen-1 (also known as entactin (Carlin *et al.*, 1981)) is a 150 kDa glycoprotein composed of three globular domains (G1-G3) (Fox *et al.*, 1991; Timpl *et al.*, 1983). Domains G1 and G2 are connected by a flexible link, while a rod domain consisting of EGF-repeats connects G2 and G3. Binding sites for type IV collagen, perlecan and fibulin-1 are located in the G2

domain, while G3 harbors sites for laminin and fibulin-2. Nidogen-2, is a recently described 200 kDa isoform that shares many properties with nidogen-1, such as shape and binding to perlecan and type IV collagen. Laminin-binding was found to be weaker compared to nidogen-1, and fibulin-binding was not detected (Kohfeldt *et al.*, 1998). Both nidogens are widely expressed, but there are differences in expression levels in different tissues (Kimura *et al.*, 1998; Kohfeldt *et al.*, 1998).

Nidogens are ubiquitous components of BMs. Due to their capacity to bind multiple BM components simultaneously, nidogens are thought to act as linkers connecting the different BM components together. Certain cell lines are able to adhere directly to purified nidogens as well (Kohfeldt *et al.*, 1998). The classical nidogen binding site in laminins is located in a LE-repeat in domain III of the γ1-chain (Gerl *et al.*, 1991; Poschl *et al.*, 1994). The nidogen-binding site appears to be present in the recently described γ3-chain (Iivanainen *et al.*, 1999; Koch *et al.*, 1999), and one would thus predict nidogen binding to laminin γ3 as well. In contrast, nidogen does not bind to the laminin γ2-chain suggesting that laminin-5 is the only laminin isoform that does not bind nidogen (Mayer *et al.*, 1995).

6.2.3.4 PERLECAN AND AGRIN

Perlecan (heparan sulfate proteoglycan) is a very large multidomain glycoprotein that is found in virtually all basement membranes, but it is also found in other locations, such as cartilage. The protein has an elongated shape with five globular domains (I-V). The main three heparan sulfate side chains are attached to SGD sequences in domain I at the N-terminus. The other domains have binding sites for type IV collagen and nidogen. Binding to laminin is thought to be mediated mainly via nidogen. (Iozzo *et al.*, 1994) The heparan sulfate side chains, which the laminin G-domains have binding sites for, may also mediate interaction. Fibulin-2 binds to perlecan domains IV and V whereas fibulin-1 does not (Brown *et al.*, 1997; Hopf *et al.*, 1999).

Agrin is another major heparan sulfate proteoglycan which has a crucial function in acetylcholin receptor clustering in the neuromuscular junction (Gautam *et al.*, 1996). It is also expressed in many other locations, such as brain, GBM and heart muscle, but its function in these locations is less well known (Gesemann *et al.*, 1998; Gesemann *et al.*, 1995). Furthermore, there are several tissue-specific splice variants of agrin (Erickson and Couchman, 2000; Ruegg *et al.*, 1992). Although agrin can interact with laminin via its GAG chains, it also has a specific binding site in the coiled-coil region (Denzer *et al.*, 1998; Kammerer *et al.*, 1999).

6.2.3.5 FIBULINS

Fibulins are a novel group of extracellular matrix proteins with five identified members (fibulins-1 to 5), characterized structurally by a rod-like tandem array of EGF-like motifs flanked by globular domains. Fibulin-1 has four splice-variants (A-D) which differ in their C-terminus and have different binding properties (Pan *et al.*, 1993a; Pan *et al.*, 1993b; Sasaki *et al.*, 1995; Zhang *et al.*, 1994). Fibulin-2 differs from fibulin-1 by having an N-terminal rod domain and by its ability to form homodimers (Sasaki *et al.*, 1995; Sasaki *et al.*, 1997). Fibulins-3 and 4 are most similar to each other and least similar to fibulin-2 (Giltay *et al.*, 1999). Fibulin-5 has only recently been identified and has not been characterized (Kowal *et al.*, 1999).

Fibulins are found in basement membranes and microfibrils connecting the BM to the rest of the ECM, but so far their functions are poorly known. Data for binding properties is available so far for fibulins-1 and 2, and they appear have a wide and complex binding repertoire. Fibulins-1 and 2 bind to nidogen-1 but poorly to nidogen-2 (Kohfeldt *et al.*, 1998; Sasaki *et al.*, 1995). Both fibulins bind directly to laminin-1 and fibulin-2 also to laminin-5 (Pan *et al.*, 1993a; Utani *et al.*, 1997).

6.2.3.6 LAMININS AND BASEMENT MEMBRANE ASSEMBLY

As briefly mentioned above, certain laminins are able to self-polymerize into networks, which are major structural elements of basement membranes. The assembly occurs by association of the N-terminal globular domains (VI-domains) at the ends of the short arms (Fig. 5) (Paulsson, 1988; Yurchenco and Cheng, 1993). Laminin-5 lacks these domains completely, and consequently it is unable to polymerize. Other isoforms, lacking only one of the three VI-domains, are able at least to co-polymerize into networks formed of non-truncated laminin (Cheng *et al.*, 1997). It is not entirely clear how these isoforms are associated to one another in the BM assembly, but apparently, they can crucially contribute to BM formation, as illustrated in paper IV. Laminin-5 is a special case in that it can be covalently linked to laminins-6 and -7 (Champliaud *et al.*, 1996).

Laminin-1 can self-polymerize in the absence of other proteins *in vitro* at a critical concentration in the order of 70-140 nM (Colognato and Yurchenco, 2000; Yurchenco *et al.*, 1985). The polymerization requires calcium and occurs at high rather than low temperatures, and is readily reversible (Yurchenco and Cheng, 1993). It is unlikely that concentrations required for polymerization are found *in vivo* without some specific concentrating mechanism, but the nature of this mechanism is not conclusively established. Laminin binding to lipid bilayers resulted in enhanced polymerization (Kalb and Engel, 1991). One recent report described concentration-independent laminin polymerization in acidic conditions, and proposed that low local pH near cell membranes could promote laminin polymerization (Freire and Coelho-Sampaio, 2000). Laminin polymerization and BM assembly are disturbed when laminin receptors such as α-DG or β1 integrins are inactivated by gene targeting (Bloch *et al.*, 1997; DiPersio *et al.*, 1997; Henry and Campbell, 1998; Sasaki *et al.*, 1998a), and it has been proposed that laminin polymerization *in vivo* is a receptor-mediated process. Cell surface receptors would bind and locally concentrate the laminin molecules and thus facilitate controlled polymerization at the cell membrane (Colognato *et al.*, 1999; Colognato and Yurchenco, 2000).

There is genetic evidence indicating that laminin is required for BM assembly. Embryos and embryoid bodies devoid of laminin, due to inactivation of the γ1 chain gene, do not form proper BMs (Smyth *et al.*, 1999). In cultured cells, inactivation of laminin synthesis with antisense strategy also lead to failure of BM formation (De Arcangelis *et al.*, 1996). Interestingly, nidogen binding to laminin is not essential for BM formation (Kim and Wadsworth, 2000; Mayer *et al.*, 1998). It is not known how laminin facilitates the assembly of the rest of the BM. Type IV collagen can polymerize *in vitro* independent of laminin, so

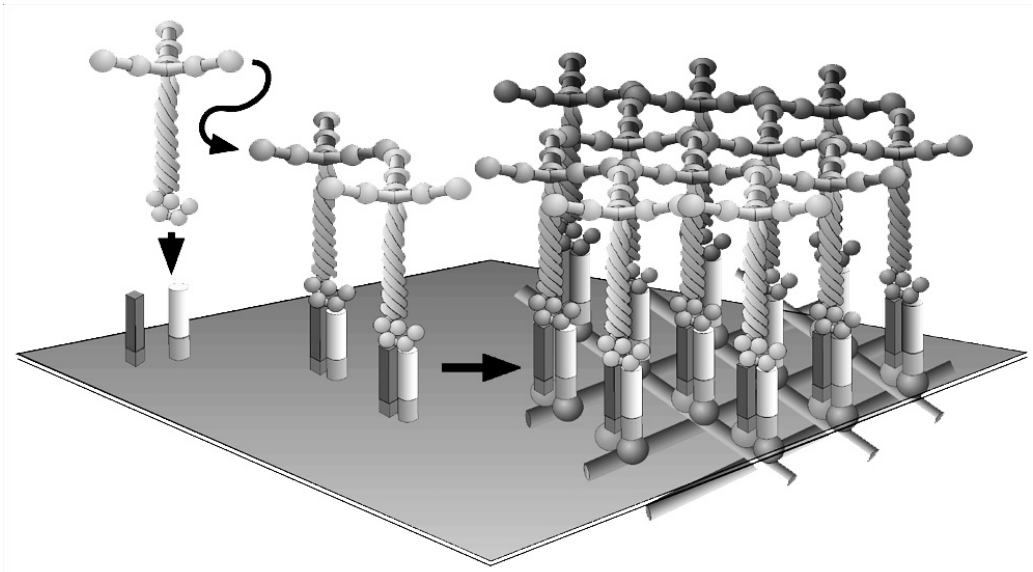

Figure 5. Model of receptor-facilitated laminin assembly. Laminin binding to cell surface receptors brings the laminin molecules closer and helps network formation. Adapted from Colognato and Yurchenco, 2000.

why would laminin be needed *in vivo*? One possibility would be that laminin is needed to aid the assembly at physiological concentrations of type IV collagen and other components, but can be disposed of – at least to some extent – at artificially high concentrations. Alternatively, laminin presence could give a feedback cue to the cells to make the proper components for BM, or to express receptors necessary for BM assembly.

6.2.4 WHERE AND WHEN LAMININS ARE FOUND: TISSUE DISTRIBUTION

The tissue- and developmental distributions of laminins and their constituent chains have been studied using immunohistochemistry, northern analysis and *in situ* -hybridization. For most chains and heterotrimers, the studies have not been exhaustive, but clear differences are observed among the members of the laminin family. It is apparent that the expression pattern is complex both spatially in different tissues, and temporally during development. The same BM can contain different laminin isoforms in different parts, as demonstrated in developing kidney (Miner *et al.*, 1997). One more complicating factor follows from the heterotrimeric nature of laminins. Finding an α, β and γ chain in the same location does not necessarily prove that they also are assembled into a trimer together. Unfortunately, trimer-specific antibodies do not exist.

The most ubiquitous laminin chains seem to be the β1 and γ1, which are found in most BMs (Kallunki *et al.*, 1992; Klein *et al.*, 1990). In the skeletal muscle, the β2 chain is present at the neuromuscular junction BM while β1 is restricted to the extrasynaptic muscle BMs. Perineural, but not endoneural BMs are rich in the β2 chain (Patton *et al.*, 1997). In the kidney, a developmental switch from β1 to β2 occurs during development of the GBM. Arteriolar smooth muscle cells also undergo developmental transition from β1 to β2. In pathological intimal thickenings, where the smooth muscle cells proliferate, expression of β1 becomes detectable again (Glukhova *et al.*, 1993). The finding that β2 is expressed in muscle arterioles but not in capillaries or venules (Patton *et al.*, 1997), might reflect β2 expression in arteriolar smooth muscle cells. The novel γ3 chain is found in non-BM locations in nerve, brain, testes and certain epithelia (Iivanainen *et al.*, 1999; Koch *et al.*, 1999).

Laminin-5 and its component chains (α3β3γ2) are specific to epithelia such as skin, lung, intestine and certain parts of the kidney (Aberdam *et al.*, 1994a; Kallunki *et al.*, 1992). The laminin α3 splice variants α3A and α3B exhibit tissue-specific variation in expression levels, and are not mutually exclusive (Doliana *et al.*, 1997). Since most antibodies and probes used recognize both variants, the exact patterning is still incompletely studied. The splice variant γ2* has a more restricted expression that γ2 (Airenne *et al.*, 1996; Airenne *et al.*, 2000).

It seems that the α-chains exhibit the highest tissue specificity. The most restricted one is the α1-chain, which is expressed in the adult mainly in a subset of epithelia. It is found in the kidney, reproductive organs and parts of the nervous system. During development, the α1 chain is found already in pre-implantation embryos (Falk *et al.*, 1999; Nissinen *et al.*, 1991; Virtanen *et al.*, 2000). Since the laminin isoform first discovered was laminin-1 (α1β1γ1), which includes the ubiquitous β1 and γ1-chains, immunostaining with polyclonal antibodies initially showed rather uniform expression of laminin in all BMs. When chain-specific antibodies were first developed, it was seen that in the mouse, the α1 chain has a restricted expression pattern. In the human, however, the α1-chain expression appeared widespread. Much later, it was shown that the particular human α-chain antibody (4C7) was actually against the α5-chain, resolving the discrepancy (Tiger *et al.*, 1997). As a consequence, it was long believed that laminin-1 is a typical laminin despite its tumor origin. Laminin α2 is the predominant α-chain in skeletal and cardiac muscle. It is also found in the endoneurium of peripheral nerves, brain and kidney mesangium (Miner *et al.*, 1997; Patton *et al.*, 1997; Vuolteenaho *et al.*, 1994).

The most widely expressed α-chains seem to the most recently discovered α4 and α5. Laminin α4 is found in lung alveolar septa, subendothelial BMs in capillaries and other vessels, in cardiac muscle and the perineurium of peripheral nerves (I). The expression patterns of laminin α4 are discussed in more detail below under Results and Discussion. Laminin α5 is found in most epithelia both during development and in adult. Lung alveolar septa, kidney GBM and epithelial BM in skin contain laminin α5. Arteriolar BMs have laminin α5 both during development and in the adult, and also placental blood vessels are rich in α5. In capillaries, the situation is different, as laminin α5 appears only much later, at a few weeks of age. In addition, laminin α5 is found in the perineurium of peripheral nerves and in the neuromuscular junction. (Miner *et al.*, 1998; Miner *et al.*, 1997; Patton *et al.*, 1997; Sorokin *et al.*, 1997b)

6.2.5 WHY DO WE HAVE LAMININS: BIOLOGICAL FUNCTIONS

Biological functions of laminins have been difficult to study because they are a part of very intricate systems: the extracellular matrix and the basement membrane. These in turn cannot be easily isolated in native form for *in vitro* assays. Protein-protein interactions have been studied, but with such a multipart system with complex proteins there is a substantial risk of detecting biologically non-relevant interactions. While studies in cell- and organ culture systems have been useful, diseases or animal models where laminin gene is mutated or missing have provided more specific knowledge of *in vivo* function.

There are numerous studies reporting a biological effect by laminin on cells. Cell attachment (Johansson *et al.*, 1981; Terranova *et al.*, 1982), cell differentation (Klein *et al.*, 1988), cell migration (McCarthy *et al.*, 1983) and neurite outgrowth (Manthorpe *et al.*, 1983) are classical examples. Laminins have also been implicated in pathological processes such as cancer metastasis (Terranova *et al.*, 1983).

Epithelial morphogenesis during development has been widely studied in organ culture systems. Epithelial branching in development of many organs such as kidney, lung and many glands, depends on interactions between epithelium and the underlying mesenchyme. In organ culture systems, it has been possible to study molecular details of this interaction. Addition of antibodies to integrin α6, nidogen and different laminin fragments disrupts morphogenesis. Similarly, addition of excess laminin or laminin fragments causes perturbations. (Ekblom *et al.*, 1998; Ekblom *et al.*, 1994; Kadoya *et al.*, 1997; Klein *et al.*, 1988; Sorokin *et al.*, 1990)

In the lethal skin blistering disease, junctional epidermolysis bullosa, mutations have been found in all of the component chains of laminin-5 (α3β3γ2) (Kivirikko *et al.*, 1995; Pulkkinen *et al.*, 1994a; Pulkkinen *et al.*, 1994b). The disease is characterized by disruption of epithelial-matrix linkage as hemidesmosomes are detached from the underlying basement membrane. Mouse models lacking α3 (Ryan *et al.*, 1999) or β3 (Kuster *et al.*, 1997) have been established, and the murine phenotypes closely resemble the human disease.

Laminin α2 is mutated in certain types of congenital muscular dystrophy (CMD). CMDs are heterogenous autosomal recessive disorders, that cause severe early hypotonia and weakness, markedly delayed motor development and contractures, often associated with joint deformities. Histological findings include large variations in muscle fibre size, as well as some necrotic and regenerating fibres and a marked increase in endomysial collagen tissue. In some patients, the laminin α2 -deficient type of CMD is caused by splice site- and nonsense mutations in the laminin α2-chain gene, presumably leading to truncated protein (Helbling-Leclerc *et al.*, 1995; Tome *et al.*, 1994). The phenotype of mouse mutants is similar to the human disorder. Two spontaneous mouse mutants have been known for a long time, and have now been recognized as α2-deficient. The *dy*^{2J} strain (Meier and Southard, 1970) has a deletion in domain VI (Xu *et al.*, 1994), resulting in somewhat milder phenotype than *dy*-strain (Michelson, 1955), where the α2 protein is nearly totally absent (Sunada *et al.*, 1994). Two independent targeted null mutants have also been produced (Kuang *et al.*, 1998a; Miyagoe *et al.*, 1997). The muscle phenotype has been successfully rescued using transgene coding for laminin α2 under the creatine kinase promoter. Since the creatine kinase promoter is muscle-specific, there are still abnormalities in the nervous system (Kuang *et al.*, 1998b).

Inactivation of laminin α5 in mice lead to embryonic lethality at day E14-17. The α5 null embryos had defects in anterior neural tube closure, kidney development and limb development. The presumed cause of lethality was reported to be placental defects (Miner *et al.*, 1998; Miner and Li, 2000)

The laminin β2 chain has been shown to play a regulatory role in motor nerve terminal formation (Noakes *et al.*, 1995a). It is also needed in the normal adult kidney filtration barrier. Mice lacking the β2 chain develop normal kidneys, but soon after birth they start to suffer from massive proteinuria and die, although the filter barrier structure and composition appears normal (Noakes *et al.*, 1995b).

The laminin γ1-chain is found in most basement membranes, and the outcome of its inactivation is early embryonic lethality from failure of endoderm differentiation. Interestingly, it was found that γ1 null embryos and embryoid bodies failed to form BMs altogether, suggesting that laminin is required for BM assembly (Smyth *et al.*, 1999).

6.2.6 WHAT WAS ALREADY KNOWN OF LAMININ α4 PRIOR TO THIS STUDY

Prior to this study, only two articles had been published on the laminin α4 chain. The human gene was found and localized to chromosome 6q21 by Richards and co-workers, who also published a partial cDNA sequence and measured the mRNA size to be 6.45 kb (Richards *et al.*, 1994). The following year, the complete cDNA sequence was published by Iivanainen and co-workers (Iivanainen *et al.*, 1995a).

The translation product of the human laminin α4 gene contains a 24-residue signal peptide preceding a 1792-residue mature chain with a calculated mass of 199 306 Da. The domain structure is much more similar to that of the α3-chain than those of the α1-, α2- or α5-chains. The C-terminal domain has the five repeat motifs typical to the other laminin α chains, and the coiled-coil domain (I/II) has heptad repeats similar to those in other laminin chains. In contrast to the "classical" and "long" chains, the α4 chain lacks domains IVb-VI, and has only one rod-like segment which is preceded by a short N-terminal domain (Figure 3). The rod-like segment consists of three complete EGF-like repeats flanked by incomplete ones. This sequence is closest in sequence homology to the domain IIIb of the α1 chain (Iivanainen *et al.*, 1995a).

Expression of the laminin α4 chain in human had been studied using northern analysis and *in situ* -hybridization analysis. In human adults, the expression was found to be strongest in the

heart, and somewhat weaker expression was observed in lung, ovary, placenta and liver. There was even weaker expression in small intestine, colon, kidney, skeletal muscle, testis, prostate and pancreas. In northern analysis, there was no sign of expression in the brain. In the fetus, there was strong expression in lung, and weaker expression in kidney (Iivanainen *et al.*, 1995a).

6.2.7 EXTRA INFORMATION ON LAMININS

6.2.7.1 HOW LAMININS ARE NAMED: NOMENCLATURE

A "systematic" nomenclature has been applied to cope with the complexity of laminin isoforms, genes and trimer combinations (Burgeson *et al.*, 1994). Splice-variants such as α3A/α3B and γ2/γ2* were not known at the time. Although this nomenclature has been adopted widely, it is subject to criticism. Theoretically possible laminin trimers number well over 100, and although the actual number seems to be much lower, it still is too large to be conveniently handled in this manner.

Table II: Nomenclature of laminin chains and their genes

Current chain name	*Previously used names*	*Gene name*
α1	A, Ae	LAMA1
α2	M, Am	LAMA2
α3A	200 kDa, A1k	LAMA3
α3B	-	LAMA3
α4	-	LAMA4
α5	-	LAMA5
β1	B1, B1e	LAMB1
β2	s, B1s	LAMB2
β3	140 kDa, B1k	LAMB3
γ1	B2, B2e	LAMC1
γ2	B2t	LAMC2
γ2*	-	LAMC2
γ3	-	LAMC3

Table III: Nomenclature of laminin trimers (extended from (Burgeson *et al.*, 1994))

Current name	*Chain composition*	*Previously used names*	*References*
Laminin-1	α1β1γ1	EHS-laminin	(Chung *et al.*, 1979; Timpl *et al.*, 1979)
Laminin-2	α2β1γ1	merosin	(Engvall *et al.*, 1990)
Laminin-3	α1β2γ1	s-laminin	(Green *et al.*, 1992)
Laminin-4	α2β2γ1	s-merosin	(Engvall *et al.*, 1990)
Laminin-5	α3β3γ2	kalinin, nicein, epiligrin	(Carter *et al.*, 1991; Rousselle *et al.*, 1991; Verrando *et al.*, 1991)
Laminin-6	α3β1γ1	k-laminin	(Marinkovich *et al.*, 1992)
Laminin-7	α3β2γ1	ks-laminin	(Champliaud *et al.*, 1996)
Laminin-8	α4β1γ1	-	(Miner *et al.*, 1997)
Laminin-9	α4β2γ1	-	(Miner *et al.*, 1997)
Laminin-10	α5β1γ1	-	(Miner *et al.*, 1997)
Laminin-11	α5β2γ1	-	(Miner *et al.*, 1997)
Laminin-12	α2β1γ3	-	(Koch *et al.*, 1999)

6.2.7.2 KNOWN LAMININ CDNAS

Table IV: Known laminin chains (naming after nomenclature by (Burgeson *et al.*, 1994))

Chain	*Species*	*References (cDNA)*
α1	Man	(Haaparanta *et al.*, 1991; Nissinen *et al.*, 1991)
	Mouse	(Sasaki *et al.*, 1988)
α2	Man	(Vuolteenaho *et al.*, 1994)
	Mouse	(Bernier *et al.*, 1995)
	Drosophila	(Martin *et al.*, 1999)
α3A,B	Man	(Doliana *et al.*, 1997; Ryan *et al.*, 1994)
	Mouse	(Galliano *et al.*, 1995)
α4	Man	(Iivanainen *et al.*, 1995a; Richards *et al.*, 1994)
	Mouse	(Frieser *et al.*, 1997; Liu and Mayne, 1996)
α5	Man	(Durkin *et al.*, 1997), partial sequence
	Mouse	(Miner *et al.*, 1995)
	Drosophila	(Garrison *et al.*, 1991)
β1	Man	(Pikkarainen *et al.*, 1987)
	Mouse	(Sasaki *et al.*, 1987)
	Drosophila	(Montell and Goodman, 1988)
β2	Man	(Iivanainen *et al.*, 1995b; Wewer *et al.*, 1994)
	Mouse	(Noakes *et al.*, 1995a)
	Rat	(Hunter *et al.*, 1989)
β3	Man	(Gerecke *et al.*, 1994)
	Mouse	(Utani *et al.*, 1995)
β4	Man	(Olson, 1997; Sulston, 1998), partial sequence
γ1	Man	(Pikkarainen *et al.*, 1988)
	Mouse	(Sasaki and Yamada, 1987)
	Drosophila	(Chi and Hui, 1989)
γ2, γ2*	Man	(Airenne *et al.*, 1996; Kallunki *et al.*, 1992)
	Mouse	(Sugiyama *et al.*, 1995)
γ3	Man	(Koch *et al.*, 1999)
	Mouse	(Iivanainen *et al.*, 1999)

6.2.7.3 CHROMOSOMAL LOCALIZATIONS OF LAMININ GENES

Table V. Identified laminin genes and their chromosomal locations in man and mouse.

Gene	*Species*	*Chromosomal location*	*References*
LAMA1	Man	18p11.3	(Nagayoshi *et al.*, 1989)
	Mouse	17 38.0 cM	(Kaye *et al.*, 1990)
LAMA2	Man	6q22-23	(Vuolteenaho *et al.*, 1994)
	Mouse	10 20.0 cM	(Sunada *et al.*, 1994)
LAMA3	Man	18q11.2	(Ryan *et al.*, 1994)
	Mouse	18 3.0 cM	(Aberdam *et al.*, 1994b)
LAMA4	Man	6q21	(Richards *et al.*, 1994)
	Mouse	10 25.0 cM	(Miner *et al.*, 1997)
LAMA5	Man	20q13.2-3	(Durkin *et al.*, 1997)
	Mouse	2 106.0 cM	(Miner *et al.*, 1997)
LAMB1	Man	7q22	(Pikkarainen *et al.*, 1987)
	Mouse	12	(Seldin *et al.*, 1989)
LAMB2	Man	3p21	(Wewer *et al.*, 1994)
	Mouse	9 60.0 cM	(Porter *et al.*, 1993)
LAMB3	Man	1q32	(Vailly *et al.*, 1994)
	Mouse	1 104.0 cM	(Aberdam *et al.*, 1994b)
LAMB4	Man	7q22-q31.2	(Sulston, 1998)
LAMC1	Man	1q31	(Fukushima *et al.*, 1988)
	Mouse	1 81.1 cM	(Kaye *et al.*, 1990)
LAMC2	Man	1q25-q31	(Kallunki *et al.*, 1992)
	Mouse	1 81.1 cM	(Aberdam *et al.*, 1994b)
LAMC3	Man	9q31-34	(Koch *et al.*, 1999)
	Mouse	Unknown	

A useful source for up-to-date information on laminin genes can be found at National Center for Biotechnology Information web site:
http://www.ncbi.nlm.nih.gov/LocusLink/list.cgi?Q=laminin&ORG=&V=0

6.3 BLOOD VESSEL FORMATION

The processes of blood vessel formation, angiogenesis and vasculogenesis, have been under intense study for a number of years. Vasculogenesis is the *de novo* formation of a primitive network of vessels, and occurs mainly during development. Formation of blood vessels by sprouting, intussusception or division of existing vessels is called angiogenesis. In the adult, angiogenesis is thought to be the main mechanism by which vessels form, although vasculogenesis does occur even in the adult to some extent. All types of vessels originate as capillaries; they can then further grow and differentiate into larger veins or arteries. Angiogenesis is of major importance in many common pathological conditions, such as cancer, diabetes complications and various ischemic and inflammatory diseases. Already in 1971, it was proposed that inhibiting angiogenesis could prevent tumor growth (Folkman, 1971). On the other hand, after stroke and myocardial infarction, angiogenesis can be vital for salvaging the ischemic tissues and it is required for proper wound healing. This chapter will mainly deal with angiogenesis by sprouting, which we have studied in laminin α4 null mice (IV). Angiogenesis has been recently reviewed (Carmeliet, 2000; Carmeliet and Jain, 2000).

Angiogenesis can be initiated in several different conditions. Tissue hypoxia (ischemia), tissue injury, inflammation or, under experimental conditions, addition of exogenous growth factors (e.g. vascular endothelial growth factor VEG or fibroblast growth factor-2) stimulate an angiogenic response. The first step is thought to be permeabilization of vessel walls and leakage of plasma proteins into the extravasal space. VEGF can induce such a response, which also involves NO. Formation of fenestrations and vesiculo-vacuolar organelles, as well as downregulation of cell-cell adhesion contribute to increased permeability. The leaked proteins, fibrin in particular, provide a temporary scaffold for endothelial cell migration. (Eriksson and Alitalo, 1999; Gale and Yancopoulos, 1999)

Following stimulus, endothelial cells begin to proliferate and migrate out into the stroma. Circulating endothelial cell precursors may also contribute. To break out of the vessels and migrate out, endothelial cells need to break up their contacts with neighboring cells, a process that is mediated in part by angiopoietin 2 (Gale and Yancopoulos, 1999). The cells also need to dissolve the preexisting underlying BM and other matrix molecules. Proteases such as plasminogen activator, matrix metalloproteinases, and chymases are involved in the degradation of the matrix (Bajou *et al.*, 1998; Heymans *et al.*, 1999; Werb *et al.*, 1999; Vu *et al.*, 1998).

When outside, the endothelial cells keep proliferating and invading the tissue. They can migrate long distances guided by the angiogenic stimulus gradient. The cells line up forming a string. Then, the cells start forming tight junctions and a lumen forms. When the growing capillary encounters another vessel, a connection is formed allowing blood to circulate. The cells also lay down a new BM, which we have observed to be rich in laminin-8 (IV). Recently it was shown that angiogenesis could be inhibited in several experimental models by interfering with BM assembly (Petitclerc *et al.*, 2000).

In order to remain stable, endothelial cells need accessory cells such as pericytes in capillaries, or in the case of larger vessels, smooth muscle cells. PDGF-BB is essential for pericyte recruitment (Lindahl *et al.*, 1997), and signaling by TGF-β1 and angiopoetin 1 is involved in interactions between smooth muscle and endothelial cells (Carmeliet and Jain, 2000). Maturation of the endothelial network involves remodeling. The process of remodeling is poorly understood, but it involves elimination of excess vessels leading to a structurally uniform, organized network.

The main methods to study angiogenesis *in vivo* that can be applied to null mice are the cornea angiogenesis assay (Kenyon *et al.*, 1996), intravital microscopy through a chronically implanted window (Jain *et al.*, 1997), and oxygen-induced retinal neovascularization (Smith *et al.*, 1994). We used the cornea assay, which involves implanting a pellet that slowly releases an angiogenic growth factor that then causes angiogenic sprouting from the existing limbus vessels at the periphery of the cornea. The sprouting vessels invade the normally avascular corneal matrix towards the source of the growth factor. Since the cornea in translucent, the growing capillaries can be readily observed. Weaknesses of this model are that the cornea is not the normal matrix for the vessels to grow in, and that the artificial stimulus may have properties different from normal angiogenesis.

7 AIMS OF THE PRESENT STUDY

Laminin α4 is a recently described laminin chain. This study was part of a project aimed at characterizing the structure, expression and biological function of laminin α4 and heterotrimers containing this chain. The following specific goals were set:

1. Clone and sequence the mouse laminin α4 cDNA
2. Study laminin α4 expression in mice using northern blotting, *in situ* hybridization and immunostaining
3. Produce and purify recombinant laminin-8
4. Study the interactions between cells and laminin-8 by using the recombinant laminin-8
5. Produce genetically engineered mice lacking laminin α4
6. Study the biological function of laminin α4 by analyzing the phenotype of mice lacking it

8 RESULTS AND DISCUSSION

8.1 CLONING OF MOUSE LAMININ α4 AND TISSUE DISTRIBUTION (I)

Six overlapping cDNA clones were isolated and characterized in this study. Together, they covered a total of 5 824 nucleotides, including 208 bp of a 5' UTR and 168 bp of a 3' UTR region, respectively (GenBank accession number U59865). The open reading frame of 5 448 nucleotides coded for a 1 816-residue laminin α-type chain, characterized by the presence of a carboxyl terminal G domain with five internal repeat motifs. The predicted sequence of the first 24 residues is characteristic of a hydrophobic signal peptide which is present in all other laminin chains, and the amino acid sequence around position 24 is characteristic for a signal peptidase cleavage site (Nielsen *et al.*, 1996; von Heijne, 1986). Thus, the mature mouse laminin α4 chain proper contains 1 792 residues. The calculated mass of the entire translation product is 201 818 Da and that of the processed α4 chain 199 164 Da. The human and mouse chain amino acid sequences have 88.1% identity. Of the 44 cysteins present in the human chain, 43 were conserved. Likewise, 18 out of 19 potential N-glycosylation sites were conserved in mouse. The mouse cDNA has been published independently by two other groups (Frieser *et al.*, 1997; Liu and Mayne, 1996). Aligning these three sequences shows that in 2 nucleotides both sequences differ from our sequence, indicating acceptable sequencing accuracy (1 error/3 000 bp). Of course, the differences could be due to polymorphisms as well.

Although the overall length of the murine and human chains is the same, two balancing 2-residue gaps are located in the G-domain. At the time of publication, the exact borders of the LG modules were not known. When compared to domain borders as presented by Timpl *et al.*, 2000, our prediction that the two amino-acid deletion would be in the spacer separating LG1-3 and LG4-5 appears to have been correct. In contrast, the two amino-acid insertion we predicted to be located in the border of G-domain and coiled-coil region seems to be in fact located in the LG1 module. When compared to other LG-modules it seems that there is rather a deletion in the human chain and instead of an insertion in the mouse chain. It can be seen from the crystal structure that the two amino-acids reside in a loop connecting two β-strands (C and D). Apparently the deletion of these two amino acids in human chain is compatible with normal folding and function.

Expression of the laminin α4 chain was studied using northern analysis. As expected from results on human tissues (Iivanainen *et al.*, 1995a), tissue-specific expression was observed. Using RNA from whole mouse embryos, the level of expression was low at embryonic day 7,

but it later increased being highest at day 15. In the adult, strong expression of a 6 kb mRNA was seen in lung and in skeletal and heart muscle. Weaker expression was seen in all other tissues studied, *i.e.* brain, spleen, liver, kidney and testis. In testis, a second weak band of about 5 kb was also observed. By *in situ* hybridization of fetal and newborn tissues, expression of the laminin α4 chain was mainly localized to mesenchymal cells. Strong expression was seen in the villi and submucosa of the developing intestine, possibly in endothelial cells of capillaries. The expression seen in the external root sheath of vibrissae follicles was also possibly located in capillaries. Strong expression was observed in the mesenchymal stroma surrounding the branching lung epithelia. Distinct expression was observed in the nuclei of both cardiac and skeletal muscle fibers, and in capillaries of the muscle. In the developing kidney, intense but transient expression was seen. Expression was first observed in mesenchymal condensates next to ureter buds. Strong expression was seen in early kidney tubules differentiating from mesonephric condensates. The expression then diminished following maturation of the proximal nephron into distinct glomerular and tubular structures, and was absent in adult kidney.

Immunohistological staining with affinity-purified polyclonal antibodies was used to localize the laminin α4 chain. In lung, strong staining was observed in alveolar septa. In peripheral nerves, staining was located in the perineurium and Schwann cells. In cardiac muscle, strong staining was observed in capillaries, and less intensely lining the muscle fibers. In adult skeletal muscle, staining was restricted to capillaries, but in embryonic and newborn skeletal muscle also the muscle fibers themselves stained positively.

Since our publication, other investigators have also studied laminin α4 expression (Frieser *et al.*, 1997; Lefebvre *et al.*, 1999; Miner *et al.*, 1997; Patton *et al.*, 1997; Ringelmann *et al.*, 1999; Sorokin *et al.*, 2000; Sorokin *et al.*, 1997a). These investigators have provided more detailed information on laminin expression, especially in the kidney and the neuromuscular junction, but in general the results are in good agreement.

8.2 LAMININ-8 IN PLATELETS (II)

Permeabilized, but not intact platelets were found to be labeled by an anti-laminin γ1 antibody as measured by immunofluorescence flow cytometry. Western blotting of platelet lysate indicated presence of polypeptides reacting with antibodies against laminin α4 (180 kDa major and 140 and 200 kDa minor bands), β1 (230 kDa) and γ1 (220 kDa). Using an anti-laminin β1 affinity column, platelet laminin could be isolated. It appeared in SDS-PAGE as four major bands (230, 220, 200 and 180 kDa). Using amino-acid sequencing the 230 kDa,

220 kDa and 180 kDa were identified as laminin β1, γ1 and α4 chains, respectively. The 200 kDa band could not be sequenced. It could be a contaminant, a bona fide laminin-associated protein, or an unknown laminin chain associating with the β1 chain. It was concluded that platelets contain laminin-8. Interestingly, some of the laminin α4 chain was not disulfide-bonded to the β1 and γ1 chains, which were disulfide-linked to each other. Later, laminin-8 has been shown to be synthesized by megakaryocytic cells, the precursors of platelets (Geberhiwot *et al.*, 2000).

The platelets secreted 20-35% of their laminin-8 when stimulated. The secreted laminin was indistinguishable in western blotting from lysate laminin. The amount of laminin was calculated to be about 1 fg/cell, or about 700 molecules/cell. This can be compared to amount of fibronectin in platelets, about 3.5 fg/cell. The laminin-8 secreted following vascular injury and platelet activation could contribute to healing processes.

Platelets were found to adhere similarly to the isolated platelet laminin-8 and recombinant laminin-8 (III). Since the adhesion could be blocked by antibodies against either integrin α6 or integrin β1, it was concluded that platelets adhere to laminin-8 mainly using integrin α6β1.

8.3 PRODUCTION AND CHARACTERIZATION OF RECOMBINANT LAMININ-8 (III)

Human embryonic kidney cells (HEK293) were sequentially transfected with expression constructs coding for the component chains of laminin-8 (α4β1γ1). A fusion FLAG-tag was engineered into the C-terminus of the laminin α4-chain to facilitate purification. The cells required simultaneous expression of all three chains for efficient secretion. After selection, the best clones expressed several milligrams of recombinant laminin-8 per liter of medium.

The recombinant laminin-8 was purified using an anti-FLAG antibody column. The laminin was bound to the column directly from the medium and eluted competitively with FLAG-peptide. In this single step, a highly purified protein was obtained. Further purification and removal of free FLAG-peptide was achieved in anion-exchange chromatography using Q-column, and the preparation was desalted with gel-filtration. When needed, the protein was concentrated using ultrafiltration.

The purified protein was analyzed in SDS-PAGE. Under non-reducing conditions the purified protein hardly entered the gel, which was to be expected as the predicted molecular weight for the mature trimer is very high (570 kDa). A minor fraction of the purified trimer appeared as non-covalently associated. In this minor fraction, the β1 and γ1 chains appeared as

covalently associated dimers, whereas the α4 chain was non-covalently associated. Under reducing conditions, the protein appeared as a broad band at around 200 kDa, which reacted on western blots with α4, EHS, β1, γ1 and anti-FLAG antibodies. The expected molecular weights for mature α4, β1 and γ1 polypeptides are 200, 195 and 174 kDa, respectively. Heavy glycosylation common to laminins might account for the slightly larger molecular weights observed in SDS-PAGE. The β1 and γ1 chains of laminin-1 purified from EHS tumor showed similar or slightly slower mobility than those of recombinant laminin-8. The recombinant laminin-8 protein appeared Y-shaped in rotary shadowing electron microscopy with two short and one long arm, as expected. In many cases, a very short (5-10 nm) rod-like stub could be seen at the junction of the arms. The G-domain could sometimes be seen as consisting of two moieties, probably corresponding for LG-modules 1-3 and 4-5.

To identify integrin receptors mediating adhesion to recombinant laminin-8, cell adhesion was assayed in the presence of different blocking anti-integrin antibodies. The effect of heparin on cell adhesion was also tested, since α-DG-mediated binding to certain laminin isoforms has been shown to be inhibitable by heparin.

The binding of HT-1080 fibrosarcoma cells to recombinant laminin-8 was completely abolished by antibodies against integrin α6 and β1 subunits. Antibodies against integrins α1, α2, α3 and α5 resulted in no or small effects on adhesion to the laminins. The presence of these integrins on the cells was verified by the ability of the antibodies to block cell adhesion to type IV collagen or fibronectin. Heparin had little effect.

The role of α6β1 and α6β4 integrins in binding to recombinant laminin-8 was verified using K562 cells transfected to express these integrin complexes. K562-cells expressing integrins α6β1 or α6β4 bound to recombinant laminin-8 while those expressing α3β1 did not. Adhesion of α6β1 expressing cells could be completely blocked by either α6 or β1 antibodies. Binding of cells expressing both α6β1 and α6β4 could be completely blocked by monoclonal antibodies against integrin α6 but only partially by integrin β1 antibodies indicating that α6β4 is also capable of mediating cell adhesion to recombinant laminin-8.

Since laminin-8 is found in vascular endothelia, immortomouse brain capillary endothelial (IBE) and bovine adrenal microvascular endothelial (BCE) cells were also used to study the adhesion of endothelial cells to recombinant laminin-8. The BCE cells express at least integrins α6β1, α6β4, α1β1, α2β1, α3β1, α5β1, αvβ1, αvβ3 and αvβ5 (Klein *et al.*, 1993), whereas IBE cells have been reported to express integrins α3, α5 and β1 but not α1, α2 or α6 (Kanda *et al.*, 1999). The antibody against integrin α6 was found to inhibit binding of BCE cells to recombinant laminin-8 completely, while binding to laminin-1 was only

moderately reduced. In contrast, integrin β1 antibody completely blocked binding to laminin-1, but only partially to recombinant laminin-8, suggesting involvement of α6β4 integrin in BCE cell adhesion to recombinant laminin-8. Due to unavailability of integrin β4 subunit-blocking antibodies against the bovine antigen, this could not be tested directly. The adhesion of IBE cells to recombinant laminin-8 was moderately reduced by anti-integrin α6 but binding to laminin-1 was not affected. Significant binding still remained after blocking of the α6 integrin, but the receptors responsible could not be identified due to lack of mouse-specific function blocking antibodies. Heparin binding did not inhibit binding by either cell line.

In conclusion, recombinant laminin-8 was produced as a native trimeric protein and integrins α6β1 and α6β4 were found to be major mediators of cell adhesion to laminin-8. Binding via integrins α1β1, α2β1, α3β1 and α5β1 was not detected. The integrin specificities were found to differ somewhat from that of laminin-1. Considering expression patterns of laminin-8 and integrin α6β1 it is likely that the former is a ligand for the latter *in vivo* as well.

8.4 STUDIES OF MICE LACKING THE LAMININ α4 (IV)

The laminin α4 chain gene was inactivated using gene targeting. Complete gene inactivation was seen in northern blotting and with immunostaining using a laminin α4-specific antibody. The ratio of -/- to +/+ newborn pups was close to the expected Mendelian ratio. During the first two days, mortality was slightly increased, and the pups were slightly smaller, but the -/- mice that survived beyond the perinatal period had a similar mortality rate and life span as littermate controls. Newborn null mice were lethargic, pale and icteric, and diffuse subcutaneous and intramuscular hemorrhages were observed. Hemoglobin values revealed severe anemia in the -/- mice, but other blood parameters were normal suggesting that bleedings were causing the anemia. The hemorrhages gradually disappeared during the first days of life and the anemia was no longer present in pups after 1-2 weeks of age, nor in the adults.

Immunostainings of newborn muscle sections with antibodies against laminin-1, laminin β1, laminin γ1, type IV collagen and nidogen showed that these proteins were drastically reduced in null capillaries, while integrin α6 and perlecan were present in normal amounts. In contrast, the composition of muscle BMs was normal. In electron microscopy, newborn -/- muscle capillary BMs had a more loose and discontinuous pattern than in controls. Beyond 1-3 weeks of age, the staining intensities for all the BM components were identical to the controls. This normalization coincided with the appearance of the α5 chain in the capillary BMs.

Angiogenesis in the adult was studied in adult mice with the cornea angiogenesis assay (Kenyon *et al.*, 1996). *Lama4* -/- mice showed earlier and more intensive sprouting than controls at 2-3 days after implantation of the FGF-pellet. At 3-4 days after insertion, grossly distorted blood vessel architecture, with dilated vessels and hemorrhages, could be seen. The branching pattern appeared irregular and uncoordinated. Some animals developed foci of hemorrhage and intense swelling of the cornea.

The hemorrhages and structural defects observed in BM of the capillaries indicate that laminin-8 is required for the stability of newly formed capillaries. In the capillaries of skeletal muscle, laminin α4 has been first detected at E11 and is retained at this site throughout development and into adulthood (Patton *et al.*, 1997), II. Laminin α5 is the only other laminin α chain known to occur in capillary basement membranes of muscle and most other tissues, appearing late in development, at around 3-4 weeks postnatally (Patton *et al.*, 1997; Sorokin *et al.*, 1997b). Since the laminin β2 chain has not been found in capillaries (Patton *et al.*, 1997), laminin-8 is presumably the only laminin trimer in the capillary BMs of neonatal muscle. Laminin-8 is bound by α6β1 and α6β4 integrins (II, III) that are typically found on endothelial cells. It is, therefore, possible that the laminin-8 in capillary BMs is bound to the integrins on one hand, and type IV collagen via nidogen on the other, providing a link between endothelial cell surface and type IV collagen molecules necessary for nucleation of the type IV collagen network. Apparently, perlecan deposition is independent of the other components. Alternatively, laminin-8 may function as a signaling molecule, necessary for the induction of expression of other BM components. The fact that the vascular phenotype in null mice disappeared during the first weeks of life could be explained by initiation of expression and deposition of LN-10 (α5β1γ1) into the capillary BMs, which probably then facilitates type IV collagen and nidogen deposition, restoring BM structure.

8.5 SHOULD SOMETHING HAVE BEEN DONE DIFFERENTLY

Cloning and sequencing the murine laminin α4 chain certainly had an educational function. The main utility of the sequence has been the construction of in situ-probes, but the complete sequence would not have been needed for this purpose.

The production of recombinant laminin took a long time. One major source for difficulties was instability of expression. As a starting material, we obtained cells form the lab of Peter Yurchenco that were transfected to express mouse laminin β1 and γ1 chains, and I would only have to transfect with the human α4 construct. Unfortunately, the expression of the γ1 chain was always lost during clone expansion without me noticing it until rather late. What

caused the confusion was the extreme sensitivity and nonlinear signal strength inherent to the chemiluminescent western system. Even with the little γ1 expression that remained, in addition to endogenous laminin, seemingly considerable amounts of laminin protein was found in the medium, while in fact it was only a few micrograms per liter. Thus all seemed promising, but when it came to preparative scale, the purification yields were pathetic. Regrettably, it was very easy to deceive oneself when trying to estimate quantitative amounts in chemiluminescent western blotting. A good guideline when trying to make large amounts of recombinant protein would be "If it can not be seen with traditional alkaline phosphatase-NBT, it is not worth seeing." Using a less modern system probably would have saved considerable amount of blood, sweat and tears.

8.6 WHAT STILL NEEDS TO BE DONE

A project that aims to elucidate the biological function of a molecule can hardly ever be complete. Much remains to be done before the biological function of laminin-8 can be said to be thoroughly studied.

As seen in paper III, endothelial cells adhere well to laminin-8. Whether this applies only to capillary endothelial cells, which were studied here, or also other endothelial cells such as arterial endothelial cells, remains to be studied. It appears likely that other processes involving endothelial cells such as migration, differentiation and proliferation could be promoted by laminin-8. On the other hand, we have preliminary data suggesting that vascular smooth muscle cells adhere better to other laminin isoforms (Lundmark *et al*., unpublished). A major complication in balloon angioplasty, restenosis, is thought to be caused by excessive proliferation of smooth muscle cells. Furthermore, endothelial cells have been found to downregulate SMC proliferation (Bauters, 1998). If laminin-8 could be used therapeutically to promote re-endothelialization and suppress smooth muscle cell proliferation after vessel injury remains to be shown.

It is be very important to verify that the recombinant laminin-8 has the same biological activity as naturally occurring laminin-8, but to make the comparison, highly purified laminin-8 from natural sources is be needed. There are, at present, several potential factors that could contribute to differences. First, Talts *et al.*, 2000 have reported that the LG4-LG5 domains are proteolytically cleaved off the rest of the molecule *in vivo* and are not detected in immunostainings. The recombinant laminin-8 in not processed in this way, and this might cause a functional difference. Second, there is a FLAG epitope tag at C-terminal. Third, glycosylation might be different. Fourth, the recombinant molecule was a species hybrid. Work aiming to produce a fully human molecule is ongoing. There is preliminary data

(Geberhiwot *et al*., unpublished) that suggests a quantitative functional difference between recombinant laminin-8 and platelet-purified laminin-8.

Leukocytes are another group of cell types that probably come to contact with laminin-8 during endothelial transmigration. They also contain laminin-8 intracellularly, but the function of the intracellular laminin is unknown. It was recently shown that laminin-8 supports migration of monocytic cells, while laminin-10/11 did not (Pedraza, 2000). Laminin-8 also provides a co-stimulatory signal for T-cell proliferation while laminin-1 does not (Geberhiwot, 2000). These findings suggest that laminin-8 has a role in leukocyte physiology. The recombinant protein and the laminin α4 deficient mice provide tools for further work in this field.

Our findings in laminin α4 null mice demonstrate a distinct role for laminin α4 in capillary development. Although we could show that laminin α4 was needed for capillary BM formation, the mechanism by which the laminin facilitates BM formation remained unclear. Is it the molecular interactions between laminin and the other components that is a requirement for BM assembly or is the laminin a signaling molecule? One interesting detail to study in this context would be pericyte recruitment. Does laminin α4 play a role in it? Studying wound healing and tumor-related angiogenesis in the null mice would also be interesting.

One of the first things noticed in the knock-out mice was a movement problem affecting the hind limbs. So far, the underlying cause has remained elusive. The severity was highly variable in hybrid genetic background but more constant in animals back-crossed to Bl/6. The abnormality could be consistently detected in a beam-walk experiment, and does not seem to be progressive. Muscle histology showed a very low percentage of abnormal fibers, but physiological measurements of mutant muscles could not distinguish them from normal ones. Neuromuscular junctions are structurally abnormal in mutant mice (Patton *et al.*, unpublished), but again the physiological findings suggest adequate function. Dorsal root ganglia, where the sensory neuron cell bodies reside, are morphologically abnormal in mutant mice, but we have not been able to show any defects in sensory function. No obvious histological defects were found in the brain either.

There are also a number of other observations on the laminin α4 knock-out mice that we have not had an opportunity to study in greater detail. It would seem that old (over 8 months) -/- mice are leaner than their normal littermates. Since laminin-8 was the only laminin found in cultured adipocytes (Niimi *et al.*, 1997), one could hypothesize that lack of laminin α4 causes

defects in adipocytes. Of course, the putative weight loss could be due to some secondary mechanism.

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