Biological Roles of Laminins 8, 9 and 10

by

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Front cover: Rotary shadowing electron microscopy of recombinant laminin 10.
In memory of all the individuals of the species Mus musculus, who brought us the new knowledge presented in this thesis.
SUMMARY

In order to form multicellular organisms, cells need attachment, either directly to each other through cell-cell interactions, or indirectly via a complex mixture of macromolecules called the extracellular matrix. Basement membranes are specialized forms of this matrix. They can be seen in the electron microscope as a thin flat structure underlying epithelial or endothelial cells, or surrounding certain individual cells such as muscle, nerve and fat cells. The basement membranes provide a structural framework for individual cells, or groups of cells, delineating them from the surrounding tissues, and anchor the cells through specific interactions with cell surface receptors. These interactions are of vital importance for the development of a cell as well as for its maintenance, and in many cases, even for its survival. Basement membranes are evolutionary old inventions and can be found in very primitive organisms. Most mammalian basement membranes have a type IV collagen-network and a laminin-network that are thought to be interconnected via a linker molecule called nidogen. Dystroglycan and the integrin family of receptors are the most well characterized cell surface receptors for laminins.

Laminins are large glycoproteins comprised of three individual polypeptide chains (αβγ) and to date, 5 α, 4 β and 3 γ genetically distinct variants are known. Different chain variants combine into the laminin isoforms, and 14 have been identified in vivo so far. The different isoforms have partially overlapping expression patterns, which are complex both temporally and spatially. This study mainly focuses on the biological roles of laminin 8 (α4β1γ1), laminin 9 (α4β2γ1) and laminin 10 (α5β1γ1).

In order to study the biological role of the laminin α4 chain, its gene was targeted in mouse embryonic stem cells. The Lama 4 null mice were viable and fertile but displayed transient hemorrhages at birth, a subtle motor impairment as adult mice, as well as resistance to obesity. The hemorrhages in the newborn null animals were located in soft tissues. Mild fetal hemorrhages were also seen, but they were never as extensive as in the newborn. We therefore hypothesized that the hemorrhages were the result of microvessel damage during delivery. Electron microscopy analysis revealed defects in the basement membranes of microvessels in muscle from newborn animals. Furthermore,
immunostaining of endothelial basement membranes showed that the amount of type IV collagen and nidogen were significantly reduced. It was concluded that laminin α4 is important for endothelial basement membrane formation.

The Lama 4 null mice also exhibited a mild motor impairment. Using neurophysiologic testing, we found muscle function to be intact and muscle histology revealed no obvious signs of dystrophy or myopathy. A detailed analysis of the neuromuscular junctions (NMJs) found these to be generally properly formed in Lama 4 null mice, but to have interesting specific defects in the apposition of the active zones to the junctional folds. In the NMJ, the laminin β2 and γ1 chains are expressed; leading to the conclusion that laminin 9 (α4β2γ1) is of importance for the localization of synaptic specializations in the NMJ.

Resistance to diet-induced as well as age-related obesity was another finding in Lama 4 null mice. The difference in the weight of males became significant after 10 weeks on a high-fat diet and was not due to a decrease in food intake. The adipocyte size was reduced to half of that of controls. The null animals had normal levels of serum lipids, except for cholesterol, which was not elevated to the same extent as in control animals on high-fat diet. Furthermore, nulls showed increased spontaneous activity, but no increase in resting metabolic rate. The hyperactivity could be part of a neurological syndrome, and is likely to contribute to the lean phenotype.

The cDNA encoding the human laminin α5 chain was obtained through screening of λ phage cDNA libraries and PCR amplification of cDNA mixes. The cDNA sequence was used to make a full-length laminin α5 expression vector, which was used to transfect a human embryonic kidney cell line. This cell line was also transfected with vectors expressing the laminin β1 and γ1 chains, enabling the cells to produce all the three chain components of laminin 10. The resulting cell line was shown to produce high amounts of recombinant laminin 10, which could be purified using affinity chromatography. The protein was shown to promote cell adhesion as well as cell migration. Using function-blocking antibodies, integrin α3β1 was identified as a main mediator of adhesion to laminin 10 in HT-1080 cells, as well as in human saphenous vein endothelial cells.
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**ABBREVIATIONS**

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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AchR</td>
<td>acetylcholine receptor</td>
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<tr>
<td>BM</td>
<td>basement membrane</td>
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<tr>
<td>CMD</td>
<td>congenital muscular dystrophy</td>
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<tr>
<td>DG</td>
<td>dystroglycan</td>
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<tr>
<td>DRG</td>
<td>dorsal root ganglia</td>
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<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>EHS</td>
<td>Engelbreth-Holm-Swarm</td>
</tr>
<tr>
<td>ES cells</td>
<td>embryonic stem cells</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
</tr>
<tr>
<td>GAG</td>
<td>glycosaminoglycan</td>
</tr>
<tr>
<td>G-domain</td>
<td>globular domain</td>
</tr>
<tr>
<td>Hb</td>
<td>hemoglobin</td>
</tr>
<tr>
<td>HSPG</td>
<td>heparan sulfat proteoglycan</td>
</tr>
<tr>
<td>HSVECs</td>
<td>human saphenous vein endothelial cells</td>
</tr>
<tr>
<td>LE-module</td>
<td>laminin-type epidermal growth factor-like module</td>
</tr>
<tr>
<td>LG-module</td>
<td>laminin G-domain module</td>
</tr>
<tr>
<td>LN-module</td>
<td>laminin-type N-terminal module</td>
</tr>
<tr>
<td>MuSK</td>
<td>muscle-specific receptor tyrosine kinase</td>
</tr>
<tr>
<td>N-linked</td>
<td>asparagine linked</td>
</tr>
<tr>
<td>NMJ</td>
<td>neuromuscular junction</td>
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<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
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<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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</table>
INTRODUCTION

Laminins are a family of basement membrane proteins and to date, 14 isoforms have been identified in vivo. During the past decade, knowledge has accumulated on the specific biological roles of the many different isoforms, e.g. through gene targeting experiments and by studies with native and recombinant laminins. The contribution of this thesis work to the field includes the phenotypic characterization of mice lacking the α4 chain, and the production and characterization of the properties of recombinant laminin 10 (α5β1γ1). Recent successful attempts to produce these large and complex molecules in vitro hold promise for the future of tissue engineering and stem cell technology, since laminins are known to fundamentally influence differentiation and maintenance of tissues.

During my thesis work, I have mainly worked on the laminin α4 and α5 chains. The human α5 sequence was used to generate and study recombinant laminin 10. The laminin α4 knock out mice lack all currently known laminin isoforms that contain this chain; laminins 8 (α4β1γ1), 9 (α4β2γ1) and 14 (α4β2γ3). Tissues where laminins 8 and 9 are expressed have been studied in this thesis, while the potential effects of the absence of laminin 14, which is known to be expressed in the retina, has not been studied. The focus of this thesis is therefore limited to studies of the biological roles of laminins 8, 9 and 10.

When analyzing the phenotype of mice lacking a particular gene it is crucial to know the in vivo expression pattern of the respective gene, in order to focus the analysis. The results of a phenotypic analysis of a mouse with an inactivated gene can generate useful information on its biological role, but can also provide clues as to what a human phenotype lacking the same gene would exhibit. However, there are differences between mice and men. In order to predict such differences or similarities, it is of use to know the exact expression patterns in the two species. I have therefore put emphasis on what is known to date about the localization of different laminin chains, in particular the α4 chain, in the Review of the literature section. In addition to an overview about laminins, I have also included a brief overview of the research fields where a role for laminin α4 has been established or is implied, namely in NMJ development, angio- and adipogenesis.
LIST OF PAPERS AND MANUSCRIPTS PRESENTED IN THIS THESIS


III Thyboll, J., Moverare, S., Kortesmaa, J., Soininen, R., Bergström, G., Ohlsson, C., Rozell, B., Tryggvason, K. Resistance to obesity in hyperactive laminin α4 deficient mice. *Manuscript*

REVIEW OF THE LITERATURE

Basement membranes

Basement membranes (BMs) are composed of extracellular matrix (ECM) components and can be seen by electron microscopy as thin electron dense structures. They are found beneath the endothelium and various epithelia of an organism. Certain cells are also surrounded by a BM, e.g., muscle cells, fat cells, pericytes and Schwann cells, while others, for instance fibroblasts, are not. In some organs, the BM is part of a specialized structure with a specific function, such as the glomerular filtration barrier and the blood-brain barrier. A BM is usually defined by its appearance through either the light- or the electron microscope. The structure was originally divided into the lamina lucida, a less electron dense layer closest to the cell, and the lamina densa, further away from the cell. In non-fixed frozen tissues, the lamina lucida is usually not apparent. The appearance after fixation is likely to be an artifact resulting from the alterations of tissues that occur when the tissue is subjected to dehydration.

Laminin, type IV collagen, nidogen and perlecan are examples of molecules found in most BMs (Timpl and Brown, 1996). The BM provides a structural framework for cells and tissues. In addition, the different molecules in a certain BM interact with cells through cell surface receptors. These interactions influence the cells profoundly by affecting cell survival, migration, differentiation and organization. It was recently shown that the formation of a laminin network on the surface of a cell can reorganize the cytoskeleton via interactions with cell surface receptors (Colognato et al., 1999). Among the best studied cell surface receptors for BM components are the integrins and dystroglycan. Interaction with these cell surface receptors seems to be vital for BM formation, since the absence of e.g. integrin β1 or α-dystroglycan in mice leads to very early embryonic lethality with failure of the formation of BMs (Aumailley et al., 2000; Henry and Campbell, 1998; Williamson et al., 1997). The BM also influences the cells by sequestering growth factors in the vicinity of a cell, which has been shown e.g. for the heparan sulfate proteoglycans (HSPGs) (Park et al., 2000).
Recently, much attention has been paid to the properties of fragments derived from BM proteins. For example, endostatin, which is a cleavage product from type XVIII present in blood vessel BMs, has been reported to be anti-angiogenic (O'Reilly et al., 1997). Another research field where BM components are most likely to be of use is the emerging stem cell field. Growing embryonic stem cells (ES cells) on ECM substrates, e.g. type IV collagen, in combination with different growth factors, has been shown to have specific effects on differentiation (Yamashita et al., 2000).

**Figure 1. A model of basement membrane structure in muscle.** The laminin network is bound to the cell surface by α-dystroglycan and integrins, and nidogen links the type IV collagen network to laminin. **Left**, features specific for neuromuscular junctions (NMJ). **Right**, features of extrasynaptic muscle BM. Modified from an image kindly provided by Peter Yurchenco.
**Laminins**

**Anatomy of laminins**

To date, 14 different laminin isoforms have been identified by immunoprecipitation studies from tissue material (Colognato and Yurchenco, 2000; Libby et al., 2000). They all share a common structure with three different subunits, termed α, β and γ chains, which are joined together into a heterotrimer through a long α-helical coiled-coil region. This region constitutes the back-bone of the cruciform-like shape of these molecules. The cDNA sequences of five different α, four β and three γ chains have been reported in man (Table I). Alternative splicing is known to occur in the case of two chains (Airenne, 2000; Galliano et al., 1995; Miner et al., 1997; Ryan et al., 1994). The nomenclature of laminin isoforms is confusing, and therefore, the chain components of a particular isoform are written in brackets. The official nomenclature is shown in Table II.

The different laminins can be divided into different classes by appearance, or predicted appearance, in rotary shadowing electron microscopy. The first identified laminin was laminin 1 (α1β1γ1), which was isolated from the mouse soft tissue tumor called the Engelbreth-Holm-Swarm (EHS) tumor (Timpl et al., 1979). Some laminin isoforms are similar to laminin 1 and are sometimes called “classical”. Others lack one (“topless”) or more of the short arms (“truncated”, Fig. 2). The ones that have a longer α chain than the original α1 chain could be called “long” (Colognato and Yurchenco, 2000).

Laminins are large modular glycoproteins and an entire molecule has not been crystallized. The methods used to study intact molecules includes rotary shadowing electron microscopy, atomic force microscopy, hydrodynamic methods and circular dichroism spectroscopy (Chen et al., 1998; Engel, 1992). The functions of different regions of the molecules have been studied by subjecting laminins to limited proteolysis, and the resulting fragments have been used for mapping sites at which interaction with other molecules take place. Different domains are defined based on sequence analysis, and designated with Roman numerals (Sasaki et al., 1988). The domains are further subdivided into modular units, like for instance domains III and V, which contain
laminin-type epidermal growth factor-like (LE) modules. The binding site for nidogen, a repeat of three LE-modules in the short arm of γ1, has been crystallized (Baumgartner et al., 1996; Fox et al., 1991; Paulsson et al., 1987; Stetefeld et al., 1996). Other domains include the laminin-type N-terminal modules (LN-modules) located at the ends of the short arms, which are important for polymerization of laminin, and the globular domain (G-domain), where several binding sites are located. The G-domain is further subdivided into five laminin G-domain modules (LG-modules), and is located at the C-terminus of the α-chain at the “base” of the cross. The LG-domains 4-5 of laminin α2 (α2β1γ1) was recently crystallized (Tisi et al., 2000).

Laminins are ancient molecules and can be found in the primitive polyp Hydra vulgaris (Sarras et al., 1994). The fruit fly Drosophila melanogaster and the worm Caenorhabditis elegans have two laminin α chains, one “classical” and one “long”, which have a high degree of similarity to the human form, and they also have full-sized β1 and γ1 chains (reviewed in (Hynes and Zhao, 2000). Homologues of all the human chains, except the β4, has been published in Mus musculus (for references see Table I).
Figure 2. **Upper:** Laminin heterotrimer structures. Some binding sites that have been mapped to specific part of the molecule (mostly laminin 1) are shown. Adapted from Colognato and Yurchenco, 2000. **Lower:** Domain structures of laminin chains. Structurally conserved or homologous domains are indicated by identical symbols. Modified from (Kortesmaa, 2000)
<table>
<thead>
<tr>
<th>Chain</th>
<th>Species</th>
<th>References (cDNA)</th>
</tr>
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<tbody>
<tr>
<td>α1</td>
<td>Man</td>
<td>(Haaparanta et al., 1991; Nissinen et al., 1991)</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>(Sasaki et al., 1988)</td>
</tr>
<tr>
<td>α2</td>
<td>Man</td>
<td>(Vuolteenaho et al., 1994)</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>(Bernier et al., 1995)</td>
</tr>
<tr>
<td></td>
<td>Drosophila</td>
<td>(Martin et al., 1999)</td>
</tr>
<tr>
<td>α3A,B</td>
<td>Man</td>
<td>(Doliana et al., 1997; Ryan et al., 1994)</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>(Galliano et al., 1995)</td>
</tr>
<tr>
<td>α4</td>
<td>Man</td>
<td>(Iivanainen et al., 1995a; Richards et al., 1994)</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>(Frieser et al., 1997; Liu and Mayne, 1996)</td>
</tr>
<tr>
<td>α5</td>
<td>Man</td>
<td>(Durkin et al., 1997), partial sequence</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>(Miner et al., 1995)</td>
</tr>
<tr>
<td></td>
<td>Drosophila</td>
<td>(Garrison et al., 1991)</td>
</tr>
<tr>
<td>β1</td>
<td>Man</td>
<td>(Pikkarainen et al., 1987)</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>(Sasaki et al., 1987)</td>
</tr>
<tr>
<td></td>
<td>Drosophila</td>
<td>(Montell and Goodman, 1988)</td>
</tr>
<tr>
<td>β2</td>
<td>Man</td>
<td>(Iivanainen et al., 1995b; Wewer et al., 1994)</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>(Noakes et al., 1995a)</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>(Hunter et al., 1989)</td>
</tr>
<tr>
<td>β3</td>
<td>Man</td>
<td>(Gerecke et al., 1994)</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>(Utani et al., 1995)</td>
</tr>
<tr>
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<td>Man</td>
<td>(Olson, 1997; Sulston, 1998), partial sequence</td>
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<td>Man</td>
<td>(Pikkarainen et al., 1988)</td>
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<td>Mouse</td>
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<tr>
<td></td>
<td>Drosophila</td>
<td>(Chi and Hui, 1989)</td>
</tr>
<tr>
<td>γ2, γ2*</td>
<td>Man</td>
<td>(Airenne et al., 1996; Kallunki et al., 1992)</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>(Sugiyama et al., 1995)</td>
</tr>
<tr>
<td>γ3</td>
<td>Man</td>
<td>(Koch et al., 1999)</td>
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<tr>
<td></td>
<td>Mouse</td>
<td>(Iivanainen et al., 1999)</td>
</tr>
<tr>
<td>Current name</td>
<td>Chain composition</td>
<td>Previously used names</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>Laminin 1</td>
<td>α1β1γ1</td>
<td>EHS-laminin</td>
</tr>
<tr>
<td>Laminin 2</td>
<td>α2β1γ1</td>
<td>Merosin</td>
</tr>
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<td>Laminin 3</td>
<td>α1β2γ1</td>
<td>s-laminin</td>
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<tr>
<td>Laminin 4</td>
<td>α2β2γ1</td>
<td>s-merosin</td>
</tr>
<tr>
<td>Laminin 5</td>
<td>α3β3γ2</td>
<td>kalinin, nicein, epiligrin</td>
</tr>
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<td>Laminin 6</td>
<td>α3β1γ1</td>
<td>k-laminin</td>
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<td>Laminin 7</td>
<td>α3β2γ1</td>
<td>Ks-laminin</td>
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<tr>
<td>Laminin 8</td>
<td>α4β1γ1</td>
<td>-</td>
</tr>
<tr>
<td>Laminin 9</td>
<td>α4β2γ1</td>
<td>-</td>
</tr>
<tr>
<td>Laminin 10</td>
<td>α5β1γ1</td>
<td>-</td>
</tr>
<tr>
<td>Laminin 11</td>
<td>α5β2γ1</td>
<td>-</td>
</tr>
<tr>
<td>Laminin 12</td>
<td>α2β1γ3</td>
<td>-</td>
</tr>
<tr>
<td>Laminin 14</td>
<td>α4β2γ3</td>
<td>-</td>
</tr>
<tr>
<td>Laminin 15</td>
<td>α5β2γ3</td>
<td>-</td>
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</table>
Synthesis of laminins and network formation

BM components are synthesized and secreted by the cells they surround or underlie. The BM can even be synthesized by several different cell types acting in concert, e.g. in the case of developing mouse intestine, where both the mesenchymal and the epithelial cells synthesize laminins that become deposited into the subepithelial BM (Lefebvre et al., 1999). Laminins are synthesized in the endoplasmic reticulum. The trimers are held together by the α helical coiled-coil region, and the specificity of chain assembly is determined by interactions of residues in the core of the coil, as well as by interchain ionic interactions (Beck et al., 1993; Beck et al., 1990; Hunter et al., 1990; Hunter et al., 1992). Furthermore, the assembled heterotrimers are stabilized by disulfide bonds, and they undergo N-linked glycosylation before secretion (Cooper et al., 1981; Peters et al., 1985). In the case of laminin 1 (α1β1γ1), it has been shown that the expression of all three chains is required for the secretion of the β1 and γ1 chains, while the α1 chain can be secreted as a monomer to some extent (Yurchenco et al., 1997).

Many laminins undergo proteolytic cleavage in the G-domain, e. g. in laminin 8. Using monoclonal antibodies against the α4 LG 4-5, it was shown that this fragment is lacking from the mature endothelial BM of mouse tissues, showing that cleavage at this site occurs in vivo (Talts et al., 2000). Proteolytically processed laminin 5 (α3β3γ2) has been found specifically in tumors and tissues undergoing remodeling, and this form of laminin 5 promotes migration of epithelial cells, which indicates that sites that cryptic sites interact with specific receptors (Giannelli et al., 1997; Goldfinger et al., 1998).

Once secreted, the laminins may form a network polymer as part of the BM, a process likely to be mediated in vivo by cell surface matrix receptors (Bloch et al., 1997; DiPersio et al., 1997; Henry and Campbell, 1998; Sasaki et al., 1998). Laminin can polymerize in vitro, a process that is calcium-dependent and that involves interactions between domain VI of the short arms of the laminin molecules (Paulsson, 1988; Yurchenco and Cheng, 1993; Yurchenco et al., 1985). It has been proposed that the polymerization in vivo is facilitated by laminin binding to cell surface receptors. This would increase the local concentration to that needed for polymerization to take place.
(Henry and Campbell, 1998; Sasaki et al., 1998). Furthermore, it has been shown that the ligation of these receptors with a polymerized laminin can lead to profound changes in the cells by influencing the organization of the cytoskeleton (Colognato et al., 1999).

Because of their size and complexity, laminins have been difficult to produce recombinantly. Laminin 1 (α1β1γ1) and laminin 8 (α4β1γ1) have been successfully produced (Kortesmaa et al., 2000; Yurchenco et al., 1997).

**Figure 3. Model of receptor-facilitated laminin assembly.** Laminins can polymerize spontaneously in vitro, but in vivo it is likely that this process is facilitated by binding of the C-terminus of the laminins to cell surface receptors, thereby increasing the concentration of laminins locally. Adapted from Colognato and Yurchenco, 2000.
Interactions with other molecules

Cell surface receptors

Laminins have been shown to interact with numerous molecules. The integrin family of receptors and dystroglycan are the most thoroughly studied of the cell surface receptors. The integrins are heterodimeric receptors, each member being composed of different $\alpha$ and $\beta$ chains, combining into more than 20 integrins in vivo (van der Flier and Sonnenberg, 2001). Through interactions with ECM molecules they provide a physical adhesion link for cells, as well as transducing signals via e.g. focal adhesion and mitogen activated kinases. A list of integrins shown to interact with laminins is shown in Table III. The $\alpha6\beta1$ and $\alpha7\beta1$ integrins seem to be quite specific for laminin, with a few exceptions. In contrast, the $\alpha1\beta1$ and $\alpha2\beta1$ integrins are promiscuous and bind a variety of ligands, including laminin 1 ($\alpha1\beta1\gamma1$), laminin 2/4 ($\alpha2\beta1\gamma1/\alpha2\beta2\gamma1$) and several types of collagens. The $\alpha7\beta1$ integrin is expressed in muscle, particularly in developing myotubes, and has been shown to interact with laminin 1 and the muscle laminins 2/4. Consequently, inactivation of the $\alpha7$ integrin leads to muscular dystrophy (Mayer et al., 1997). Integrin $\alpha6$ null mice, which lacks the $\alpha6\beta1$ and $\alpha6\beta4$ integrins, die shortly after birth, suffering from severe skin blistering due to the absence of $\alpha6\beta4$ from hemidesmosomes (Georges-Labouesse et al., 1996). The mice also displayed brain abnormalities, but many organs were unaffected by the absence of these integrins, predicted to be essential for e.g. kidney development (Sorokin et al., 1990). As mentioned before, integrin $\beta1$-null embryos fail to develop beyond the implantation stage, due to lack of BM formation (Aumailley et al., 2000).

Another important laminin receptor, dystroglycan, is an adhesion receptor (Winder, 2001) in the dystroglycan glycoprotein complex (DGC, see Fig. 1), which is part of the link between the ECM and the cytoskeleton of muscle cells. It interacts with the C-terminal G-domain of several laminins with different affinities, in a heparin-sensitive manner. The interaction between carbohydrate moieties of dystroglycan and laminin $\alpha2$ LG5 was recently mapped when the crystal structure of this domain was
solved (Hohenester et al., 1999). The dystroglycan null embryos die early, and show ruptures of the extraembryonic Reichert’s membrane (Williamson et al., 1997).

A few other receptors for laminins have been identified, e.g. HNK-1, B-CAM and LAR (El Nemer et al., 1998; Hall et al., 1997; O’Grady et al., 1998). The first protein to be proposed as a laminin receptor was the 67kDa elastin/laminin receptor (Mecham et al., 1989; Rao et al., 1983), but its role as an in vivo receptor has not been thoroughly established.

Table III: Laminin interactions with integrins.

<table>
<thead>
<tr>
<th>Laminin isoform</th>
<th>Integrin</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laminin 1</td>
<td>α1β1</td>
<td>(Ignatius and Reichardt, 1988; Tomaselli et al., 1988)</td>
</tr>
<tr>
<td></td>
<td>α2β1</td>
<td>(Languino et al., 1989)</td>
</tr>
<tr>
<td></td>
<td>α3β1</td>
<td>(Wayner and Carter, 1987)</td>
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<td>α6β1</td>
<td>(Sonnenberg et al., 1988)</td>
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<td>α6β4</td>
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<td>α7β1</td>
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<td>α9β1</td>
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<td>αvβ3</td>
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<td>Laminin 2/4</td>
<td>α1β1, α2β1</td>
<td>(Colognato et al., 1997)</td>
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<td></td>
<td>α3β1</td>
<td>(Delwel et al., 1994)</td>
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<td>α6β1</td>
<td>(Delwel et al., 1993)</td>
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<td></td>
<td>α7β1</td>
<td>(Yao et al., 1996)</td>
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<tr>
<td>Laminin 5</td>
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<td>(Carter et al., 1991; Delwel et al., 1994)</td>
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<td></td>
<td>α6β1, α6β4</td>
<td>(Delwel et al., 1993; Niessen et al., 1994)</td>
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<td>not α7β1</td>
<td>(Yao et al., 1996)</td>
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<tr>
<td>Laminin 8</td>
<td>α6β1, α6β4</td>
<td>(Geberhiwot et al., 1999; Kortesmaa et al., 2002; Kortesmaa et al., 2000; Sixt et al., 2001a; Sixt et al., 2001b)</td>
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<tr>
<td>Laminin 10/11</td>
<td>α3β1, α6β1*, α6β4*</td>
<td>(Kikkawa et al., 2000; Kikkawa et al., 1998).</td>
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* The result of paper IV of this thesis is not in agreement with these findings; see results and discussion concerning this paper.
Interactions with other extracellular matrix proteins

Type IV collagen is a network-forming collagen present in BMs, with the capacity to polymerize (Fig. 1). The role of collagens in maintaining structural integrity is well established. The type IV collagen network of BMs does not interact directly with the laminin network, but are thought to be interconnected via other molecules such as nidogens (Fig 1). Two isoforms of nidogen have been described, nidogen-1 (Carlin et al., 1981) and nidogen-2 (Kohfeldt et al., 1998), with mostly overlapping expression patterns. Both isoforms bind type I and IV collagen, perlecan and laminins, although nidogen-2 binds to the specific nidogen binding site in the laminin γ1 chain with lower affinity (Kohfeldt et al., 1998).

The HSPG perlecan is present in BMs as well as in cartilage, and has been shown to interact with nidogen and type IV collagen. Interaction between the heparan sulfate side chains and the G-domain of laminins have been demonstrated (Battaglia et al., 1992; Talts et al., 1999). Perlecan is expressed already in the pre-implantation embryo and has therefore been hypothesized to be of fundamental importance, e.g. for adhesion of the embryo to the uterine wall (Smith et al., 1997). However, mice lacking the perlecan gene develop normally until E 10-12, when the majority of the embryos die due to hemorrhages into the pericardial sac resulting from disruptions of the myocardium, possibly due to compromised structural integrity of the BMs of the heart. The surviving embryos showed severe chondrodysplasia and brain abnormalities and died around birth (Costell et al., 1999).

Another BM proteoglycan is agrin, which interacts with a binding-site in the coiled-coil region of laminins (Kammerer et al., 1995). It has an important role in NMJ formation and will be discussed further below. The chondroitin sulfate proteoglycan Bamacan is also widely expressed in BMs, but its function is less well known (Wu and Couchman, 1997).

Five isoforms of the extracellular protein fibulin have been identified and localized to BMs, as well as to the adjacent microfibrills of the ECM. (Giltay et al., 1999; Kowal et al., 1999; Pan et al., 1993a; Pan et al., 1993b). Fibulin-1 and -2 have been shown to bind laminin 1, nidogen-1 and weakly to nidogen-2, but only fibulin-1 binds to
perlecan (Brown et al., 1997; Hopf et al., 1999; Kohfeldt et al., 1998; Pan et al., 1993a; Sasaki et al., 1995).

**The importance of different BM components and their receptors for BM assembly**

A number of recent studies have further strengthened the notion that the interaction of laminins with cell surface receptors is necessary for laminin polymerization and BM assembly. The dystroglycan null embryos show defects in BM formation, and furthermore, embryoid bodies generated from dystroglycan null ES cells lack BMs (Henry and Campbell, 1998). A recent study using ES cells lacking either dystroglycan, β1 integrin or perlecan, shed further light on the complex mechanisms of BM formation. Exogenously added laminin 1 (α1β1γ1) was shown to organize into well structured linear arrays and plaques on the surface of ES cells. (Henry et al., 2001). Dystroglycan null ES cells failed to aggregate laminin 1 altogether, underlining the importance of the dystroglycan-laminin interaction. On the other hand, laminin 1 did form organized structures on the surfaces of β1-integrin null cells, but mostly in small plaques and not into well structured linear arrays, such as those seen on wild-type cells. This further supports a role for β1 integrins in the organization of BMs. On perlecan null ES cells, laminin 1 aggregated, but the organization into plaques and linear arrays on the cell surface was defective. This would imply a more limited role for perlecan in BM formation, but an important role in organizing laminin into more complex structures. This is in line with the fact that most BM developed normally in perlecan null embryos (Costell et al., 1999).

A central role for nidogen in BM formation has been proposed, but genetic evidence exist that nidogen is not a crucial player. The nidogen binding site in the laminin γ1 chain has been mutated in ES cells, but this did not lead to failure of BM membrane formation (Mayer et al., 1998). It was also shown that nidogen is expendable in BM formation of *C. elegans*, but may be involved in nerve positioning (Kim and Wadsworth, 2000).

The presence of laminins seems to be of vital importance for the assembly of BMs. This is a bit puzzling, since type IV collagen has the ability to both self assemble
and interact directly with cell surface receptors. Despite this, embryos lacking the laminin \( \gamma_1 \) chain die very early due to failure of BM formation (Smyth et al., 1999). Embryoid bodies generated from the \( \gamma_1 \) deficient ES cells also lacked BMs, and showed irregular deposition of type IV collagen and perlecan. Although nidogen has been shown to bind to type IV collagen and perlecan in vitro, it was not deposited into the ECM, but secreted into the medium. Another study used the antisense strategy to abolish laminin \( \alpha_1 \) expression in CaCo2 cells cultured on top of fibroblasts. These CaCo2 cells also failed to deposit type IV collagen and nidogen into BMs (De Arcangelis et al., 1996). A possible explanation for these observations could be that laminin assembly and its subsequent interactions with cell surface receptors are transmitting signals necessary for the expression of the relevant receptors for type IV collagen and nidogen (Colognato and Yurchenco, 2000).

**Tissue distribution and biological roles of different laminin chains**

The expression of different laminin chains have been studied with Northern hybridizations, RNase protection analysis and *in situ* hybridizations, and the tissue distribution has been characterized by immunostainings. The distribution patterns of laminin isoforms in BMs are complex. The laminin isoforms in the BM of a particular structure can vary at different developmental stages, and many times several isoforms are present in the same BM. Some general conclusions can be made from the numerous studies of the distribution of laminin chains. The \( \beta_1 \) and \( \gamma_1 \) chains are present in most BMs. Of the \( \alpha \) chains, the \( \alpha_1 \) chain have a very restricted expression pattern in some epithelial BMs, while the \( \alpha_4 \) and particularly the \( \alpha_5 \) chains, are widely distributed. The \( \alpha_2 \) is mainly expressed in tissues of mesodermal origin, such as heart and skeletal muscle, while the \( \alpha_3 \) is expressed in epithelial BMs.

**The \( \beta \) and \( \gamma \) chains**

The \( \alpha_1, \beta_1 \) and \( \gamma_1 \) chains are expressed already in the pre-implantation embryo (Shim et al., 1996). The \( \gamma_1 \) chain is the most ubiquitous laminin chain, present in almost all BMs
of developing and mature tissues, and the inactivation of its gene in mice leads to very early embryonic lethality at 5.5 days post-coitum (Smyth et al., 1999). As mentioned earlier, the embryos lack BMs, leading to retention of primitive endoderm cells in the inner cell mass and failure of parietal yolk sac formation. The β1 chain (Klein et al., 1990) has a somewhat more restricted distribution than the γ1 chain, and the β2 chain (Iivanainen et al., 1995b; Wewer et al., 1994) was reported to have an even more restricted expression pattern in mouse (Durbeej et al., 1996; Glukhova et al., 1993; Patton et al., 1997; Sanes et al., 1990). However, a recent study using antibodies raised against recombinantly produced domain IV of the β2 chain as well as to domain IV of β1, indicate that both chains are present in many BMs (Sasaki et al., 2002). In a few cases the expression is confined to one of the two β chain variants. For instance, the β2 chain is present in the synaptic BM and in the perineurium, sites which the β1 chain is absent from. The β1 chain is present in the developing glomerular basement membrane (GBM), while the β2 is abundant in the mature GBM (Noakes et al., 1995b). Mice lacking the β2 chain have defects in NMJ development and suffer from massive proteinuria and die during postnatal week 2-3 (Noakes et al., 1995a; Noakes et al., 1995b).

All three chain components of laminin 5 (α3β3γ2) are expressed in epithelia of the kidney and respiratory-, GI-, and urinary tracts, as well as in skin and the developing tooth (Aberdam et al., 1994a; Kallunki et al., 1992). Mutations in any of the three chain components of laminin 5 in man leads to the postnatally lethal skin blistering disease junctional epidermolysis bullosa (Aberdam et al., 1994b; Kivirikko et al., 1995; Pulkkinen et al., 1994a; Pulkkinen et al., 1994b), as does inactivation of the α3 gene or β3 gene in mice (Kuster et al., 1997; Ryan et al., 1999). Two splice variants of the γ2 chain have been described, termed γ2 and γ2*(Airenne, 2000). The mRNA of the shorter γ2* form has a more restricted expression pattern than the γ2 (Airenne et al., 2000). The γ2 chain is expressed in many cancer forms of epithelial origin and has been shown to serve as a marker for the invading front of some carcinomas (Pyke et al., 1995; Skyldberg et al., 1999). The novel γ3 chain is expressed in some epithelia and in the retina (Iivanainen et al., 1999; Libby et al., 2000), but was also found in non BM locations on the apical surfaces of ciliated epithelia, e. g. in the oviduct (Koch et al., 1999). No studies of the human laminin β4 chain has been published (Olson, 1997).
The α chains

As mentioned before, laminin 1 (α1β1γ1) was the first laminin identified, and the first polyclonal antibodies were raised against this laminin. The initial immunofluorescence studies showed a widespread distribution of laminin 1 in BMs (Timpl et al., 1979). When chain specific antibodies were produced, immunofluorescence studies of human tissues showed the α1 chain to be widely expressed in the adult (Engvall et al., 1990), in disagreement with in situ studies showing a restricted expression pattern (Vuolteenaho et al., 1994). The reason behind the conflicting data was later shown to be that the antibodies used for these studies were actually reacting with the α5 and not the α1 chain (Church and Aplin, 1998; Kikkawa et al., 1998; Tiger et al., 1997).

A detailed study of the distribution of the α1 chain in fetal and adult human tissues using immunofluorescence was recently published (Virtanen et al., 2000), and it was shown that the α1 chain is present in the fetus at 16 weeks in epithelia of the developing kidney, budding bronchial tubules, testis and epididymis as well as in developing glands in the gastrointestinal tract and hair buds of the skin. In adult tissues, laminin α1 was found in the proximal tubules of the kidney, in various glandular epithelia of the GI-tract, thyroid and mammary glands, as well as in the endometrium of the uterus and in testis. Positive staining was also seen in capillaries in the brain, and this was later shown to be due to the presence of laminin α1 in the BM of the astrocytic endfeet surrounding vessels in the brain, and not to presence in endothelial BM (Sixt et al., 2001a). The distribution of laminin α1 has been studied in developing and adult mice (Durbeej et al., 1996; Ekblom et al., 1990; Falk et al., 1999; Frojdman et al., 1995; Gu et al., 1999; Klein et al., 1990; Miner et al., 1997; Sorokin et al., 1997a). In general, the expression patterns are in agreement with the findings in human tissues, with a few exceptions. The function of the laminin α1 chain has not been studied with gene targeting experiments, but many studies interfering with the function of laminin 1 (α1β1γ1) have been performed. For example, experiments using the addition of anti laminin 1 antibodies to lung explant or embryonic kidney mesenchyme cultures have identified laminin 1 as a crucial factor for branching morphogenesis in lung (Schuger et al., 1990; Schuger et al., 1991), and for kidney development (Sorokin et al., 1992). Furthermore, the addition of
laminin fragments that interfere with laminin polymerization, to such organotypic cultures, disturbs BM membrane assembly at the epithelial-mesenchymal interface and leads to failure of epithelial cells to polarize, and perturbs bronchial smooth muscle cell arrangement (Schuger et al., 1998).

The mRNA of the laminin α2 chain is expressed in cardiac muscle, pancreas, lung, spleen, kidney, adrenal gland, skin, testis, meninges, choroid plexus, and certain other regions of the brain of human fetal tissues (Vuolteenaho et al., 1994). No thorough study using immunolocalization of this α chain has been performed on human tissues, but it has been localized to the BMs of the myocytes, Schwann cells (Leivo and Engvall, 1988), and the developing kidney and it has been found in the mesangium of the mature kidney (Virtanen et al., 1995). In mice, it has been localized to the BMs of developing and adult skeletal muscle, heart muscle, endoneurium of peripheral nerves, the mesangium and some tubular BMs of the kidney (Miner et al., 1997; Patton et al., 1997; Sorokin et al., 2000) as well as in blood vessels of heart and skeletal muscle (Talts et al., 2000). It is also present in the intestine during late gestation and in crypt regions of the mature tissue (Lefebvre et al., 1999).

Mutations in the human gene encoding the laminin α2 chain has been detected in a group of muscular diseases named congenital muscular dystrophies (CMD) (Helbling-Leclerc et al., 1995; Tome et al., 1994). In the severe forms of this disease, hypotonia and weakness become evident shortly after birth, and the early motor development is delayed and is associated with contractures. The pathological findings in these patients include dystrophic changes of the skeletal muscle, decreased motor nerve conductivity and white matter abnormalities of the CNS (Philpot et al., 1999; Shorer et al., 1995). There are naturally occurring mouse models of the human CMD; the dy and dy2j strains (Meier and Southard, 1970; Michelson, 1955). In the dy strain, the laminin α2 is almost totally absent (Xu et al., 1994). Immunostaining of the muscles from the dy2j mice shows that the α2 chain is present in the tissues, but laminin 2 (α2β1γ1) isolated from these mice show defective polymerization due to a mutation in domain VI (Colognato and Yurchenco, 1999; Sunada et al., 1995). This example demonstrates the importance of polymerization of laminin for proper function in vivo.
Targeting of the α2 gene has been performed by two independent groups (Kuang et al., 1998; Miyagoe et al., 1997), and the resulting mice, that lack expression of the α2 chain totally, resembles the spontaneous mutants but have shorter life spans. In one of the cases the expression in muscle was rescued, and these animals displayed milder motor dysfunction than the total nulls. The remaining dysfunction could be explained by nervous system defects (Kuang et al., 1998).

There are two splice variants of the α3 chain (α3A and α3B, (Ferrigno et al., 1997; Galliano et al., 1995; Miner et al., 1997; Ryan et al., 1994) and studies of mRNA expression found both forms in some epithelia, while only the α3B was found in some tissues, e.g. in the developing brain (Doliana et al., 1997; Galliano et al., 1995). As mentioned in the previous chapter, mutations in the α3 chain leads to epidermolysis bullosa (Kivirikko et al., 1995), and mice lacking the gene for this chain also show severe skin blistering (Ryan et al., 1999).

The laminin α4 chain was cloned first in man (Iivanainen et al., 1995a; Richards et al., 1996; Richards et al., 1994) and shortly thereafter in mouse (Frieser et al., 1997; Iivanainen et al., 1997; Liu and Mayne, 1996). Immunolocalization of the laminin α4 has been studied in some detail in both man and mouse. In mice, expression is seen in developing as well as mature blood vessels, around developing skeletal muscle fibers, in the developing kidney, in peripheral nerves, in bone marrow, in lung alveolar septa and around cardiomyocytes (Gu et al., 1999; Iivanainen et al., 1997; Miner et al., 1997; Patton et al., 1997; Ringelmann et al., 1999; Sorokin et al., 2000; Sorokin et al., 1997a; Talts et al., 2000). In the intestine it is present in the mucosal connective tissue and smooth muscle layer (Lefebvre et al., 1999). The expression of laminin α4 in the NMJ, adipose tissue and blood vessels are especially important for the interpretation of the phenotypic manifestations in mice lacking this chain, and will be discussed in detail in separate sections.

A thorough study of human tissues using antibodies raised against the recombinantly produced laminin 8 (α4β1γ1) was recently performed (Petäjäniemi et al., 2002). The α4 chain was found in BM of myotubes of the developing heart, skeletal and smooth muscle. In the adult it was absent from the mature skeletal muscle cell BM, but still present around cardiomyocytes and smooth muscle cells. It was localized to specific
parts of the developing nephron, e. g. associated with invading capillaries in the comma-shaped stage, but adult kidney stained positive in the mesangium and in some tubules, and not in the GBM. In the gastrointestinal canal, it was found in the BMs of some glands and in the smooth muscle layers and it was present in BMs of acini of the salivary gland and exocrine pancreas. BMs of capillaries and of smooth muscle cells in fetal and adult lung were clearly immunoreactive, as were myoid cells of the testis. The BMs of adipocytes and of axons of developing and mature nerves were positive. All endothelial BMs of fetal and adult tissues contained the α4 chain, except the capillaries of the glomerulus. One striking discrepancy between the staining patterns of mouse and man could be noted in the skin, where the epithelial BM was positive in man, although weakly, while this is not the case in mice. The α4 chain is also found in non-BM locations; in the connective tissue stroma of the endometrium (Petäjäniemi et al., 2002) and in various blood cells, for example thrombocytes and lymphoid cells (Geberhiwot et al., 2001; Geberhiwot et al., 1999).

The α5 chain is the most recently identified laminin α chain, and it is widely distributed in developing and mature BMs. This α chain is present in most BMs of the embryo at E 8.5, but later during development its distribution becomes somewhat more restricted, and it is not present in the early stages of blood vessel BM development. It is only seen in the BMs of larger vessels of the embryo and the newborn mouse (Durbeej et al., 1996; Miner et al., 1998; Sorokin et al., 1997a; Sorokin et al., 1997b). In the adult mouse it is still present in BMs of the lung, skin and intestinal epithelial and smooth muscle cell BMs, in virtually all kidney BMs, and it is also present in smaller blood vessels (Lefebvre et al., 1999; Miner et al., 1997; Patton et al., 1997; Sorokin et al., 1997a; Sorokin et al., 1997b).

The absence of this important α chain leads to defective development of several organs and intrauterine death at latest E 17, and the phenotype includes exencephaly, failure of digit separation and placental defects (Miner et al., 1998). Its prominent role in kidney development is underlined by the fact that the α5 nulls have severely defective glomerulogenesis and sometimes renal agenesis (Miner and Li, 2000).
**BMs and development of the neuromuscular junction**

The term neuromuscular junction (NMJ) refers to the specialized structure found at the contact site between motor nerve and muscle cell, where the neuromuscular signal is transmitted. It is comprised of the motor nerve terminal, which is capped by a Schwann cell, and the specialized structure of the postsynaptic muscle cell membrane, which forms a shallow gutter where the nerve terminal resides. Acetylcholine is the central neurotransmitter at this site, and it is concentrated in synaptic vesicles in the motor nerve terminal. The nerve terminal is polarized, with synaptic vesicles clustered into dense structures, called active zones, which lie apposed to invaginations of the muscle cell membrane, called junctional folds. A BM lies between the nerve terminal and the muscle cell membrane, and this also lines the junctional folds. This synaptic BM contains type IV collagen, nidogen, and several laminin isoforms. Two other important components of the synaptic BM are agrin (Cohen and Godfrey, 1992) and neuregulin (Goodearl et al., 1995). The postsynaptic BM also harbors high concentrations of the receptor for acetylcholine, AChR.

During development of the NMJ, motor axons, closely followed by accompanying Schwann cells, innervate the developing muscle at the stage when myoblasts fuse to form myotubes (Sanes and Lichtman, 1999). The AChRs are expressed by the myotube and is diffusely distributed in the plasma membrane. However, upon contact with the axon terminal, they become concentrated at the site of contact through clustering of the existing AChRs, as well as through transcriptional regulation. Agrin was found to be a central molecule for the clustering process, as demonstrated by the fact that mice lacking agrin exhibit severe defects of post synaptic specialization with few AChR clusters (Gautam et al., 1996). Agrin is a HSPG (Denzer et al., 1995; Tsen et al., 1995) that exist in two different splice forms, and the one expressed by nerve terminals is essential for AChR clustering (Burgess et al., 1999). A transmembrane protein tyrosine kinase expressed by muscle cells, muscle-specific receptor tyrosine kinase (MuSK), has been proposed as a receptor for agrin, since mice lacking this receptor show similar defects of
AChR clustering as agrin deficient mice (DeChiara et al., 1996). However, agrin does not bind MuSK directly, which has led to the assumption that MuSK is part of a receptor complex interacting with agrin (Glass et al., 1996).

The different laminin isoforms seem to play important roles in NMJ formation. During development, the muscle BM expresses the laminin α2, α4 and α5 chains, while the mature muscle BM only contains α2. The α4 and α5 chains are however retained within the synaptic BM, so the mature structure contains the α2, α4, α5, β2 and γ1 chains (Patton et al., 1997). This would make the presence of laminins 4 (α2β2γ1), 9 (α4β2γ1) and 11 (α5β2γ1) likely in the synaptic BM. Myotubes synthesize β1 and β2 laminins, but localizes the latter to AChR rich sites (Martin et al., 1995). However, a

Figure 4. Schematic drawing of the NMJ. The nerve terminal harboring synaptic vesicles reside in a shallow gutter in the muscle cell membrane. The vesicles are concentrated to regions named active zones, which lie apposed to invaginations of the muscle cell membrane; junctional folds. The BM residing between the nerve terminal and the muscle cell is called the postsynaptic BM, which extends into the junctional folds.
recent study found the β2 chain in the BM outside the synaptic cleft, although at much lower concentrations than in the cleft (Sasaki et al., 2002). Inactivation of the laminin β2 gene in mice led to severe defects with few active zones in the nerve terminals and invagination of the synaptic cleft by Schwann cells (Noakes et al., 1995a), showing that the synaptic laminins are required for synaptic differentiation.

**Laminins in angiogenesis and adipogenesis**

Angiogenesis is the formation of new blood vessels from preexisting ones. This takes place in the developing embryo, but also in mature tissues during the regular variations in the vascular bed of the endometrium, and in tissues subjected to disease or undergoing repair (Carmeliet and Jain, 2000; Smith, 2001). A large number of molecules capable of stimulating or inhibiting this process have been identified in recent years, and this has raised much hope of influencing many disease processes such as cancers and inflammatory diseases.

Vascular endothelial growth factors (VEGFs) and fibroblast growth factor (FGF) have been shown to play important roles for instance in angiogenesis models, where an uninjured vasculature is subjected to factors initiating the development of sprouts (Carmeliet, 2000; Cross and Claesson-Welsh, 2001). Such models include the cornea angiogenesis assay in mice, where a pellet with a particular growth factor is implanted into the avascular cornea, which become invaded by new vessel sprouts (Cao and Cao, 1999; Cao et al., 1998). Initial increases in permeability of the preexisting vessels are of importance, leading to leakage of plasma proteins forming a plug, which is used as a scaffold for the endothelial cells that start to form the sprout (van Hinsbergh et al., 2001). Two aspects of endothelial BMs are of importance in the formation of sprouts. First, the BM of the preexisting vessel has to be broken down, and then a new BM has to form around the sprout, re-enforcing the new structure. Supportive cells, pericytes, are also recruited to the new vessel, a step where PDGF-B has been shown to be of vital importance (Lindahl et al., 1997). As discussed previously, BM proteins have the capacity to regulate the accessibility of growth factors (Iozzo and San Antonio, 2001),
something that may be of importance for the recruitment of the pericytes. That BM components are of importance for regulating the process of angiogenesis was established when an angiogenesis inhibitor, endostatin, was found to be a fragment generated from the proteolytic processing of the BM component collagen XVIII (O'Reilly et al., 1997). The NC1 domains of type IV collagen chains have also been shown to have angiostatic effects (Colorado et al., 2000; Petitclerc et al., 2000).

Laminins are present in the BM of blood vessels. At E 11, laminin α4 is the only known α chain clearly expressed by vessels, accompanied by the β1 and γ1 chains (Patton et al., 1997). Laminin α5 is seen in developing larger vessels, and after birth its expression is turned on in capillaries, clearly seen by immunostainings at three weeks of age (Sorokin et al., 1997b). Using immuno electron microscopy, the laminin α2 chain was recently shown to be present in capillaries of the adult mouse heart (Talts et al., 2000), where it has not been found before (Miner et al., 1997). The β2 chain has previously been reported to be primarily expressed in larger vessels (Hunter et al., 1989; Patton et al., 1997; Sanes et al., 1990), but a recent study using an other antibody showed the β2 chain to be present also in capillaries of adult tissues (Sasaki et al., 2002). In the vasculature of the brain, a specific distribution of the laminin chains is seen. The endothelial cells are surrounded by astrocytic endfeet, which have their own BM that contains laminins α1 and α2, while the endothelial BM contain the α4 and α5 chains. There is one important exception, the postcapillary venules, a site where transmigration of leukocytes takes place upon inflammation. These vessels contain only the laminin α4 chain and not the α5 (Sixt et al., 2001a).

The injection of a mixture of ECM components (so called “Matrigel”) and FGF into the subcutis of mice leads to the formation of a solid plug, into which blood vessels grow. This has been used as a model for studying angiogenesis, but has also been shown to be a model for studying the formation of fat tissue, adipogenesis (Kawaguchi et al., 1998). After a few weeks, the ECM plug becomes converted into fat tissue. With obesity emerging as a major threat to health in many countries, the interest in adipose tissue is growing. Much effort is put into understanding the mechanisms that control fat tissue development and its central cell players; the adipocyte and its precursor; the preadipocyte. In addition to being a storage cell for triglycerides, the adipocyte is an
important player in the complex systems that regulate energy balance, together with cells of other organs such as liver, skeletal muscle and the hypothalamus. The adipocyte is involved in an intricate network of endocrine, paracrine and autocrine signals. An explosion in the research field occupied with these signals took place the past decade, resulting in the identification of numerous factors secreted by the adipocyte. These secreted proteins influence a variety of processes such as appetite, energy expenditure, inflammatory processes and the coagulation system and includes e.g. leptin, adipisin, insulin-like growth factor-1, interleukin-6, prostaglandins, plasminogen activator inhibitor-1 and tissue factor (Fruhbeck et al., 2001). The origin of this important cell type remains to be thoroughly studied, but several studies indicate that adipocytes originate from multipotent cells capable of differentiating into myocytes, osteoblasts and chondrocytes, in addition to adipocytes (Konieczny and Emerson, 1984; Poliard et al., 1995; Taylor and Jones, 1979).

Several preadipocyte cell lines have been established, that can be induced in vitro into an adipocytic phenotype, characterized by the expression of adipocyte specific genes, e.g. 3T3-L1 cells. Two factors involved in growth arrest and the induction of adipocyte specific genes are PPAR-γ and C/EBP-α, and their interplay with different endocrine factors have been extensively studied (Gregoire et al., 1998). On the other hand, the role of the ECM in the differentiation process is largely unknown. Upon differentiation the cells undergo changes in shape, and the type of ECM components produced switch from type I and III collagens to type IV collagen, nidogen and laminin (Aratani and Kitagawa, 1988). The specific isoform of laminin secreted by 3T3-L1 cells undergoing in vitro differentiation was shown to be laminin 8 (α4β1γ1) (Niimi et al., 1997). EHS laminin favored adhesion and spreading of the preadipocytic population of stromal vascular cells isolated from pig (Hausman et al., 1996). Furthermore, it promoted differentiation into an adipocyte phenotype, while type IV collagen and fibronectin had no effects.

The laminin α4 chain has recently been found in the BM surrounding individual adipocytes, for example in the vessel wall of the mouse aorta (Talts et al., 2000) and the adventitial layer of the pulmonary trunk in man (Petäjäniemi et al., 2002).
AIMS OF THE PRESENT STUDY

Laminins are BM proteins with properties influencing cell phenotype and behavior, in addition to being structural components. Many isoforms were identified during the past two decades. The aims of the studies in this thesis were to provide basic knowledge about the recently identified laminin isoforms 8, 9 and 10, whose biological roles have been partially or completely unknown. The aims were:

1. To clone and sequence the human laminin α5 chain, in order to use this for the production of recombinant laminin 10.

2. To study the biological consequences of the lack of the laminin α4 chain through phenotypic characterization of mice lacking the gene encoding this chain. This included:
   a. Histopathological characterization of a hemorrhaging phenotype in newborn laminin α4 deficient mice, and clinical investigations with the aim to determine the cause of the hemorrhages.
   b. Clinical characterization of a motor dysfunction in laminin α4 deficient mice and elucidation the mechanism underlying it.
   c. Studying the resistance to obesity that the laminin α4 deficient mice exhibited, and to discriminate between possible defects in food intake, energy expenditure or adipogenesis.
METHODS

The methods used in this thesis are described in detail in the papers included. A summary of the methods and where to find the description is given in the table IV. A summary of the antibodies used in the different papers are shown in Table V.

**Table IV** A summary of methods used in this thesis.

<table>
<thead>
<tr>
<th>Methods</th>
<th>Described in papers</th>
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RESULTS AND DISCUSSION

*Analyses of a neonatal hemorrhaging phenotype in laminin α4 deficient mice (I)*

The deletion of exons 1 and 2 as well as part of the promoter region resulted in complete inactivation of the laminin α4 gene, demonstrated by the absence of α4 mRNA in Northern analysis and lack of immunoreactivity of tissues with a laminin α4-specific antibody. *Lama4* null mice were born at approximately Mendelian ratios, and were pale and icteric. Upon careful examination of the newborn pups, hemorrhages could be seen under the skin, particularly in the soft tissues surrounding the lower limbs, and in the head. Mild hemorrhages were also seen in the heart and meninges and, occasionally, blood was seen in the colorectum or intraperitoneally. In order to investigate if the *Lama4* null mice were anemic, blood was pooled from a whole litter of null or wild type animals, and whole blood and serum parameters were analyzed. The hemoglobin (Hb) values of the null newborn pups were ~50% of those of controls and the reticulocyte count and serum bilirubin levels were elevated. The presence of elevated reticulocyte counts indicated normal production of erythrocytes. The Hb values were also studied in more detail, by separately measuring all the individuals in a litter containing all the genotypes. This study confirmed that the Hb values of null pups were decreased to ~50% (\( P = 1.8 \times 10^{-18} \)), and that there were no differences between the heterozygote and wild-type littermates (128 ± 3.6, \( n = 22 \) wild-types; 126 ± 2.1, \( n = 49 \) heterozygotes and 67 ± 2.3, \( n = 24 \) nulls; average in mg/dL ± SEM, unpublished). These findings clearly showed that the newborn *Lama4* null mice are anemic, and indicated that the anemia is due to hemorrhages in soft tissues.

The hemorrhages and anemia probably accounted for the slightly increased perinatal mortality, which was 6% in *Lama4* nulls compared to <1% in littermate controls. The hemorrhages were cleared in a few days, and no signs of hemorrhaging were seen thereafter in *Lama4* nulls, which had normal life spans.
In order to determine whether the hemorrhages were caused by birth trauma, embryos at two different stages were examined. Significantly milder hemorrhages than those seen in newborn pups were observed at E11, especially in the head. At E18, just prior to birth, mild hemorrhaging could be seen along the vertebral column. Thus, hemorrhages were present before birth, but they were greatly aggravated by the passage through the birth canal. In order to examine whether there are abnormalities of blood coagulation in *Lama4* null animals, we determined the aPTT, PT and bleeding time. Adult animals were studied, since there is a high risk of contaminating the sample with tissue proteins that affect the coagulation assay, if blood is taken from newborns after decapitation. No coagulation abnormalities were detected in adult nulls, and thus it is unlikely that a coagulation defect in the newborns is the reason for the hemorrhages in soft tissues. We therefore investigated whether there were defects in the microvasculature. Immunostaining of muscles from newborn *Lama4* null mice with antibodies against several BM components revealed that the staining intensity for type IV collagen and nidogen was drastically reduced, while perlecán staining was normal. The capillaries of newborn muscle normally contain laminin α4, β1 and γ1, but in null animals there was no staining for α4 and very little for β1 and γ1. Furthermore, by immunostaining it was found that the laminin α1, α2, α3 or α5 chains were not upregulated. In skin overlying muscle from wild type newborns, smaller vessels appeared to contain low amounts of laminin α2. The intensity of the α2 immunoreactivity in the null animals appeared slightly increased compared to wild types. However, the changes were so small that it was not possible to convincingly verify them (unpublished). As mentioned, the laminin α2 chain was recently found by immuno-EM staining to be present in the capillary BM of adult mouse heart (Talts et al., 2000), a location where it has not been found previously using conventional immunofluorescence (Miner et al., 1997). The reason why this finding has escaped detection could be due to the higher resolution of immuno-EM. Further studies will reveal if the α2 chain is present in capillaries of other tissues as well.

Electron microscopy of capillaries from the muscles of newborn null mice revealed that BMs were more discontinuous and less well organized than in the littermate controls. These data indicated that laminin α4 is important for the development of the
capillary BM and thereby for the stability of newly formed vessels. Also, in the cornea angiogenesis assay, newly formed vessels in the *Lama4* null mice had a tortuous, dilated appearance and were leaky.

The mechanism by which laminin α4 is involved in microvessel maturation remains unknown. Two possibilities are conceivable. First, laminin 8 (α4β1γ1) may be important for the assembly of the BM. Laminin 8 is able to interact with cell surface receptors and the type IV collagen network, via nidogen. These interactions may be crucial for the BM assembly. Secondly, as also mentioned in the review of the literature, interaction of laminin 8 with the cell surface receptors may initiate signaling, which results in the induction of the expression of other BM components or their receptors. The expression of the laminin α5 chain is induced soon after birth, and therefore, the deposition of laminin 10 (α5β1γ1) into the capillary BMs may be the reason why the vascular phenotype in null mice disappeared during the first weeks of life.

Mice lacking fibulin-1 also display hemorrhages at birth. These hemorrhages are also localized to soft tissues, and are much more severe than the *Lama4* null mice (Kostka et al., 2001). Since fibulin-1 is an ECM protein associated with BMs, there might be a biologically relevant connection between the two molecules. We stained muscles from newborn *Lama4* null animals with a fibulin-1 antibody, but the staining pattern was not different from that of controls (unpublished).

Since the recruitment of pericytes to the newly formed vessels is of crucial importance for the stabilization of the vessels, it is reasonable to ask whether the fragility of microvessels in *Lama4* nulls is secondary to the absence of pericytes. No quantification of pericytes was performed, but upon EM analysis of the BMs in newborn muscle, pericytes were seen surrounding capillaries. A pilot study of the retinal vasculature of 2-3 week old mice revealed normal staining patterns for smooth muscle actin, a marker for supportive cells of the vasculature (unpublished). Still, a partially defective recruitment of pericytes to the vessels of the tissues showing severe hemorrhages remains a possibility. On the other hand, if such defects were found, it would not prove a direct link between the absence of BM components and defective pericyte recruitment, since the migration of pericytes could be physically impaired by excessive leakage and distortions of architecture in the *Lama4* null vasculature. Future in
vitro studies of pericyte behavior on recombinant laminins could shed light on the specific role of laminins in pericyte recruitment.

**Defective motor control and NMJ abnormalities in Lama4 null mice (II)**

The initial analyses of animals in the hybrid genetic background (B6; 129) revealed that Lama4 nulls displayed abnormal motor behavior, but the severity was variable. The animals either clasped or spastically overstretched their hind limbs in an abnormal manner when suspended by the tail. The penetrance of the phenotype was 100 % in the congenic B6 strain, and the variation in severity was less than in the hybrid background. The null animals performed significantly worse on a beam walk test, but the motor impairment did not obviously interfere with feeding or mating behaviors, and was difficult to detect for an untrained observer. These rather mild and unspecific neurological symptoms could be the result of defects in the central nervous system, as well as of abnormalities in peripheral sensory or motor nerves.

When analyzing the paravertebral hemorrhages in newborns, we noted that the dorsal root ganglia (DRG), where the nerve cell bodies of sensory neurons reside, were sometimes misshaped and groups of ganglion cells could occasionally be seen in the paravertebral muscles. However, a pilot study using DiI-tracing showed no abnormalities of sensory afferents projecting into the ganglia (unpublished). We assumed that the misshaped ganglia, which were frequently seen caudally, could be secondary to the sometimes severe hemorrhaging that was seen along the vertebral column. This interpretation would also explain why the majority of DRGs looked normal. However, we are not able to exclude that these abnormalities affect sensory input function in Lama4 null animals.

No defects were detected in muscles or nerves of young animals, but older males seemed to accumulate more inclusion bodies in the muscle fibres than control animals. Detailed analysis of the NMJ revealed specific defects in the Lama4 nulls. The NMJs were slightly longer and broken up into a larger number of varicose segments than in controls. On the ultrastructural level, only 23 % of active zones were directly apposed to
junctional folds in null animals, while 78% were directly apposed in controls. The same phenomenon could be seen in young mice (two weeks of age), excluding the possibility that a more severe defect, which is then partially corrected, exists in earlier stages of NMJ development. As previously shown, laminins $\alpha_2$, $\alpha_4$, $\alpha_5$, $\beta_2$ and $\gamma_1$ are expressed in the synaptic BM (Patton et al., 1997), and no changes in the staining pattern of the other chains except for the absence of laminin $\alpha_4$ was seen in Lama4 nulls. The synaptic BM structure was also intact, which excludes the possibility that disrupted BM or compensatory changes in the expression of other laminin chains were responsible for the observed defects.

A more detailed analysis showed that laminin $\alpha_4$ was concentrated at the sites flanking junctional folds in control animals, while $\alpha_2$, $\alpha_5$ and $\beta_2$ were present throughout the synaptic BM (Fig. 6 in paper II). This suggests that laminin 4 ($\alpha_2\beta_2\gamma_1$) and laminin 11 ($\alpha_5\beta_2\gamma_1$) are present in all parts of the synaptic BM, while laminin 9 ($\alpha_4\beta_2\gamma_1$) is concentrated between folds. Mice lacking the $\beta_2$ chain, and thereby all synaptic laminins, have much more severe defects of the NMJ, with few active zones as well as few junctional folds and Schwann cell invagination of the synaptic cleft (Noakes et al., 1995a). A detailed analysis of the NMJ showed that the dy/dy mice, that have a mutation in the laminin $\alpha_2$ chain, showed that the active zones were apposed to junctional folds in these mice. Taken together these data indicate that the synaptic laminins have distinct functions, and suggest that laminin 9 could have an instructive role in the apposition of active zones with junctional folds.

Physiological testing of muscles from null animals revealed that the observed NMJ defects had no effect on the contractile function of isolated muscles in the unfatigued state, or during fatigue and recover. Since NMJ defects that are severe enough to cause motor dysfunction are also usually associated with defective fatigue recovery, it seems unlikely that the specific defects of the NMJ in Lama4 nulls are the sole explanation for the dysfunctional motor-pattern.
**Lama4 null mice are resistant to obesity (III)**

A rather unexpected finding was made when studying aging *Lama4* null mice. The male null animals did not seem to develop age-related obesity, as their littermate controls did. We studied this closer, and followed the weight and food intake of animals on the congeneric B6 background. The animals were kept in cages where nulls were housed either together with littermate heterozygotes, or nulls and wild-type mice in separate cages. Indeed, in males, a small significant difference in weight was seen already at the age of ~4 months. The difference then increased in magnitude, and at 6 months of age, the weight of null males leveled out at ~30-35 grams. Both heterozygote and wild-type littersmates continued to increase in weight and weighed around 50 g at 15 months of age. A small difference was also seen in the weight of females, but neither *Lama4* nulls nor control females developed obesity to the same extent as male controls did.

Even when fed a high-fat diet, the *Lama4* nulls did not increase their weight to more than ~35 g on average. However, there was more variation than on standard food, with a few animals weighing ~40 g. A specialized X-ray method, designed to determine body composition, confirmed that the amount of body-fat was decreased in null animals, as did weighing of individual fat pads and an abdominal CT.

A lowered food intake could possibly explain the difference in weight of the animals. However, it was not the case that *Lama4* were eating less than controls. On the contrary, the null animals seemed to have a tendency towards higher food intake. We therefore went on to investigate the energy expenditure of the animals. Oxygen-consumption and spontaneous physical activity were measured in specially designed cages. The null animals were clearly more active than their littermate controls, and consequently had increased oxygen consumption. However, at times of low activity, no difference in O$_2$-consumption could be seen, indicating that there was no difference in resting metabolic rates. The serum lipid profile of *Lama4* null mice was indistinguishable from that of controls on standard and high-fat diet, with the notable exception of cholesterol on high-fat diet. Interestingly, the cholesterol levels were not elevated to the same extent as in littermate controls fed the same diet, but currently we do not understand
why this was the case. Further studies of sterol metabolism should provide additional clues.

Lama4 null animals display motor abnormalities (paper II) and the hyperactivity could be part of a neurological syndrome in these mice. The newborn Lama4 animals had elevated serum bilirubin levels, which is known to be harmful for the developing human brain, and is known to cause a condition termed kernicterus. This is due to the accumulation of bilirubin in the basal ganglia which leads to motor abnormalities. The levels of bilirubin in newborn Lama4 nulls were not as high as those considered being harmful for humans, but on the other hand, it is difficult to compare toxic levels between species. However, we did not detect any obvious structural defects in the basal ganglia upon routine staining and histological examination. The increased activity of Lama4 null mice could also be a consequence of, instead of explanation for, the lean phenotype. Analyses of activity in young mice, before the onset of obesity in controls, may shed light on this issue.

As mentioned in the review of the literature, laminins have been implicated as being important for the differentiation of adipocytes. However, we could exclude the possibility of laminin 8 (α4β1γ1) being necessary for differentiation, since the appearance of Lama4 null adipocytes was normal, although a more subtle role in differentiation could not be ruled out. In a pilot study, Lama4 null mouse embryonic fibroblasts subjected to adipocyte-inducing factors accumulated intracellular lipids (unpublished), but a more thorough analysis of differentiation was not performed.

A growing number of knock-out studies have identified genes involved in the regulation of food intake and energy expenditure. In a few of these studies, gene inactivation has led to lean phenotypes (Crowley et al., 2002). In several of these, specific alterations of adipocyte function have been demonstrated. For instance, inactivation of the translation inhibitor 4E-BP1, was shown to lead to an increased metabolic rate due to induction of termogenesis (Tsukiyama-Kohara et al., 2001). Furthermore, mice lacking perilipin, an intracellular protein surrounding lipid droplets in adipocytes, showed increased lipolysis (Tansey et al., 2001). Lack of the carboxylase ACC2 increases fatty acid oxidation rates, thereby resulting in a lean phenotype (Abu-Elheiga et al., 2001). Targeting of the gene encoding the TGF-β family member myostatin resulted in a
phenotype which closely resembles that of \textit{Lama4} nulls. These mice are resistant to age-related obesity, and do not show decreased food intake or increased resting metabolic rate (McPherron and Lee, 2002). However, spontaneous activity was not measured in these mice, and the mechanism behind the phenotype remains unknown.

**Cloning of the human laminin $\alpha5$ chain and the production of laminin 10 (IV).**

Screening of cDNA libraries resulted in clones covering $\sim 70\%$ of the human laminin $\alpha5$ sequence. The remaining parts of the sequence were obtained by PCR amplification from cDNA libraries and the 5’ end was obtained with 5’ rapid amplification of cDNA ends. The coding sequence of the full-length cDNA of $11,088$ base pairs encodes a polypeptide of $3696$ amino acids (GenBank\textsuperscript{TM} accession number AF443072).

cDNAs were generated and sequenced from four different sources. When the same nucleotide difference resulting in amino acid substitution was found in at least two of them, we assumed these to be polymorphisms. Four of the polymorphisms were located in domain IIIa, which has LE-modules, and one in the G domain (Fig. 4).

The clones generated during the sequencing of the human laminin $\alpha5$ were used to make a full-length construct containing a FLAG-epitope. This was used to transfect HEK293 cells, which first had been sequentially transfected with constructs expressing the human laminin $\beta1$ and $\gamma1$ chains. Recombinant laminin 10 ($\alpha5\beta1\gamma1$) was purified from the medium with an anti-FLAG antibody column.

In accordance with the expected structure, rotary shadowing EM revealed the recombinant laminin 10 protein as having three short arms and one long arm. In SDS-PAGE under non-reducing conditions the majority of the recombinant laminin 10 appeared as disulfide cross-linked heterotrimer, while a minority consisted of the $\alpha5$ chain non-covalently associated with a disulfide-linked $\beta1/\gamma1$ heterodimer.
Figure 4. cDNA-derived amino acid sequence of the human laminin 5 chain (upper) and its alignment with the laminin 5 chain mouse chain (lower). Domain boundaries are depicted, and RGD sequences are boxed. The potential cleavage site for the signal peptide is indicated by a solid triangle (Predicted by PSORT II). The five possible polymorphisms are shown as bold italic characters above the human sequence. The sequence submitted to GenBank by the Human Genome Project (accession NM_005560) only differed from our sequence at a few sites, indicated above our sequence. At these sites, our sequence did not differ from the genomic sequences reported by Celera.
Western blotting of the conditioned medium revealed an intact form of the α5 chain and a smaller form, from which a C-terminal fragment of ~40 kD harboring the FLAG-epitope had been cleaved off. This is likely to be the result of protelytic cleavage between domains LG3 and LG4 as reported for other laminins (Talts et al., 1998).

The production of recombinant laminin 10 made it possible to compare its effects on cell behavior to those of laminin 1 (α1β1γ1) and 8 (α4β1γ1), of which the latter has also been produced earlier in our lab (Kortesmaa et al., 2000). The HT-1080 fibrosarcoma cell line and two endothelial cell types (immorto mouse brain endothelial, IBE, cells and human saphenous vein endothelial cells, HSVECs) were studied. Laminin 1 and recombinant laminin 8 were less effective than recombinant laminin 10 in promoting cell adhesion. recombinant laminin 10 was also the most potent to promote HSVEC migration. The cell adhesion was dependent on divalent cations, but the addition of heparin had no effect. Function-blocking antibodies against various integrins were used to identify potential receptors for recombinant laminin 10. This study showed that

**Figure 5.** HEK293 cells were sequentially transfected with plasmids expressing the different chains, resulting in the production of trimeric laminin 10. For each step, the antibiotics indicated were used for selection.
integrins α3β1 and α2β1, but not α6 or αv integrins, are mediating the cell adhesion to recombinant laminin 10. These findings differ from those obtained in a study using a mixture of laminin 10/11 (α5β1γ1/α5β2γ1) purified from tissues. In that work it was found that laminin 10/11 interacts with integrin α6β1 and α6β4 but not α2β1 (Kikkawa et al., 2000). Furthermore, in a recent study, recombinantly produced domain IVa of laminin α5 were shown to bind integrin αvβ3 (Sasaki and Timpl, 2001). This finding, together with those of our study, suggest that the binding site of integrin αvβ3 is not exposed in the intact laminin 10.

In many previous studies concerning laminin 10, a mixture of laminin 10/11 purified from tissues was used. It has been shown that this preparation contains partially digested protein (Sixt et al., 2001b). This is a problem if one tries to identify biological roles of the intact laminin 10 molecule. Recombinant laminin 10 provides a new pure source of this laminin, enabling us to study the interactions of the intact trimer.
CONCLUDING REMARKS

This work has provided important new knowledge about the laminins 8 ($\alpha_4\beta_1\gamma_1$) and 9 ($\alpha_4\beta_2\gamma_1$). Analyses of mice lacking the $\alpha_4$ chain identified a complex phenotype including disturbed microvessel maturation, defective NMJs and resistance to obesity.

The presence of $\alpha_4$-containing laminins in the endothelial BMs of developing vessels seems to be of crucial importance for the proper formation of this BM. Since the $\beta_1$ and $\gamma_1$ chains are expressed in developing microvessels, it is likely that laminin 8 ($\alpha_4\beta_1\gamma_1$) is the laminin isoform important in this context. (However, a recent study showed a more widespread expression of the $\beta_2$ chain than that previously reported (Sasaki et al., 2002). It is therefore possible that low amounts of laminin 9 ($\alpha_4\beta_2\gamma_1$) is also present in developing microvessels, although this has not been examined). Since the amount of type IV collagen was decreased in the BMs of $Lama4$ null mice, one possibility is that laminin 8 is needed in the assembly of the BM. It is worth noting that, in spite of defects in the endothelial BMs, angiogenesis seemed to proceed normally, with the exception that the vessels were more fragile. This argues against a central role for the BM in the formation of a vessel, but for a role in the subsequent maturation. However, an important fact to notice is that the endothelial BM was defective in the absence of laminin $\alpha_4$, and not absent. Perlecan deposition seemed to proceed normally, and the presence of this HSPG may still be critical for the developing blood vessel, especially as a growth-factor regulator.

Our findings raise the question what the roles of the different laminin isoforms may be in the early phases of angiogenesis. Studies of the development of retinal vessels, which are remodeled during the first three postnatal weeks in mice, have shown that pericytes migrate in from larger vessels to smaller ones, thus stabilizing the structure. Before this process is completed, there seems to be a window allowing for plasticity. In line with this notion it has been proposed that the endothelial cells deposit a provisional ECM during this period (Benjamin et al., 1998). It is very likely that laminin 8 is part of this provisional BM. Since laminin 8 ($\alpha_4\beta_1\gamma_1$) is a truncated laminin, lacking one of the short arms, it is possible that it is unable to form polymers (Yurchenco and Cheng, 1993). It may therefore take part in the formation of a more
flexible BM of developing endothelial cells that provide some structural support while allowing for remodeling. It is intriguing that the laminin α5 chain is clearly present in smaller vessels at around three weeks of age, a time point when pericytes coverage is about to be completed and the vessels become stabilized. Laminin 10 (α5β1γ1) is a “long” laminin with three short arms, and is very likely able to form polymers. It seems possible that this potentially polymer-forming laminin, which interacts with integrin isoforms different from those interacting with laminin 8, plays a specific role in the mature vasculature.

Angiogenesis was clearly not impaired in the Lama4 null mice. On the contrary, a tendency towards excessive sprouting was observed in the cornea angiogenesis assay. A possible role of laminin 8, in the provisional BM surrounding the growing vessel, could be as a negative regulator of sprouting. In this context, the observations that certain domains of type IV collagen are anti-angiogenic (Colorado et al., 2000; Petitclerc et al., 2000), are of interest. It was recently shown that laminin 8 lack the LG4-5 domains in the mature BM (Talts et al., 2000). It is tempting to speculate that this part of laminin 8 is proteolytically cleaved away after laminin 8 is secreted into the immature BM of developing vessel, and could possess antiangiogenic activity.

The studies of NMJs of the Lama4 null mice indicated that the different laminin isoforms of the synaptic BM have distinctly different roles. Since the β2 and γ1 chains are expressed in the synaptic BM, it is likely that the specific defects in synaptic specialization seen in Lama4 null mice were due to the absence of laminin 9 (α4β2γ1). However, the details of how this laminin isoforms plays an instructive role in the development of the NMJ remain to be elucidated.

The mechanisms underlying the obesity resistance in Lama4 null mice are not currently known. Increased spontaneous activity could be the main reason why the null animals don’t develop obesity, but increased activity could instead be secondary to the leaner, more “fit” status, of the aging Lama4 null mice. More detailed analyses of the behavior of younger mice, before the onset of obesity in controls, will provide important information to help resolve this issue. Another exiting finding to study further is the lower levels of cholesterol in Lama4 null mice fed a high-fat diet.

In the field of vascular surgery, a number of non-biological materials are used in the reconstruction and repair of vessels subjected to various disease processes. Once inserted into the body, endothelial cells cover the surfaces of these “vascular
grafts”; a process called endothelialization. Proper in growth of endothelial cells has been shown to be of importance for inhibiting thrombus formation on the surface, as well as for reducing the neointimal thickening. This work has resulted in the full sequence of the human laminin α5 chain and a source of pure laminin 10 in larger quantities, and this laminin isoform showed migration promoting activity on endothelial cells isolated from humans. This laminin could potentially be used to coat vascular grafts in order to improve the endothelialization, and thereby to reduce the frequency of complications following vascular surgery.
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