Laminin 332 processing impacts cellular behavior

Patricia Rousselle¹* and Konrad Beck²

¹SFR BioSciences Gerland-Lyon Sud; Institut de Biologie et Chimie des Protéines; UMR 5305, CNRS; Université Lyon 1; Lyon, France; ²Cardiff University School of Dentistry; Cardiff, UK

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Abbreviations: ECM, extracellular matrix; LG, laminin globular domain; pre-laminin, precursor-laminin; MMP, matrix metalloproteinase; mTLD, mammalian tolloid; BMP-1, bone morphogenic protein 1; JEB, junctional epidermolysis bullosa; pGt, protein data bank

Laminin 332 and Its Maturation Events

Laminin 332, composed of the α3, β3 and γ2 chains, is an epithelial-basement membrane specific variant. Its main role in normal tissues is the maintenance of epithelial-mesenchymal cohesion in tissues exposed to external forces, including skin and stratified squamous mucosa. After being secreted and deposited in the extracellular matrix, laminin 332 undergoes physiological maturation processes consisting in the proteolytic processing of domains located within the α3 and the γ2 chains. These maturation events are essential for laminin 332 integration into the basement membrane where it plays an important function in the nucleation and maintenance of anchoring structures. Studies in normal and pathological situations have revealed that laminin 332 can trigger distinct cellular events depending on the level of its proteolytic cleavages. In this review, the biological and structural characteristics of laminin 332 domains are presented and we discuss whether they trigger specific functions.

Laminins are large extracellular glycoproteins that are important components of all basement membranes. They are involved in several biological processes, including self polymerization, binding to the extracellular matrix (ECM) and cellular interactions.¹,² All laminins are composed of three different gene products, termed α, β and γ chains assembled into a cross-shaped heterotrimer αβγ. The three chains assemble within the endoplasmic reticulum through their C-terminal domains to form a triple stranded α-helical coiled coil rod.³,⁴ Sixteen laminin isoforms of different subunit composition selected from five individual α chains (α1 to α5), three β chains (β1 to β3) and three γ chains (γ1 to γ3), are known with variable cell and tissue specific expression, and they are differentially recognized by cellular receptors.⁵ All laminin α chains possess a large globule at the carboxyl-terminal end that consists of five similar domains LG1 to LG5 each containing about 200 residues.⁶,⁷

Abbreviations:

ECM, extracellular matrix; LG, laminin globular domain; pre-laminin, precursor-laminin; MMP, matrix metalloproteinase; mTLD, mammalian tolloid; BMP-1, bone morphogenic protein 1; JEB, junctional epidermolysis bullosa; pGt, protein data bank

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Laminin 332 processing and integration into the skin basement membrane. In skin, laminin 332 is synthesized by keratinocytes as a high molecular weight precursor protein of 460 kDa. After secretion and deposition into the ECM, the α3 and γ2 chains undergo maturation events consisting in specific processing to smaller forms.⁶,¹¹ The 190–200 kDa α3 chain (α3200) can be successively processed at both C- and N-terminal extremities producing 165 (α3165) and 145 kDa (α3145) maturation products. The 155 kDa γ2 chain (γ2155) is processed in the N-terminal region leading to a 105 kDa product (γ2105).

Processing of the α3 chain consists of cleavage of the C-terminal globular domains 4 and 5 (LG45) within the spacer between LG3 and LG4 (Fig. 1A and B).¹²,¹³ In vitro studies using human laminin 332 have revealed that enzymes involved in the processing include plasmin,¹² MMP-2, MT1-MMP and the C-proteinase family of enzymes, especially mammalian tolloid (mTLD) and the bone morphogenic protein 1 (BMP-1).¹⁴,¹⁵ In addition, thrombin was shown to have the potency to cleave LG45 specifically.¹⁶ Alternatively, there may be other mechanisms that control the rate of laminin α3 LG45 processing, such as the tissue plasminogen proteolytic cascade.¹² N-terminal amino acid sequencing of human LG45 purified from the conditioned medium of either primary or immortalized human keratinocytes and of the human gastric adenocarcinoma cells STKM-1 revealed that the α3 chain is cleaved between Q₁₃₇ and D₁₃₈ within the
hinge region between LG3 and LG4 suggesting that a proteolytic cleavage site of the LG45 domain matches the minimal consensus sequence LLQD (Fig. 1B). It is not known what endopeptidase catalyzes the hydrolysis of this cleavage sequence, and the existence of additional proteolytic cleavage sites has been suspected both in the hinge and the adjacent regions of the LG3 and LG4 domains. This hypothesis is compatible with the spacer length of the laminin α3 chain, which is longer than those of the α1, α2 and α5 chains. Punctual mutations within the spacer region and/or deletion of the sequence LLQD did not protect the α3 LG3-LG4 linker from cleavage. As proteolytic processing of LG domains occurs in all α chains except α1, the substitution of the α3 LG3-LG4 hinge with that of the α1 chain was an elegant way to render the laminin α3 chain uncleavable. This was also the case when a 46 amino-acid portion within the α3 LG3-LG4 hinge was deleted. These LG45-uncleavable α3 chain constructs were expressed in human or mouse skin keratinocytes deficient for expression of the α3 chain, and in both cases the resulting LG45-uncleavable heterotrimeric laminin 332 was deposited in the ECM. Information gained from the structure of the mouse α2 chain LG45 domain pair revealed an unusual path of the LG3-LG4 linker as it forms an inter-domain disulfide bridge to LG5. Being integrated into the LG45 tandem the linker is therefore an integral part of the structure, a feature that is conserved in all laminin α chains. It is tempting to speculate that proteinases may have easy access to the LG3-LG4 cleavage sites located within a well-exposed linker.

Further cleavage occurs in the N-terminal LE region of the α3 chain releasing the full short arm which might be important for laminin 332 function (Fig. 1A). A mutation causing an N-terminal deletion of 226 amino acids in the human α3A isoform was identified in the laryngo-oncho-cutaneous syndrome, a rare autosomal recessive disorder characterized by chronic production of vascularized granulation tissue. Cultured keratinocytes obtained from patients revealed that heterotrimeric laminin 332 carrying the mutant α3 chain is assembled and secreted. The mutated laminin was regularly expressed in the skin basement membrane and only very subtle ultrastructural changes were seen such as focal widening of the lamina lucida in places where hemidesmosome plaques were smaller. These studies suggest that the laminin α3 N-terminal domain may be a key regulator of the granulation tissue response during wound healing.

In human skin laminin 332, the processing of the γ2155 chain leads to the γ2105 subunit and was shown to be achieved by BMP-1 and mTLD metalloproteinases. The N-terminal processing is complex as it occurs within a disulfide-linked loop of the L4 domain followed by reshuffling of disulfide bonds for release of the cleaved fragments. The immunohistochemical analysis of laminin 332 in mTLD/BMP-1-deficient mouse skin revealed a strong expression of the precursor γ2 chain confirming that these enzymes are involved in the γ2 chain maturation in vivo. In contrast, the processing of the LG45 domain in the α3 chain seemed, at least partially, unaffected reinforcing the hypothesis that several enzymes are involved in this maturation process.

Laminin 332 was shown to be the major component of anchoring filaments in skin where it mediates cell adhesion via interaction of the α3 carboxyl-terminal LG1–3 triplet domain with both αβ3 integrins, while the N-terminal short arms connect to basement membrane components. Laminin 332 can be incorporated into the basement membrane through at least two mechanisms. The first involves cross-linking of laminin 332 with laminin 311 (α3β1γ1) in the skin basement membrane. The complex of laminin 311 with laminin 332 is most likely to derive from an interaction of domain LN in the β3 chain of laminin 332 with domain LE of the α3 chain short arm in laminin 311 (see below). As seen on rotary shadowing electron micrographs of the complex, the LN domain of the short arm interacts with a laminin 311 domain near the intersection of the laminin 311 short arms. These complexes are most likely stabilized by a disulfide bridge between an unpaired cysteine in domain LN of the β3-chain and domain LE of processed α3 in laminin 311. A similar complex between laminin 332 and laminin 321 (α3β2γ1) is found in the basement membrane of the amnions. According to the 3-arm interaction hypothesis of laminin polymerization, the dimers could self-associate. The second mechanism reports a direct interaction between anchoring filaments and anchoring fibrils. Anchoring fibrils are disulfide bond stabilized dimers of type VII collagen. Monomeric laminin 332 as well as the laminin 332/311 dimer directly bind the N-terminal globular domain NC1 of type VII collagen. The interaction is likely to occur within the short arm of the β3 and/or γ2 subunit.

Other processing events in laminin 332. In addition to the laminin 332 maturation aimed at regulating the skin basement membrane structural integrity, other studies have reported two specific migration-inducing MMP-2 cleavages of the laminin γ2 N-termini producing γ2 chain fragments of 100 and 80 kDa. These γ2 processing could also be carried by MT1-MMP in colon and breast carcinoma cells. Both enzymes, that were first shown to cleave rat laminin 332, were proposed to also cut down human laminin 332 in both physiological and pathological situations such as tumorigenesis, MMP-3, -12, -13, -19 and -20 were also shown to process the γ2 chain inducing epithelial cell migration. Migration events might therefore result from the interaction of newly exposed laminin domains with cell surface signaling receptors. Rat recombinant γ2Leb1-LEb4 was shown to bind and activate the EGF receptor to stimulate cell migration. Studies with cancer cells have reported that MMP-7, MT1-MMP and hepsin cleave laminin β3 leading to increased migration. The molecular mechanisms of laminin 332 in squamous carcinoma have been reviewed in detail by Marinkovich.

Laminin 332 Sequence-Structure Relationship

The structure of the heterotrimeric laminin 332 molecule consisting of disulfide-linked α3, β3 and γ2 chains was first revealed by rotary shadowing electron microscopy, which showed a rod 107 nm in length terminated by one large and two small globules at opposite ends which correspond to the C-terminal
Figure 1. Structure of human laminin 332 and its physiological maturation process. (A) Laminin 332 is composed of three subunits α3A, β3 and γ2. Each chain is composed of different domains that are indicated. Domain L4 in the γ2 chain corresponds to an LE domain with an ~180 residues insert between the third and fourth cysteine of the canonical 8-Cys pattern. LE4' and LEb4' of the α3A and γ2 chain are truncated LE repeats containing only the first four and six cysteines, respectively, of the pattern. Based on the odd number of cysteines, β3LN, α3ALE4', β3LE6 and γ2LEb1 (after maturation), and the coiled coil of α3 have free SH groups as indicated. The coiled coil is stabilized by disulfide bonds at the N-terminus (dotted lines) though the connectivity is yet unknown. The large LG structure located at the C-terminal end of the α3 chain contains five repeating LG domains. The first three repeats (LG1–3) interact with α3β1, α6β1 and α6β4 integrins while the last two (LG45) contain binding sites for syndecan-1 and -4. Laminin 332 is synthesized as a precursor molecule that undergoes maturation by proteolytic processing at the α3 chain N- and C-terminus as well as at the γ2 chain N-terminal extremity. The cleavage sites are indicated by arrows as well as enzymes involved identified so far. (B) Schematic structures of LG. The five LG domains contain numerous cysteines that are numbered. Disulfide bridged and free cysteines (SH) within the human sequence are indicated. At the bottom, an alignment of the human and mouse LG3–LG4 linker is shown. Each sequence displays the same cleavage site (underlined) and a cysteine (printed in inverse font) that forms an inter-domain disulfide bond to LG5. The sequence P₁₃₋₁₅PFLMLKGSTR supposed to be crucial for integrin α3β1 binding is double underlined.
LG and N-terminal LN and LE/L4 domains, respectively (Fig.1A). This length is consistent with the later determined sequences which indicate that ca. 570 residues per chain contribute to the three stranded β-helical coiled coil structure giving a length of ~80 nm, and ~7% tandem LE domains accounting for a further 20 nm. Circular dichroism spectroscopy revealed an β-helical content of ~30% similar to that determined for laminin 111 indicating that most of this secondary structure contribution relates to the coiled coil. Assembly of the laminin 332 trimer occurs in the endoplasmic reticulum where first a disulfide linked β3-γ2 dimer is formed to which the α3 chain aligns via the coiled coil. Trimer formation is a prerequisite for transport to the Golgi complex and secretion but does not require N-linked glycosylation.

Laminin β3 LN domain. So far no high resolution structures of laminin 332 domains have been solved. However, based on sequence similarity, some insight into the molecular organization of various domains can be anticipated from homology modeling. Recently, the crystal structure of the N-terminal LN domain together with four adjacent LE modules of the mouse laminin β1 chain has been solved (pdb code: 4a4q). The corresponding regions of human laminin β3 can be fitted to this structure (Fig. 2) suggesting that LN (residues 18–248; accession number NP_002219) forms a β-sandwich consisting of eight β-strands folded into a jelly roll motif. The first LE domain (res. 249–314) is tightly integrated into LN, which starts with a disulfide-bonded reverse turn connected by C21-C26 and the N-terminal region (res. 18–28) associates to LE1. Pro278, which is conserved in all laminin LN sequences, fits into a pocket of LE1. Further disulfide bonds can be expected between C47-C69, C56-C66, and C153-C173 (Fig. 2A). This leaves C67, which is unique for β3 LN domains and surface accessible (Fig. 2B), in a reduced state making it available for covalently binding to other proteins like laminin 331. The N-linked glycosylation sites found within β1 LN at N120 and in γ1 LN at N39/N132 are absent in the β3 chain. The critical residues D106 and T114 of γ1 LN involved in calcium binding, might be important for laminin polymerization, are replaced by Ser6 and Thr56 in the β3 chain indicating that this site will not be functional.

Laminin 332 LE and L4 domains. A common feature of LE modules is their connectivity of the eight cysteine residues as C1-C3, C2-C4, C5-C6 and C7-C8. The loop formed between C5-C6 usually consists of eight residues with a glycine in position six. In human laminin 332, the following LE domains deviate from the 8-Cys pattern (Fig. 1A): β3-LE6 preceding the coiled coil contains one extra cysteine (C372) between C7 and C8 whereas the corresponding region within α3A-LE4 is truncated after the fourth cysteine (C185; accession number AAA59431) followed by an irregular cysteine (C399) just before C202/C205, which are presumably forming intrachain disulfides at the N-terminus of the coiled coil. The α3A chain is processed just after LE4 between K191 and D192 allowing L4 following domain L4 of the human 3 chain indicating that this site is truncated after the C-terminus (human β3: C1174; γ2: C1184), which based on similarity to the β1 and γ1 chains most probably form an interchain disulfide bond.

Besides its structural importance for assembly, it has recently been found that in the absence of the LG domains the laminin 111 coiled coil has anti-adhesive properties. When cultured on such truncated laminin 111, cell adhesion and spreading were inhibited; genes compatible with a pro-migratory and pro-invasive function like MMPs and various matricellular proteins were upregulated. No comparable studies have yet been performed for laminin 332, but an antagonistic interplay between coiled-coil
and LG domains (see below) could be of importance in cancer metastasis.

**Laminin α3 LG domains.** The large globule seen in electron micrographs at the tip of the long arm consists of five LG domains formed by the α chains. Domains LG4-LG5 are separated from LG1 to LG3 by a flexible linker (Fig. 1B). As these domains are involved in many interactions (see below), particular interest has been focused on their structural characterization (for a review, see ref. 7). X-ray structures have been solved for the mouse α5 LG1-LG3 triplet (pdb code: 2wjs),\(^{49}\) mouse α1 and α2 LG45 pair (2jd4,\(^{50}\) 1dyk,\(^{50}\) 1okq\(^{51}\)), and mouse α2 LG5 (1qu0\(^{52}\)) which can be used to model the corresponding α3 LG1-LG5 domains (Fig. 3). LG domains fold to a β-sandwich consisting of 14 strands, where the two sheets form a concave and convex interface. Close to the C-terminus, each LG sequence contains two cysteines ~30 residues apart, which form intradomain disulfide bonds (Fig. 3A and C). In contrast to electron micrographs suggesting close proximity of LG1 to LG3,\(^{53,54}\) the α2 LG1–3 structure shows LG1 dissociated from the LG2-LG3 pair.\(^{49}\) Previous work suggested that trimerization of the α with β and γ chains is required for the LG domain to exert its integrin binding activity, and that specifically a glutamic acid two residues

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**Figure 2.** Structural model of the human laminin β3 LN-LE1–4 domains. (A) The structure shows residues Q18 to Q464 as a cartoon with red cylinders and blue arrows indicating α-helices and β-strands, respectively. Disulfide bonded cysteines within the LE domains are depicted in ball-and-stick presentation using different colors. Domain boundaries are indicated by dashed lines. P<sub>28</sub> fits into a pocket shared by the LN and LE1 domain, and the N-terminal residues are interacting with LE1. Disulfides and the free cysteine residue of the LN domain are indicated. (B) The electrostatic surface potential is represented in the same orientation and on the same scale as in (A). Negative and positive potentials are shown in red and blue, respectively. Homology based modeling was performed using Swiss-Model\(^{106}\) in manual alignment mode with the mouse β1 structure (pdb code 4aqs\(^{46}\)) as a template.
Figure 3. For figure legend, see next page.
LG4, LG5 contain tightly bound calcium or magnesium ions and might not reflect the conformation of the native molecule. Apart from the C-terminus of the a3 chain, this residue is indeed highly conserved in the a1 and a2, but not in a3 subunits. A lack of integrin a3b1 binding activity in the absence of at least short b3 and y2 segments has been shown for a3 LG1-LG3. The absence of such crucial parts of the b and y chains could result in the open LG1-LG2 conformation (Fig. 3A and B) and might not reflect the conformation of the native molecule.

The LG3 and LG4 domains are connected by a flexible linker, which was realized very early by the proteolytic susceptibility of mouse laminin 111. Based on the C- and N-terminal residues, which can be resolved in the mouse a5 LG1–3 (2wjs) and a1 LG4–5 (2jd4) structures, respectively, for human a3 the termini of the corresponding loop can be defined as N1310 and D1352 (Fig. 1B). Close to the middle, a3 LG is processed between Q1337 and D1338. This leaves a segment P1313-PFLMLKKGSTR attached to LG3 which, based on a recombinantly overexpressed LG and synthetic peptide approach, was reported as crucial for integrin a3b1 binding. C-terminally truncated peptides, however, abolished this activity. The difference of this part in mouse a3 (PPFLMLFKSPKG, Fig. 1B), however, would suggest that this interaction is rather species specific.

Based on the structures of the a3 LG1 and a2 LG4–5 pairs, modeling predicts that human a3 C1354 within the region forms a disulfide bond with C1617 in LG5 (Fig. 3C). Corresponding cysteines within the loop and LG5 are found in all laminin a chains down to Drosophila and C. elegans. Interestingly, human a3 LG4 (C1443) and a1 LG3 contain further cysteines in a similar position, which are not conserved in mouse and rat. Within the model (Fig. 3C), the C1443 and C1587 SH groups point into the hydrophobic core of the b sandwich.

The crystal structures of mouse a5 LG1, LG2, and a1, a2 LG4, LG5 contain tightly bound calcium or magnesium ions interacting with pairs of aspartate residues. Our human a3 LG models (Fig. 3) suggest that these cation binding sites are not conserved. Although within LG1 and LG2 D287 and D1090, respectively, are present, their partner residues are changed to N910 and R1023. Within LG4 and LG5, the corresponding residues are replaced by S1417/S1481 and K1584/S1654. This does not exclude that a3 LG domains might contain other divergent cation binding sites, but so far no conclusive experimental data exist.

Due to their physiological relevance, many studies have been performed to elucidate the interaction of laminins with proteoglycans. As a model for the complex glycosaminoglycan chains, most experiments are performed with heparin. We have recently reported on the specific interaction of human a3 LG45 with syndecans and found that K1434, R1436 and K1438 are crucial for syndecan-1 binding, whereas K1440 and K1442 involved in syndecan-4 interaction are shown in green.59 The ε-amino and guanidinium groups of the lysine and arginine residues, respectively, point to the sulfate groups of the carbohydrate chains. The surface view of the model shown in (D) indicates the tight packing of the heparin fragment into the groove formed by the concave surface of the LG4 b-sandwich with positive surface potential spaced to fit the distance of sulfate groups. The template structures used for modeling were mouse laminin a2 LG1-LG3 (pdb code: 2wjs) and mouse laminin a1 LG4-LG5 (2jd4, chain B). Docking of a heparin fragment (1hpn)90 to LG4 was performed with PatchDock.107

**Functions of LG45 in Precursor a3**

A potential function for the tandem LG45 domains was initially suspected based on the ability of laminin 332 to trigger distinct cellular events depending on the level of processing of its a3 chain. A form of laminin 332 that lacked LG45 was found in mature basement membranes, where it was shown to play an important function in the nucleation and maintenance of anchoring structures through a3b1 and a2b4 integrin interactions. In contrast, laminin 332 with intact LG45 was found in migratory/remodelling situations such as epidermal repair. Indeed, laminin 332 with an a3200 chain was found in the ECM of keratinocytes migrating on collagen I or after stimulation by TGF-β1. In vivo, epidermal injury activates the transcription and deposition of laminin 332 into the provisional matrix by the leading keratinocytes in the process of epidermal outgrowth and migration at the wound edge.61,69 Note worthy, a3200 laminin 332 is found in this provisional matrix but is absent from mature basement membranes.61,62 Recently, laminin 332 comprising an a3200 chain was proposed to be involved in the invasion of squamous cell carcinomas in vivo.70 Polyclonal antibodies targeting the LG45 domains induced squamous cell carcinoma apoptosis in vivo and thus inhibited tumor proliferation.70

**Function in laminin 332/311 deposition into the ECM.** A function for LG45 in the deposition of laminin 332 in the ECM has been first hypothesized by Carter and coworkers,16 who...
showed that exogenous human α3200 laminin 332, but not α3165 laminin 332, was trapped by cultured LAMA3 deficient mouse keratinocytes and deposited within their ECM. Moreover, when these keratinocytes were transfected with constructs encoding either an LG45-uncleavable or a precleaved α3 chain, they deposited a larger amount of the LG45-uncleavable laminin 332 into their ECM as compared with the LG45 pre-cleaved laminin 332 that was preferentially secreted into the culture medium. The importance of LG45 in laminin 332 deposition was confirmed by a study reporting that a mutant laminin 332 lacking the LG45 domain, stably expressed in laminin 332 null keratinocytes derived from a patient with junctional epidermolysis bullosa (JEB) with underlying LAMA3 gene mutations, was less retained in the ECM but rather found in the culture medium as compared with the WT.70 Co-expression of LG45 in these α3165 laminin expressing keratinocytes enhanced laminin 332 deposition into the ECM, reinforced keratinocyte adhesion to the ECM and decreased migration. In these cells, LG45 co-localized with the α6 integrin subunit in stable adhesion contacts in a manner comparable to the LG45 domain of WT laminin 332 keratinocytes.70

Another study, however, found no difference in laminin 332 deposited by keratinocytes expressing either LG45 uncleavable or pre-cleaved laminin 332.79 Skin equivalents epithelialized with keratinocytes expressing the α3165 laminin 332 displayed a dermal-epidermal junction identical to that obtained with wild type keratinocytes suggesting that α3165 laminin 332 was properly deposited into the basement membrane or at least in an amount sufficient to allow dermal-epidermal cohesion.19 Besides, skin equivalents designed with keratinocytes expressing the LG45 uncleavable laminin 332 revealed absence of hemidesmosomes accompanied by an appreciable thickening of the lamina lucida likely secondary to the reduced cohesion of the tissue.19 These experiments show that LG45 cleavage appears to be a prerequisite for hemidesmosome formation in skin equivalent models.

These studies show that LG45 has the ability to integrate into the keratinocyte ECM either on its own or when present in the α3200 chain. It targets α6β4 integrin containing stable adhesion contacts suggesting that it may play a role in α6β4 integrin clustering in vitro. Although LG45 removal decreased laminin 332 deposition in the ECM, it did not fully prevent it, suggesting that other mechanisms may contribute in controlling laminin 332 deposition.

Defective α3 and γ2 processing was seen in cylindromatosis, a rare genetic human disorder characterized by the occurrence of multiple irregular benign epithelial tumors in the upper dermis.63 These nodules display a dramatically enlarged basement membrane (up to 4.3 μm thickness) as well as ultrastructural abnormalities as no clear lamina densa could be detected. The different integrin receptors are found in improper ratios as β1 integrins are upregulated while α6β4 integrin expression and hemidesmosome numbers are decreased.63 Despite a massive accumulation of laminin 332 throughout the entire basal lamina, a thin labeling of the LG45 domain detected at the interface between cindroma cells and the basal lamina suggests that the LG45 processing may have been delayed. It is not clear whether laminin 332 accumulation in the basement membrane is related to this processing defect. Only one study reported a missense mutation in the LAMA3 gene affecting LG4 in a patient with a mild non-Herlitz JEB phenotype.71 This mutation resulting in G1506E triggers an imperfect local protein folding that, without impairing trimerization of the coiled coil, causes laminin 332 intracellular accumulation within the endoplasmic reticulum. Only a small amount of the laminin 332 harboring the mutated α3 chain is secreted and physiologically processed thus providing partial adhesion functions and explaining the mild phenotype. Therefore structural changes caused by mutations of this highly conserved residue throughout laminin LG4 domains highlights potential important functions of LG45 in laminin secretion.

When a laminin α3 cDNA was transfected into HT1080 cells, the exogenous α3 chain was assembled with the endogenous β3/γ2 and β1/γ1 to produce laminin 332 and laminin 311 heterotrimers.72 Out of the two laminin isoforms found in the culture medium, laminin 311 was found primarily with a precursor α3200 chain while the laminin 332 was found with an α3165 chain.72 Sigle et al.16 showed that exogenous human α3200 laminin 311 was not trapped by cultured LAMA3 deficient mouse keratinocytes nor deposited within their ECM like α3200 laminin 332 (see above). When these keratinocytes were transfected with constructs encoding an LG45-uncleavable α3 chain, they deposited large amount of LG45-uncleavable laminin 332 within their ECM, and the LG45-uncleavable laminin 311 was found exclusively in the culture medium.16 These results suggest that the two laminin isoforms integrate within the basement membrane through different mechanisms. That α3200 laminin 311 was not deposited within the ECM first shows that LG45 could not fulfill this event on its own. It also reinforces the hypothesis that a protein domain present in laminin 332 but absent in laminin 311, possibly in the γ2 chain,73 may contribute to the laminin deposition.

Since laminin 311 forms a complex with laminin 332 in vivo, it is tempting to speculate that laminin 311 deposition within the ECM might depend on its covalent association with laminin 332. Most interestingly, co-polymers of perlecan with mature laminin 311 were identified in ECM formed by alveolar epithelial cells.74,75 Through nogenkoid linkage to the laminin 311 γ1 chain, perlecan was proposed to nucleate formation of laminin 311 fibrils.

Function in cell adhesion and migration. It has been proposed that precursor laminin 332, together with integrin α3β1, plays a central role in the polarization and migration of cells.12,66 Processing of the laminin 332 α3 chain was suggested to alter its binding ability to various integrins by differential exposure of binding sites/sequences to integrin receptors. Cell adhesion studies have shown that immobilized purified α3200 laminin 332 promotes adhesion of keratinocytes, but to a lesser extent than that obtained with mature laminin 332.79 Further analysis revealed that the α3β1 integrin was the major receptor to interact with α3200 laminin 332, while both α3β1 and α6β4 integrins were involved in cell adhesion to α3165 laminin 332.76 These findings correlate with previous results showing that cells plated on an α3200 laminin 332 rich matrix failed to form α6β4
Table 1. Heparin binding sites identified within human and mouse LG45 domains

| Laminin a3 LG45 heparin binding sites | Method used for identification | Binding partner | References |
|--------------------------------------|---------------------------------|-----------------|------------|
| Human                               |                                 |                 |            |
| N1412SFMALYLSKGR                     | Peptide                         | Syndecan-2      | 82         |
|                                     |                                 | Syndecan-4      |            |
| L1429GTDGKKLRKISKEKCNDG              | LG45/heparin cross-link         | Heparin         | 85         |
| K1443KLRIK                           | Directed mutagenesis            | Syndecan-1      | 59         |
| R1438JKSKEK                          | Directed mutagenesis            | Syndecan-4      | 59         |
| L1388GSSPSGKPKSL                      | LG45/heparin cross-link         | Heparin         | 85         |
| V1510TPKQOSLC                         | LG45/heparin cross-link         | Heparin         | 85         |
| Mouse                                |                                 |                 |            |
| K1338ARSFNVNQLLQD                     | Peptide                         | Unknown receptor| 81         |
| K1398PRLQFSLDIQT                     | Peptide                         | Unknown receptor| 81         |
| D1622GQWHSVTVSIK                      | Peptide                         | Unknown receptor| 81         |

Heparin binding sites within a3 LG45. The mechanism underlying the function of the LG45 domain in laminin 332 remains poorly understood. Several heparin-binding sites have been identified in the α3LG45 sequence (Table 1). Some of these conferred heparin-dependent cell adhesion properties, which suggested that this region in laminin 332 could interact with a heparan sulfate proteoglycan receptor. An early report described the characterization of a heparin-binding synthetic peptide corresponding to residues K1398PRLQFSLDIQT derived from the murine α1 LG4 sequence. This peptide induces adhesion of the melanoma cells B16F10 through an heparan sulfate proteoglycan type receptor that remains to be identified. A peptidic screening of the murine α3 LG45 domains allowed identification of three heparin binding sites with cell adhesion properties. Peptidic screening of the human sequence lead to the identification of a motif that included residues N1412SFMALYLSKGR, which was shown to induce syndecan-2 and -4 mediated cell adhesion, neurite outgrowth and MMP-1 and -9 secretion. Further work suggested that this motif also induced keratinocyte migration by triggering syndecan-4 clustering and subsequent β1 integrin activation. Later, three novel heparin-binding sites were identified based on cross-linking the native protein to heparin beads. Recently, through a site-directed mutagenesis approach to alter the most critical basic residues in a recombinant LG45 protein, we identified a unique heparin-binding site surrounded by a track of converging low affinity, positively charged residues. We further showed that this K1433KLRIK sequence region, which matches one of the sites identified by Vives et al., harbours distinctive syndecan-1 and -4 interaction sites (Table 1). Besides, our group has reported that syndecan-1 is the cellular receptor involved in cell adhesion to the α3 LG45 domain and this interaction may participate in keratinocyte migration by supporting the formation of actin-based cellular protrusions (Fig. 4A and B). The development of these membrane protrusions remarkably requires dephosphorylation of tyrosine residues in the cytoplasmic tail of syndecan-1, a condition essential for syndenin-1 recruitment. In the epidermis, syndecan-1 is located in the pericellular region of keratinocytes and displays a modest expression in the basal cell layer, which becomes increasingly intense in the suprabasal layers. Remarkably, syndecan-1 is strongly induced in wound edge keratinocytes during wound healing.
in keratinocytes appears to be specific to syndecan-1 as no changes have been detected for other syndecans. Moreover, syndecan-1 deficient mice display a defect in keratinocyte proliferation and migration during wound healing. In light of these data, syndecan family members stimulate interest as potential laminin 332 co-receptors. Recent data has provided evidence that when human squamous carcinoma A431 cells are plated on laminin 332, syndecan-1 forms a complex with the α6β4 integrin. This triggers Fyn-mediated phosphorylation of the β4 integrin cytoplasmic tail activating PI3K- and Akt-mediated signalling, protecting the cells against apoptosis. This is of particular interest as LG45 in α3β2 chain was proposed as an essential PI3K pathway activation promoter.

**Functions of Precursor γ2 N-Terminal Domain**

It has been shown that in human skin basement membrane, the N-terminal domain of the γ2 chain is absent suggesting that the γ2 processing could have an important physiological role. In contrast to these findings, antibodies specific for the released L4 domain were shown to label the basal epidermal cells in mouse skin. A positive signal was also found in the basement membranes of early stages of skin development suggesting that the precursor γ2 chain or its released N-terminal domain expression might persist in vivo. The analysis of mTLD/BMP-1 deficient mice revealed that although defaults in skin cohesion were not clinically apparent, ultrastructural examination of the basement membrane showed places where anchoring structures were disconnected at the level of the lamina densa with presence of rudimentary hemidesmosomes. A strong expression of the precursor γ2 chain suggested that γ2 chain maturation takes part in the basement membrane cohesion and stability. Besides, defective γ2 processing was found in the rare inherited cylindromatosis disease (see above), in which abnormal basement membranes and excessive ectopic expression of collagen VII were found. The defective processing of the γ2 chain may have resulted in a deficient interaction with its basement membrane partners collagen VII causing defaults in anchoring fibrils linkage.

Numerous studies conducted with normal breast epithelial and carcinoma cells have shown that cell migration-inducing functions of the γ2 chain rely on its proteolysis. Recently, it has been reported that a syndecan-1 interaction with the N-terminal domain in precursor γ2 could negatively regulate β4 integrin phosphorylation leading to enhanced carcinoma cell adhesion and decreased motility reinforcing the idea that the precursor γ2 chain does not support cell migration. In contrast, other reports have suggested that precursor γ2 may play a role in keratinocyte migration during the wound healing process. An interaction of its N-terminal portion with α2β1 integrin was shown to occur during TGF-β1 induced keratinocyte migration in vitro and was proposed to take place at the wound margin of the skin healing process in vivo. Keratinocytes at the edge of wounds made in early passage cultures shown to co-express the laminin 332 γ2 chain and the cell cycle inhibitor p16INK4a, displayed increased directional motility. Additional studies revealed that this keratinocyte hypermotility/growth arrest response relied on both the precursor state of the laminin 332 γ2 chain and the participation of a serum co-factor through a TGFβ2 receptor I independent mechanism. This apparent contradiction in experimental findings is likely due to tissue specific differences. A study indicated that the L4 module mediates the integration of laminin 332 into the extracellular matrix through intermolecular interactions.

The question arises whether the heparin-binding domains located within the α3 LG45 domains and the N-terminal portion of the γ2 chain are involved in laminin 332 deposition. Heparin was shown to inhibit laminin 332 deposition within the ECM suggesting that a heparan sulfate proteoglycan might be involved in binding to both precursor α3 and γ2. Null mutation in syndecan-1 has not reported defects in laminin deposition, which suggests that any of these two receptors is solely responsible for this mechanism. Generation of double syndecan-1 and -4 knockout mice would reveal whether both these receptors are implicated. Future studies should examine whether a heparan sulfate proteoglycan cell surface receptor or a basement membrane components are is implicated in precursor laminin 332 deposition. Interestingly, a recent study has reported that the perlecan colocalizes with laminin 332 at the wound margin of healing full thickness wounds generated in mice suggesting proximity of these two components during basement membrane remodelling. However, as perlecan was shown not to be essential for matrix assembly in perlecan-null mouse embryos, its presence in the ECM might not be an absolute requirement for laminin 332 deposition. Considering the important function of laminin 332 in skin homeostasis, several extracellular ligands might be involved in its deposition in the basement membrane.

**Figure 4.** Syndecan-1 mediated cell adhesion to laminin 332 LG45 domain. (A) Fibrosarcoma HT1080 cells were plated on surfaces coated with purified LG45 domain for 1 h, fixed and stained with phalloidin-FITC. The analysis of the actin staining revealed an organization of the cytoskeleton in radial arrays of microspikes, protrusive adhesions structures known to be involved in transient cell-substratum interactions. (B) Confocal image of HT1080 cells expressing GFP-syndecan-1 plated on the LG45 domain. The phase contrast image shows the typical induced cell shape characterized by the formation of spike-like actin-based protrusive structures. The receptor syndecan-1 (green) promotes cell adhesion to the laminin fragment and aligns along the actin filaments, which are organized into parallel bundles. Bars, 10 μm.
Comprehension of the physiological significance of laminin 332 maturation events remains an open issue. Numerous studies have focused on the LG45 and y2 N-terminal domains suggesting their involvement in diverse important functions such as laminin secretion, deposition and/or retention within the ECM; others have suggested a participation in epithelial cell adhesion and migration processes. However, their exact function remains unknown. While in vitro data sometimes disagree due to the cellular system’s diversity, in vivo models firmly demonstrate that defects in laminin 332 maturation impede correct basement assembly as well as hemidesmosome formation. Molecular interaction abnormalities occur at the level of cellular receptors and basement membrane components causing both functional and organisational anomalies. Molecular characterization and structural definition of laminin domains open interesting and every so often unexpected questions. From its identification in the 1990s,²,²⁶,¹⁰⁴,¹⁰⁵ to nowadays, laminin 332 has stimulated incessantly increasing interest in the scientific community due to its multifunctional properties and its involvement in human physiological processes and pathologies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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