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Chapter 2

Laminin-332 and Integrins: Signaling Platform for Cell Adhesion and Migration and Its Regulation by *N*-glycosylation

***Yoshinobu Kariya*[*1], *Yukiko Kariya*[2] *and Jianguo Gu*[3]**
[1]Department of Biochemistry,
[2]Department of Diagnostic Pathology,
Fukushima Medical University School of Medicine,
Hikarigaoka, Japan
[3]Division of Regulatory Glycobiology,
Institute of Molecular Biomembrane and Glycobiology,
Tohoku Pharmaceutical University,
Aoba-ku, Sendai, Japan

Abstract

Laminin-332 (formerly known as laminin-5) is a major component of basement membranes in the skin and other stratified squamous epithelial tissues. Laminin-332 has attracted much attention from both physicians and researchers as a disease-related and functional molecule. The study of human genetic disorders and a knockout mouse model has provided the evidence that laminin-332 is indispensable for adhesive structures in the skin. In wound healing and cancer invasion, overexpression of laminin-332 accelerates cell migration by activating PI3K, PKC, and MAPK signaling pathways. These functional activities of laminin-332 are through interactions with several cell surface receptors including integrins $\alpha 3\beta 1$ and $\alpha 6\beta 4$, and syndecans. In addition, recent studies revealed that posttranslational modifications, such as proteolytic processing and *N*-glycosylation, of laminin-332 and of integrins $\alpha 3\beta 1$ and $\alpha 6\beta 4$ are important for the

* Corresponding author: Department of Biochemistry, Fukushima Medical University School of Medicine, 1 Hikarigaoka, Fukushima 960-1295, Japan. Tel: +81-24-547-1144; Fax: +81-24-548-8641; E-mail: kariya@fmu.ac.jp.

laminin-332 activities. This review summarizes the molecular mechanisms and significance of cellular adhesion and migration through laminin-332 as well as integrins α3β1 and α6β4. Understanding of these regulatory mechanisms could lead to development of new molecular targeted therapies, particularly in oncology.

Introduction

Basement membrane (BM) is a 50–100-nm layer of specialized extracellular matrix (ECM) protein complex that underlies all epithelial cell sheets and tubes, such as epithelia, endothelia, muscle cells, fat cells, and nerve cells [1]. The major ECM components of BM are laminin, type IV collagen, nidogen, and perlecan. Self-assembly of laminin and type IV collagen individually forms insoluble networks, which are linked by nidogen and perlecan, and the resulting supramolecular structures provide BM stability and mechanical support of tissue. BMs not only maintain tissue structure but also regulate cellular functions such as cell adhesion, migration, differentiation, proliferation, and resistance to anoikis [2]. These BM-mediated cellular functions are dependent on the type of ECM components in BM.

Laminins are large extracellular glycoproteins with a molecular mass of 400–900 kDa [2]. Laminins are heterotrimers consisting of three chains, α, β, and γ, bound by disulfide bonds (Figure 1). To date, five α, four β, and three γ chains as well as alternatively spliced variants of these chains have been genetically identified to form at least 16 distinct laminin heterotrimers in mammals, although no laminin heterotrimer containing the β4 chain has been described [3]. These laminin isoforms show tissue- and development-specific expression patterns *in vivo* and lead to distinct and tissue-specific functions of BM. For example, laminin-211 (α2β1γ1; formerly laminin-2) and laminin-411 (α4β1γ1; formerly laminin-4) is predominantly expressed in BMs of developing skeletal muscles and peripheral nerves and in BMs of blood vessels, respectively. The first identified laminin, laminin-111 (α1β1γ1; formerly laminin-1), which was discovered in mouse Engelbreth-Holm-Swarm (EHS) sarcoma, is primarily expressed during very early embryogenesis [2-4].

The laminin α3 chain is predominantly expressed in the BM of skin and other stratified squamous epithelial tissues and is known to have two splicing variants: the truncated form α3A and the full-sized form α3B [5, 6]. Laminin α3A and α3B chains associate with the β3 chain and the γ2 chain to form laminin-332 (laminin-3A32; formerly laminin-5 or laminin-5A) and laminin-3B32 (formerly laminin-5B), respectively [7]. The laminin α3A chain can also associate with the β1/β2 chains and the γ1 chain, forming laminin-311 (laminin-3A11; formerly laminin-6 or laminin-6A) and laminin-321 (laminin-3B21; formerly laminin-7 or laminin-7A) [8, 9]. The laminin α3B chain is widely expressed in vascular BMs of normal tissues, probably as laminin-3B11/3B21 (laminin-6B/7B) but the expression of laminin α3B chain is lost in vessels near invasive breast carcinoma cells [10-12].

Compared with laminin-111, all short arms in laminin-332 subunits are truncated (Figure 1). This truncated structure is unique to laminin-332. Although full sized laminin short arms contain some integrins and heparin-binding sites as well as self-polymerization sites, truncated forms of laminin-332 would lose a part of these domains, and consequently would have distinct physiological properties from other laminin isoforms. In contrast, all laminin α chains have a central long coiled-coil domain and a large C-terminal comprising five tandem

laminin globular (LG) subdomains (LG1-5) [2]. The latter is the major interaction sites for cell surface receptors such as integrins, syndecans, and dystroglycans (Figure 1).

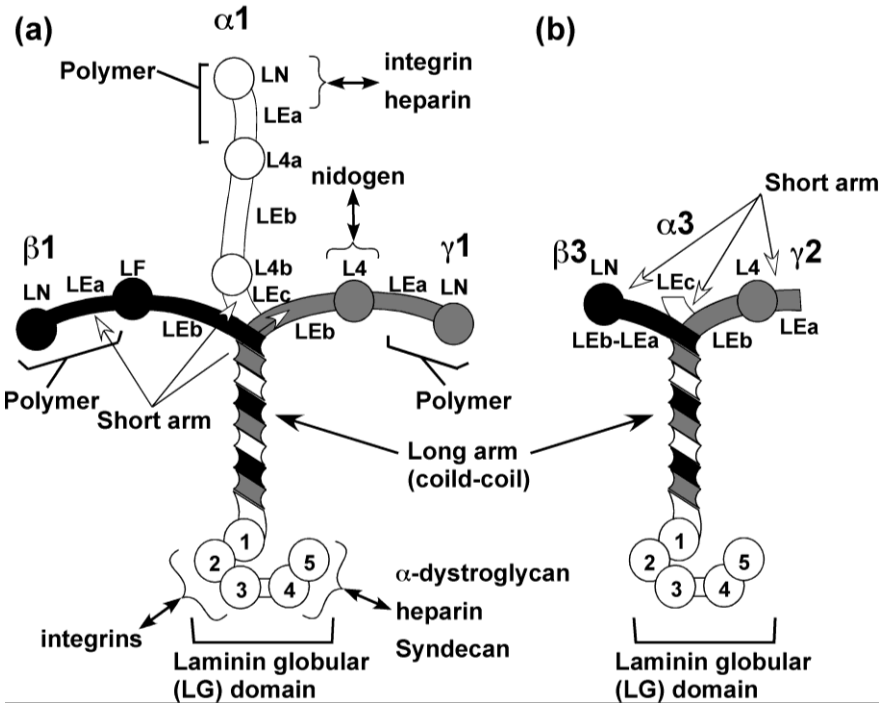

Figure 1. Schematic structures of laminin-111 and laminin-332. Laminins are heterotrimers composed of α, β, and γ chains bound by disulfide bonds. Each laminin chain contains a short arm and a long arm, forming coiled-coil domains. Only the α chain has five tandem laminin globular (LG) subdomains (LG1-5) at its carboxyl-terminal end. The amino-terminal (LN domain) and internal short arm globular (LF and L4 domains) modules are indicated by ovals. The rod-like epidermal growth factor (EGF) repeats (LE domains) are shown as lines. (a) Laminin-111 (α1β1γ1) can self-polymerize through the three LN domains, possibly requiring the participation of more distal domains in the short arms. Heparin- and integrin-binding sites are found in both the N-terminal LN domain and the C-terminal LG domain of the α1 chain. (b) Compared with laminin-111, all short arms in laminin-332 (α3β3γ2) subunits are truncated. Since the N-terminal LN domain in the laminin α1 chain contains some integrin- and heparin-binding sites as well as self-polymerization sites, laminin-332 would consequently show distinct functional activities from laminin-111. The protein binding partners of laminin-332 are described in Figures 2B and 3.

Laminin-332 in Cell Adhesion and Deposition

As shown in human genetic disorders and a knockout mouse model, laminin-332 is an essential molecule for maintaining skin tissue architecture. Laminin α3, β3, and γ2 chain knockout mice die at the neonatal stage and suffer blistering of the skin [13-15]. These phenotypes are consistent with the human severe and lethal skin blistering disease, Herlitz's junctional epidermolysis bullosa, which is caused by genetic defects of any of the laminin-332 subunits [16-19]. Laminin-332 is predominantly expressed in the BM of skin as well as

other stratified squamous epithelial tissues (Figure 2A). Skin tissue is separated into two parts epidermis and dermis by laminin-332–containing BM, and only basal keratinocytes can associate with BM (Figure 2B). Basal keratinocytes associate with laminin-332 mainly through cell surface receptor integrin α6β4, and thereby form stable adhesion complexes, termed hemidesmosomes [20, 21]. Laminin-332 can associate with integrin α6β4 and other hemidesmosome-related protein, type VII collagen through the LG1–3 domains [22] and β3 chain short arm [23, 24], respectively (Figure 2B). Genetic defects in such laminin-332 binding partners also cause other forms of epidermolysis bullosa [25-28].

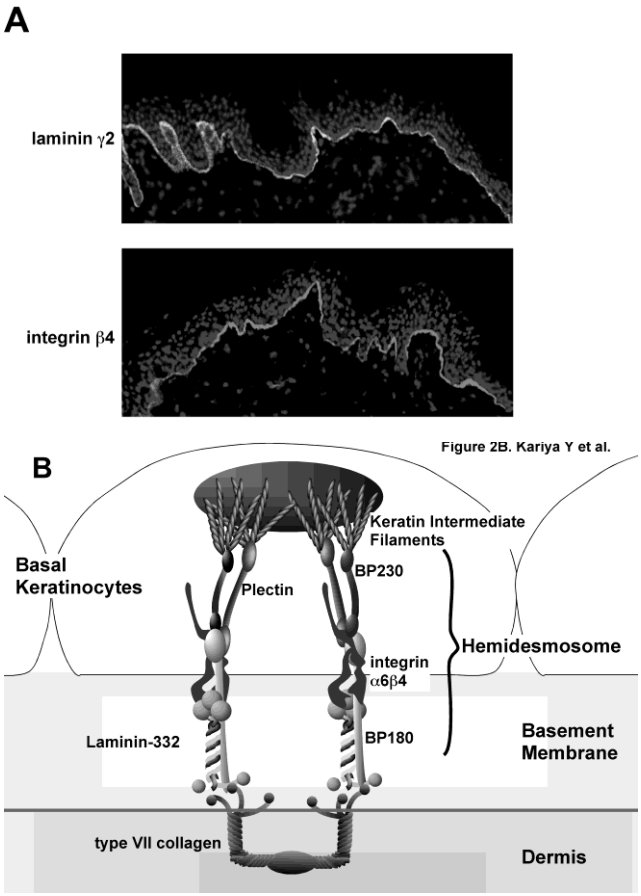

Figure 2. Hemidesmosome components in the skin. (A) Immunohistochemical staining of the laminin γ2 subunit and integrin β4 subunit in the skin. Both laminin γ2 (upper panel) and integrin β4 (lower panel) are localized in the basement membrane. Nuclei are stained with Hoechst 333432. (B) A schematic diagram of hemidesmosomes in the skin. Laminin-332 binds to integrin α6β4 and type VII collagen through the laminin globular (LG) domains 1–3 in the α3 chain and the short arm in the β3 chain, respectively. BP180 associates with laminin-332 and α6 integrin through the extracellular domain and with integrin β4, plectin, and BP230 through the cytoplasmic domain. Plectin and BP230 bind to the β4 cytoplasmic domain as well as keratin intermediate filaments. Genetic defects involving these molecular components of the hemidesmosomes cause epidermolysis bullosa.

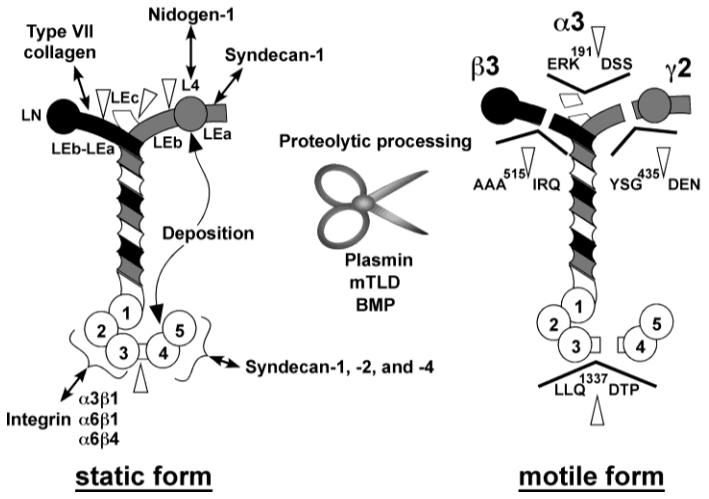

Figure 3. Regulatory mechanism of laminin-332 activities by proteolytic processing. The LG1–3 domains in the α3 chain bind to integrins α3β1, α6β1, and α6β4, and the LG4–5 domains interact with syndecans. The LEb–LEa domain in the β3 chain binds to type VII collagen. The L4 domain and LEa domain in the γ2 chain binds to nidogen-1 and syndecan-1, respectively. The precursor α3 chain (190 kDa) is cleaved in the linker region between the LG3 and LG4 domains by plasmin, mammalian tolloid (mTLD), and bone morphogenic protein-1 (BMP-1; 160 kDa). In addition to the proteolytic processing at the C-terminal G domain, in some cell lines, further proteolytic processing occurs at an N-terminal LEc domain in the α3 chain (145 kDa). The γ2 chain undergoes processing in the LEb domain by mTLD and BMP-1. Although the β3 chain is relatively resistant to proteolysis compared with the α3 and γ2 chains, the β3 chain is also cleaved at the short arm region. Arrowheads indicate the major proteolytic cleavage sites. The numbers and amino acid sequences indicate the position of amino acid residues at the cleavage sites and the amino acid sequence around the cleavage sites. The α3 LG4–5 domains and the γ2 L4 domain play critical roles in the incorporation of laminin-332 into the extracellular matrix, thereby supporting cell adhesion and suppressing cell migration (static form). Proteolytic processing of laminin-332 suppresses its deposition and its association with other molecules, thereby inducing cell migration (motile form).

Recent analysis using electron microscopy showed that the deposited laminin-332 matrix forms a mesh-like network structure and is highly polymerized [29]. Full-sized laminin chains, such as laminin α1, α5, β1, and γ1 chains, can self-assemble through the self-polymerization site in the short arm LN domains [2] (Figure 1). In contrast, because the LN domain is truncated in laminin α3 and γ2 chains, laminin-332 has been considered to be incapable of self-polymerizing and assembling into BM networks. Although the mechanism has not yet been clarified, approximately half of total laminin-332 exists in disulfide-linked complexes with laminin-311 or laminin-321 formed through the unpaired cysteine residues in their short arms [9]. Because laminins-311/321 have full-sized β1 or γ1 chains containing the self-polymerization site, laminin-332 could be integrated into a matrix network through laminins-311/321.

During wound healing, newly synthesized laminin-332 is deposited under cells as an insoluble matrix protein [30]. Deposition of laminin-332 to the matrix is facilitated by the LG4–5 domains in α3 chain [31, 32] and the L4 domain in γ2 chain [33] (Figure 3). In contrast, the short arm of the laminin β3 chain enhances the matrix assembly and cell

adhesion activity of laminin-511, another laminin isoform present in the skin [34]. Recent studies have suggested that the heparan sulfate chains on the cell surface also play an important role in the assembly of hemidesmosomes and in laminin-332 deposition [29, 35]. This may be reasonable because heparan sulfate proteoglycans bind to the LG4–5 domains in the α3 chain [36, 37] and the short arm in the γ2 chain [38], which accelerate deposition of laminin-332 into the matrix.

The laminin-332 matrix supported keratinocyte cell adhesion much more strongly than non-polymerized, purified laminin-332, whereas the laminin-332 matrix suppressed keratinocyte migration compared with non-polymerized, purified laminin-332 [29]. This strong adhesion of the laminin-332 matrix to cells is caused by the high affinity of laminin-332 to integrin α3β1, and an induction of α6β4-containing hemidesmosome-like structures in the laminin-332 matrix. These observations suggest that polymerized lamnin-332 has different physiological activities from unassembled soluble laminin-332.

Integrins α3β1 and α6β4 in Cell Adhesion

Integrins are a large family of heterodimeric transmembrane receptors comprising α and β subunits [39]. In mammals, 18 α and 8 β subunits have been characterized, and the combinations of them form 24 distinct integrins. The extracellular domains of integrin subunits associate with ECM proteins, such as laminin, collagen, fibronectin, and vitronectin, and in the cytoplasmic domain many cellular signaling molecules bind to integrins and send various cellular signaling. Thus, integrins constitute both a structural connection and a bi-directional signaling pathway that crosses the cell membrane [39, 40]. The laminin-binding integrins (α3β1, α6β1, α6β4, and α7β1) show robust association with tetraspanin proteins [41]. Tetraspanins CD151, CD81, and CD9 modulate laminin binding, affecting integrin-dependent neurite outgrowth, cell adhesion, migration, and morphology [41-43].

The main integrin receptors for laminin-332 are integrins α3β1, α6β1, and α6β4, which are highly expressed on epithelial cells as well as on cancer cells [22, 44, 45]. *In vitro* cell adhesion activity of laminin-332 through these integrins is much stronger than that of other ECM proteins such as other laminin isoforms, collagens, fibronectin, and vitronectin [46, 47]. The Glu residue at the third position from the C-terminus of the γ2 chain is critical for the association of integrins α3β1 and α6β1 with laminin-332 [48]. Keratinocytes express integrins α2β1, α3β1, and α6β4 but not α6β1 [20], and integrin α6β4 regulates α2 and α3 integrin subunit expression [49]. Integrins α3β1 and α6β4 function as cell adhesion receptors for laminin-332. The association between those integrins and laminin-332 is important for maintaining the integrity of epidermal structure and function.

Integrin α3β1-mediated adhesion to laminin-332 promotes the stabilization of leading lamellipodia in migrating keratinocytes through activation of Rac1 [50]. In contrast to the localization of integrin α6β4 in hemidesmosome, integrin α3β1 is recruited to focal contacts in cultured cells [21]. Because integrin α3β1 but not α6β4-mediated cell spreading and adhesion to laminin-332 are dependent on the cAMP-Epac-Rap1 pathway [51], the regulatory mechanisms for cell spreading and adhesion between integrins α3β1 and α6β4 are distinct. Patients with homozygous mutations in *ITGA3* gene suffer from congenital nephrotic

syndrome, interstitial lung disease, and epidermolysis bullosa [52]. Similar to the phenotypes of human patients, in integrin α3β1-deficient mice, matrix disorganization is accompanied by skin blistering [53]. However, epidermal blistering was caused by rupture of BM rather than by detachment of the epidermis from BM. Integrin α3β1-deficient keratinocytes from α3β1-deficient mice attach to laminin-332 through integrin α6β4 but spread poorly on laminin-332. Integrin α3β1 therefore is considered to function in cell spreading and migration rather than stable adhesion.

Integrin α3β1 is also found in cell–cell contacts in adherence junctions [54] and regulates cadherin–catenin complex formation [55]. Association of integrin α3β1 with tetraspanin CD151 stimulates cadherin-mediated cell–cell adhesion both by regulating the expression of PTPμ and by organizing the multimolecular association of PKCβII, RACK1, PTPμ, E-cadherin, and β-catenin [56]. Integrin α3β1 also regulates cell–cell adhesion by promoting gap junctional intercellular communication through interaction with laminin-332 [57]. Furthermore, integrin α3β1 plays important roles in proper incorporation of laminin-332 into the matrix and in the expression of the laminin-332 matrix function [53, 58]. This integrin α3β1-mediated laminin-332 deposition is regulated by the Rac activator Tiam1 [59].

Integrin α6β4 is an essential component of hemidesmosomes [20, 60] (Figure 2A and 2B). Patients with mutations in the genes encoding integrin α6β4 (*ITGA6* and *ITGB4*) suffer from epidermolysis bullosa with pyloric atresia (EB-PA), which is an autosomal recessive disorder characterized by blistering of the skin and mucous membranes and by associated congenital gastrointestinal atresia [25, 28]. Unlike other β integrins, the cytoplasmic tail of β4 integrin is more than 1000 amino acids long and contains two pairs of fibronectin type III (FNIII) repeats separated by a connecting segment [60]. This long cytoplasmic region of β4 integrin associates with hemidesmosome component molecules, plectin, BP180 (type XVII collagen or BPAG2), and BP230 (BPAG1) [60] (Figure 2B). Two missense mutations (R1225H and R1281W) in the β4 gene (*ITGB4*) lead to non-lethal phenotypes of EB-PA [25, 61]. R1225 and R1281 in the first pair of FNIII repeats are essential for binding of integrin β4 to plectin. In R1225H or R1281W β4 mutants expressing β4 null keratinocytes obtained from EB-PA patients, mutations in β4 abolish the interaction between β4 integrin and plectin and prevent the recruitment of plectin into hemidesmosomes [62]. The binding of β4 integrin to plectin is a critical step in the formation of hemidesmosomes as well as in the recruitment of BP180 and BP230 into hemidesmosomes [63].

Laminin-332 and Integrins in Cell Motility

In vitro, laminin-332 stimulates the migration of various types of cells, including cancer cells, and is known not only as a cell substrate but also as a soluble cell scattering factor [64-66]. *In vivo*, laminin-332 is overexpressed during wound healing [6] and cancer invasion [67-69] and is considered to be a key factor for such physiological events. *In vitro* cell migration, wound healing, and invasion assays using an antibody that blocks the function of laminin-332 or laminin-332–deficient keratinocytes indicated that laminin-332 is an essential molecule in cell motility [70, 71]. Furthermore, both the binding of α6β4 integrin to laminin-332 and the cytoplasmic domain of β4 integrin are required for the EGF-driven directional migration of

keratinocytes [72]. Laminin-332 promotes both cell adhesion and migration through the interaction of the LG3 domain in the C-terminus of the laminin α3 chain with integrins α3β1, α6β1, and α6β4 [22]. The apparent discrepancy between strong cell adhesion and migration activities can be explained by the presence of distinct functional sites for these two activities within the LG3 domain [22]. The association of the LG3 domain with integrins activates cellular signaling through protein kinase C, phosphatidylinositol 3-kinase (PI3K), Rac, ERK, JNK, and NF-kB, thereby promoting cell migration [65, 73-75].

Human laminin-332 is synthesized and secreted as a precursor form (190 kDa α3, 135 kDa β3 and 150 kDa γ2 chains). In both physiological and pathological conditions, such as wound healing and cancer invasion, laminin-332 undergoes proteolytic processing by some proteases after secretion by cells (Figure 3). In fact, after an injury, leading keratinocytes migrate into the wound bed where dermal collagens are exposed and secrete large amounts of laminin-332. Laminin-332 containing the precursor form of the α3 chain was found only in wound beds, whereas the mature form of laminin-332 was detected both in normal tissue, away from the wound bed, and in the provisional BM of the wound bed [30]. Proteolytic processing converts the precursor form of α3 (190 kDa) and γ2 (150 kDa) chains to the mature form of α3 (160 kDa and 145 kDa) and γ2 (105 kDa) chains, respectively [76] (Figure 3). The α3 chain of laminin-332 is cleaved in the linker region between the LG3 and LG4 domains (160 kDa) [77] by plasmin [78], mammalian tolloid (mTLD), and bone morphogenic protein-1 (BMP-1) [79, 80]. In addition to proteolytic processing at the C-terminal G domain, in some cell lines, further proteolytic processing occurs at an N-terminal LEc domain in the α3 chain (145 kDa) [7]. In human laminin-332, mTLD and BMP-1 are major processing enzymes of the γ2 chain [79, 80], which cleave the γ2 chain at the short arm LEb domain, although in rat laminin-332, matrix metalloproteinase 2 (MMP2) [81], membrane type 1-matrix metalloproteinase (MT1-MMP) [82], and neutrophil elastase [83] are also known to process the γ2 chain. Although the β3 chain is relatively resistant to proteolysis compared with the α3 and γ2 chains, the β3 chain is also cleaved at the short arm region [24, 84] by MT1-MMP [84], matrilysin-1 (matrix metalloproteinase 7; MMP7) [85], and hepsin [86].

These proteolytic conversions of laminin-332 affect its activities. Laminin-332 containing the mature form of the γ2 chain (105 kDa) increases cell migration but decreases cell adhesion, compared with laminin-332 containing the precursor form of γ2 chain (150 kDa) [81, 82, 87]. Purified recombinant laminin-332 containing the precursor form of α3 chain decreases both cell adhesion and migration compared with recombinant laminin-332 containing the mature form of the α3 chain that lacks the LG4–5 domains [32]. The main reason for the downregulation of biological activities in laminin-332 with LG4–5 domains may be decreased integrin-mediated signaling through the LG1–3 domains because of interference by the association of the LG4–5 domains with heparan sulfate proteoglycan receptors such as syndecans-1, 2, and 4 [88].

The integration of laminin-332 into ECM supports stable cell adhesion and suppresses cell migration by strong association with the integrins α3β1 and α6β4 and other matrix protein [29]. Dissociation from stable adhesion constituted by deposited laminin-332 therefore is required for inducing cell migration. The laminin-332 with LG4–5 domains is integrated into ECM more efficiently than its mature form because of the interaction of LG4–5 domains with heparan sulfate proteoglycans such as syndecans [31, 32, 37] (Figure 3).

Similarly, the N-terminal L4 domain of the γ2 chain is participated in the integration of laminin-332 into ECM [33] (Figure 3). Proteolytic processing of α3 and γ2 chains remove the LG4–5 domains from the α3 chain and the L4 domain from the γ2 chain in the laminin-332 molecule, resulting in suppression of laminin-332 deposition and its association with other molecules, thereby inducing cell migration. The LEb–LEa domain in the short arm region of the β3 chain associates with type VII collagen [23, 24], and the L4 domain and LEa domain of the γ2 chain interacts with nidogen-1 [89] and syndecan-1 [38], respectively. Because type VII collagen is the main component of anchoring fibrils and nidogen is a cross-linking protein among other matrix molecules, reduction of the association of laminin-332 with these molecules by proteolytic processing at the short arm region of the β3 [24, 84-86] and γ2 [81, 82] chains increases laminin-332–dependent cell motility.

During cell migration, the hemidesmosome assembly/disassembly is dynamically regulated [60, 90]. Serine/threonine phosphorylation of the cytoplasmic domain of β4 integrin by stimulation of growth factors, such as EGF, destabilizes hemidesmosomes [91]. Hemidesmosome formation is driven by interaction of the cytoplasmic domain of β4 integrin with plectin. The interaction between plectin and β4 integrin is regulated by serine phosphorylation of residues 1356, 1360, 1364, and 1424 [92] or threonine phosphorylation of residue 1736 [93] of the β4 cytoplasmic domain. The phosphorylation of these residues by PKC, PKA, or PKD1, which signal downstream of epidermal growth factor receptor (EGFR) results in hemidesmosome disassembly due to loss of interaction between β4 integrin and plectin, thereby inducing cell migration.

Laminin-332 and Integrins in Cancer

Many studies have reported that laminin-332 [66, 67] and its integrin receptors [94] are highly expressed in tumors, and previous *in vitro* studies of laminin-332 activities, such as cell adhesion, migration, and proliferation, indicated that laminin-332 and its integrin receptors are key factors in tumor progression [95]. The importance of laminin-332 and integrin α6β4 in tumorigenesis has been proven using an animal model of human squamous cell carcinoma (SCC). In the assay, transformed primary keratinocytes could form tumors in nude mice skin, but laminin-332–negative or β4 integrin-negative keratinocytes (derived from epidermolysis bullosa patients with *LAMB3* or *ITGB4* null mutations, respectively) could not [96]. Introduction of laminin β3 and integrin β4 cDNA expression vectors into those cells restored their tumorigenicity, suggesting that these molecules are essential for human SCC tumorigenesis. More extensive studies about laminin-332–mediated tumorigenesis have shown that the laminin α3 LG4–5 domains and the laminin β3 LEb–LEa domain are significant for SCC tumorigenesis. The LG4–5 domains activate ERK and PI3K signaling pathways that induce proliferation and inhibit apoptosis, promoting SCC tumorigenesis [97]. The association of the laminin β3 LEb–LEa domain with the type VII collagen NC1 domain, which promotes tumor cell invasion in a laminin-332–dependent manner, stimulates PI3K signaling for SCC tumorigenesis [98].

Integrin α6β4 was originally identified as a tumor-specific protein [99, 100]. Subsequent studies have reported that overexpression of integrin α6β4 was seen in several types of

invasive and metastatic carcinomas and correlated with poor prognosis [101]. Integrin α6β4 activates PKC [102], PI3K [103], and ERK [104] signaling pathways, which are linked to cell migration, invasion, and proliferation [95]. In particular, PI3K, which is an upstream regulator of Rac1, is the central factor in α6β4-dependent tumor progression [103, 105]. Integrin α6β4 associates with several receptor tyrosine kinases (RTKs), including EGFR, ErbB2, Met, and Ron [95]. These RTKs activate β4 integrin-associated Src family kinases and thereby induce the tyrosine phosphorylation of the β4 integrin cytoplasmic domain. The phosphorylation of tyrosine 1494 in the β4 cytoplasmic domain promotes breast carcinoma cell invasion through the combined activation of PI3K and Src, and anchorage-independent growth through activation of ERK1/2 [106]. The phosphorylated α6β4 integrin conversely promotes Src-family kinases-dependent phosphorylation of RTKs. ErbB2, the ligand-less member of EGFR family, associates with integrin β4 subunit through a region of the β4 cytoplasmic domain and both α6β4 and ErbB2 are required for PI3K activation and subsequent invasion in mouse NIH3T3 cells [107]. Conversely, the binding of immunoglobulin-like adhesion molecule Necl-2 (Nectin-like molecule 2) to the extracellular region of β4 integrin stabilizes the hemidesmosome structure, thereby inhibiting cancer cell motility by suppressing ErbB3/ErbB2 signaling [108].

Integrin α3β1 is also associated with tumor progression in some cancers. In hepatocellular carcinoma (HCC) cells but not in peritumoral tissue of the same HCC patients, laminin-332, Snail, and Slug are upregulated, whereas E-cadherin is downregulated and β-catenin is translocated into the nuclei. *In vitro* incubation of HCC invasive cells with purified laminin-332 induces upregulation of Snail and Slug and downregulation of E-cadherin as well as translocation of β-catenin into nuclei, resulting in a complete EMT accompanied by marked morphological change. This event is mediated through integrin α3β1 but not α6β1 or α6β4 [109]. Another group, using integrin α3-null kidney epithelial cells, demonstrated that integrin α3β1 attenuates EMT by downregulating β-catenin–Smad2 signaling when α3β1 is mainly engaged in laminin-332–rich rat 804G culture supernatant or with E-cadherin in adherens junctions [110]. These apparent contradictory findings may result from the laminin-332 source or the cell types used in each assay.

Regulation of Laminin-332 and Integrins by *N*-Glycosylation

Laminin is a heavily glycosylated molecule and between 13% and 30% of its total molecular weight is contributed by *N*-glycosylation [111]. The laminins undergo terminal glycosylation within the Golgi apparatus and then *N*-glycosylated laminins are secreted by the cells. In early studies, the carbohydrate structure and function of laminin-111 was intensively investigated because a large amount of laminin-111 can easily be prepared from mouse EHS tumor tissue. From the analysis of unglycosylated laminin-111, which is prepared from cell lysates of tunicamycin-treated cells, *N*-glycosylation of laminin-111 was revealed to be important for cell spreading and neurite outgrowth activities but not for cell adhesion activity or heterotrimer assembly [112]. However, because tunicamycin-treatment markedly inhibited secretion of laminin into the cell culture medium and unglycosylated laminin-111 was

purified from cell lysates, the activities of unglycosylated laminin-111 purified from cell lysates may not be the same as that of the secreted protein. Therefore, the significance of *N*-glycosylation in laminin molecules remained unclear for a long time.

Recent studies using laminin-332 purified from the conditioned medium of glycosyltransferase-transfected cells clearly demonstrated that *N*-glycosylation of the laminin-332 molecule affects its biological functions, such as cell adhesion, migration, and scattering [113]. In some cancers, an increase of β1,6 GlcNAc, catalyzed by *N*-acetylglucosaminyltransferase V (GnT-V), is related to cancer metastasis, whereas bisecting GlcNAc suppresses further processing by branching enzymes, such as GnT-V, resulting in downregulation of cancer metastasis. Laminin-332 purified from the gastric cancer cell line MKN45 possesses a large amount of β1,6 GlcNAc and promptly induces cell adhesion and migration. In contrast, cell adhesion and migration activities of purified laminin-332 from GnT-III overexpressing MKN45 cells was apparently decreased compared with those of control laminin-332 purified from MKN45 cells. These weakened activities were most likely derived from the impaired α3β1 integrin clustering and resultant focal adhesion formation. Thus *N*-glycosylation of laminin-332 is important for its association with integrins and the subsequent cellular signaling (Figure 4).

Integrin is also a major carrier of *N*-glycans, and proper *N*-glycosylation of integrin is important for its functional activities and for heterodimer formation. A patient with a missense mutation in *ITGA3*, which introduces an additional *N*-glycosylation motif in the integrin α3 subunit, causes fatal interstitial lung disease and congenital nephrotic syndrome. The resulting gain of glycosylation impedes heterodimerization of the α3 precursor with β1 [114]. Recent studies using molecular and cellular biology-based techniques clearly show that *N*-glycans of both integrins α3β1 and α6β4 affect various cellular functions. Introduction of bisecting GlcNAc [115] or core fucose (α1,6-fucose) [116] into integrin α3β1 in MKN45 cells or α1,6-fucosyltransferase (Fut8)-null embryonic fibroblasts downregulates cell adhesion and migration through laminin-332. In contrast, the amount of β1,6 GlcNAc branched structures on integrin α3β1 correlated with metastatic ability in melanoma cells [117]. Although the precise mechanisms are unclear, *N*-glycosylation of integrin α3β1 is modulated by tetraspanin CD151 [118]. Furthermore, an *N*-glycosylation–defective CD151 mutant did not affect interactions of CD151 with other tetraspanins or integrin α3β1 but negated its modulatory function [118]. Galectin-3–mediated association among laminin-332, integrins, and EGFR through *N*-glycans is important for their complex formation and the subsequent cellular signaling. Galectins are a family of lectins, which bind β-galactoside and are often involved in protein–protein interactions by cross-linking β-galactosides on proteins through a carbohydrate recognition domain in the galectin molecule [119]. Galectin-3 is one of the best-characterized galectins, and it is only chimeric galectin [120]. Galectin-3 is ubiquitously expressed in adult tissues but is also highly expressed in some cancers as well as in wound healing. Binding of galectin-3 to β-galactoside on integrin α3β1, α6β4, and laminin-332 supports laminin-332-induced integrin clustering and focal contact formation [71, 113]. Galectin-3–mediated association between integrin α6β4 and EGFR is involved in integrin α6β4–EGFR complex formation, which activates ERK signaling pathway [71]. These galectin-3 mediated events are canceled by introduction of bisecting GlcNAc into these interacting proteins because bisecting GlcNAc inhibits GlcNAc branch formation catalyzed by GnT-IV and GnT-V, thereby suppressing addition of poly-*N*-acetyllactosamine *N*-glycan

containing β-galactoside [71] (Figure 4). In the case of the integrin β4 subunit, expression of *N*-glycosylation-defective β4 integrin, in which asparagine (N) was replaced by glutamine (Q) in all five potential *N*-glycosylation sites of β4, decreased cell spreading, adhesion, migration, and lipid raft localization on laminin-332 in β4-null keratinocytes compared with β4-null keratinocytes expressing wild type β4 integrin [121]. Similar to the effect of *N*-glycans in CD151 on *N*-glycosylation of α3β1 [118], an *N*-glycosylation-defect in β4 integrin affects the *N*-glycosylation state of EGFR, which associates with β4 integrin through cross-linking with galectin-3–mediated *N*-glycans as well as by protein–protein interaction [121]. However, *N*-glycosylation-defective β4 integrin induced a strong association with EGFR, which is mediated only by protein–protein interaction but not by galectin-3–mediated *N*-glycan interaction compared with wild type β4 integrin [121]. Thus appropriate complex formation mediated by *N*-glycan, which would generate the adequate space between associated proteins, may have a suppressive effect on excessive protein–protein interactions and induce efficient cellular signaling and the subsequent cellular functions.

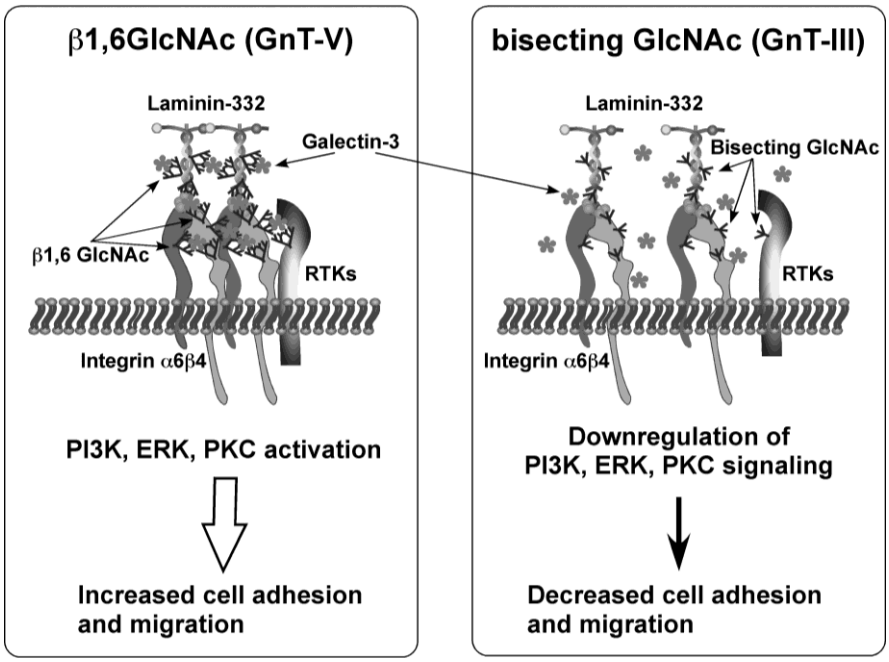

Figure 4. Model for the effect of *N*-glycans on laminin-332 and integrin α6β4-mediated signal transduction and cellular functions. With enhanced expression of GnT-V in cells (left panel), galectin-3 binds to integrin α6β4, laminin-332, and receptor tyrosine kinases (RTKs), such as EGFR, to form a complex on the cell surface responsible for cellular signaling, and cell adhesion and migration in cancer cells or normal cells such as keratinocytes. They usually express high or moderate levels of GnT-V and thus contain some poly-*N*-acetyllactosamine *N*-glycans. In this case, a β1,6GlcNAc–mediated cell signaling platform activates PI3K, ERK, and PKC signaling, thereby increasing cell adhesion and migration. In contrast, overexpression of GnT-III (right panel) results in modification of glycoproteins by bisecting GlcNAc, which inhibits GlcNAc branch formation catalyzed by GnT-V, thereby suppressing addition of poly-*N*-acetyllactosamine *N*-glycans to these proteins. Consequently, galectin-3 cannot form the signaling platform consisting of molecules such as RTKs, laminin-332, and α6β4 integrins, thereby inhibiting both cellular signaling and cellular function.

Laminin-332 in Stem Cell Biology

Human embryonic stem cells and induced pluripotent stem (iPS) cells have great potential for many applications in cellular regenerative therapy. Maintenance of these cells requires culture plate coatings. Although Matrigel is generally used as a culture substrate, its usage poses a problem for medical applications because most Matrigel components are undefined and it is prepared from mouse EHS tumors, and is not of human origin. Therefore, alternative substrates are required for culture systems. Recently, some laminin isoforms, including laminin-332, have become available as recombinant proteins [7, 122-124] and some studies have revealed that use of these recombinant laminin proteins can solve the above problems [125-129]. Further studies on the effects of recombinant laminins on cellular signaling of stem cells would not only clarify their cellular functions but would also help to establish an efficient culture system for these cells.

Conclusion

It has been more than 20 years since laminin-332 and its integrin receptors have been identified. Recent advances in investigations of these molecules provide us with new information to understand BMs and various diseases including epidermolysis bullosa and cancer. In both physiological and pathological conditions, laminin-332 and integrins associate with various binding proteins and cellular signaling molecules, and orchestrate them into a cellular signaling platform. The organized platform promotes cell adhesion, migration, and proliferation through the induction of various cellular signaling pathways, such as those of PI3K, ERK, PKC, JNK, and NFκB. The molecular constitution of the laminin-332/integrins-mediated signaling platform is altered by the proteolytic processing and *N*-glycosylation of laminin-332 as well as the phosphorylation or *N*-glycosylation of integrins. Further studies about this signaling platform are still needed to gain understanding of cell adhesion and migration under both physiological and pathological conditions. Characterization of the signaling processes mediated through laminin-332 and its associated integrins would help to design and develop new treatments and drugs for diseases as well as advance cellular therapy.

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