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Developmental and Pathogenic Mechanisms of Basement Membrane Assembly

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Abstract

Basement membranes are sheet-like cell-adherent extracellular matrices that serve as cell substrata and solid-phase agonists, contributing to tissue organization, stability and differentiation. These matrices are assembled as polymers of laminins and type IV collagens that are tethered to nidogens and proteoglycans. They bind to cell surface molecules that include signal-transducing receptors such as the integrins and dystroglycan and form attachments to adjacent connective tissues. The cell receptors, in turn, provide links between the matrix and underlying cytoskeleton. Genetic diseases of basement membrane and associated components, collectively the basement membrane zone, disrupt the extracellular matrix and/or its linkages to affect nerve, muscle, skin, kidney and other tissues. These diseases can arise due to a loss of matrix integrity, adhesion strength and/or receptor-mediated signaling. An understanding of the mechanisms of basement membrane zone assembly and resulting structure can provide insights into the development of normal tissues and the pathogenic mechanisms that underlie diverse disorders.

Keywords

Extracellular matrix; laminin; collagen; nidogen; perlecan; integrin; dystroglycan; agrin; muscular dystrophy; peripheral neuropathy; Alport's syndrome; Goodpasture syndrome; epidermolysis bullosa; Pierson syndrome

INTRODUCTION

Basement membranes (*basal laminae*) are sheet-like cell-adherent extracellular matrices (ECMs) that contribute to tissue organization and differentiation in vertebrate and invertebrate organisms. In mature tissues, they underlie epithelia and vascular endothelia; form the limiting boundaries of many organs, including the nervous system; surround individual cells or functional units in tissues such as muscle, kidney, pancreas, adipose tissue, and peripheral nerve; and mediate specialized attachments between tissues, including neuromuscular synapses and myotendinous junctions. In most if not all cases, basement membranes serve as both supportive cell substrata and solid-phase agonists.

The process of basement membrane formation is largely one of self-assembly on cell surfaces. The resulting matrix architecture is one of enmeshed polymers of laminins and type IV

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collagens that are bound to nidogens -1 and -2 and to the heparan sulfate proteoglycans agrin and perlecan. Basement membranes appear to be attached primarily through the laminins to cell surface sulfated glycolipids and transmembrane receptors. Signal transduction is mediated by matrix-ligated integrins, dystroglycan, and receptor kinases modulated by the mechanical properties of the matrix. The matrix components, receptors, and cytoskeletal binding interactions are illustrated in Figs. (1–3) and their binding interactions summarized in Tables 1–2.

A wide array of human disorders result from, or are associated with, defects in basement membrane assembly or composition. Some are acquired through autoimmune and metabolic means, including *Goodpasture syndrome* of kidney and lung, and primary complications of chronic *diabetes mellitus*, which affects the microvasculature of kidney, nerve and retina. Others arise from mutations of the genes coding for laminins, collagens or proteoglycans. These include *MDC1A congenital muscular dystrophy*, a disorder of muscle, nerve and brain; *junctional epidermolysis bullosa*; a severe blistering disease of skin and mucous membranes; *Pierson syndrome*, a disease of kidney and eye; *Alport's syndrome*, a defect of renal function and hearing; and *Schwartz-Jampel syndrome*, a myotonia and chondrodysplasia. The pathogenesis of these disorders depends upon loss of function and structure through such mechanisms as destabilization of matrix integrity, loss of cell anchorage, loss of critical ligands, and/or loss of receptor interactions.

BASEMENT MEMBRANE ARCHITECTURE

Basement membranes are found between cell surfaces and interstitium or between cells. By light microscopy they are seen in sections as periodic acid Schiff- and silver-staining linear entities that immunostain with antibodies specific for the four primary basement membrane components (laminin, collagen IV, nidogen, and perlecan). These elements topographically coincide with α - and β -dystroglycan, various integrins, and their receptor-associated partners within and immediately beneath the plasma membranes. Type XV and type XVIII collagens are concentrated at or near the interstitial face of the basement membrane. Fibronectin, a widely-distributed ECM glycoprotein, while detected in some (e.g. early embryonic) basement membranes, is usually not considered a basement membrane component *per se*.

At the ultrastructural level, single basement membranes seen in metal-impregnated thin sections typically range from ~50 to ~100 nm in thickness. They usually possess a distinctive electron-lucent layer (*lamina lucida*) sandwiched between the plasma membrane and an outer electron-dense layer (*lamina densa*). However, basement membranes appear homogenous when tissues are fixed by freeze-substitution, suggesting that the lamina lucida represents a retraction of the ECM from the cell surface during chemical fixation [1–3].

Transmission electron microscopy of thin (≤ 1 nm) high angle ($\geq 45^\circ$) platinum/carbon metal replicas has allowed investigators to visualize the supramolecular architecture of basement membranes. Using this approach, distinct type IV collagen and laminin polymeric networks were identified in the basement membrane of the placental amnion and the EHS tumor whose organization was similar to that of the corresponding polymers assembled *in vitro* [4–8]. The laminin polymer had the appearance of network of short (~35 nm) interconnecting struts whereas the collagen polymer had the appearance of branching thin fibrils, many running in parallel for short distances as coils containing two or more intertwined collagenous filaments.

THE LAMININS

This family of glycoproteins exist as α - β - γ heterotrimers. Isoforms in vertebrates are assembled from a palette of five α -, three β -, and three γ -subunits, whose expression varies between cell types and with development. New nomenclature designates laminins according to their chain

composition (e.g. laminin-111 corresponds to $\alpha1\beta1\gamma1$) [9]. Laminins have a branched structure. The N-terminal portions of the subunits are separate, forming short arms. Most short arms begin with an N-terminal globular LN domain, followed by a rod-like region consisting of multiple laminin-type EGF repeats (LE domains) in combination with two additional globular domains (L4, LF). However, the $\alpha3A$, $\alpha4$, $\beta3$ and $\gamma2$ subunits have truncated short arms, and (with the exception of $\beta3$) lack an LN domain. The more distal sequences of the three subunits participate in an extended coiled-coil domain that joins the subunits and forms a long arm. The α -subunit projects beyond the end of the coiled-coil in a series of five β -sandwich LG domains with a hinge-like region between LG3 and LG4. While there is little evidence of alternative splicing of laminin subunits, proteolytic processing mediated by furin-like and other proteases is seen in $\alpha2$, $\alpha3$, $\alpha4$, $\alpha5$, and $\gamma2$ subunits, with dissociation of the cleaved segments occurring in msot cases.

Distinct Role in Basement Membrane Formation

Studies in culture and *in vivo* suggest laminins are principally responsible for organizing basement membrane assembly on cell surfaces. Alone among basement membrane components, purified laminins are uniquely able to assemble sheet-like ECMs on cell surfaces in the absence of other components [10]. All basement membranes contain laminin as a principal component, and genetic mutations that eliminate laminin expression in specific tissues prevent basement membranes from forming in those tissues specifically. As one example, early embryos assemble two prominent basement membranes, the sub-visceral endodermal basement membrane at the embryonic plate, and Reichert's membrane, each of which contains two laminin isoforms (laminins-111 and -511). Knockout of either the $\gamma1$ or $\beta1$ subunits, which precludes expression of both laminins, resulted in peri-implantation embryos that lacked any basement membrane, leading to developmental arrest and involution [11,12]. In contrast, knockout of type IV collagen subunits [13], nidogens, perlecan, and agrin, or of compensated laminin subunits was not found to substantially affect the appearance of basement membranes until much later in development (reviewed in [14]). Thus, it appears laminins are critical to the general scaffolding of basement membranes, while type IV collagen, the combined nidogens, and perlecan enhance basement membrane stability, at least during development [13,15].

Polymerization and Scaffold Construction

Most laminins self-assemble to form a polymer, a two-step interaction consisting of a calcium-independent nucleation step followed by a calcium-dependent propagation step first identified with laminin-111 [16]. Laminin polymerization is reversible, entropy-driven, and cooperative with a critical conc. of ~ 0.14 and ~ 0.28 μM for laminin-111 and laminin-211, respectively [8,16–20]. The laminin polymer that forms is a gel whose stiffness depends upon the laminin concentration or the presence of other modifying components [21]. The laminin molecules bind to each other through the LN domain located at the end of the three short arms such that one finds at each interacting locus one α , one β and one γ LN domain [10,18]. Combined with the cooperative activation and polygonal structure of the resulting network [8], the observations indicate that polymerization is driven by the formation of a particularly stable tripartite complex of interlocked LN domains in which the α -, β -, and γ - short arms from neighboring laminin molecules are obligate partners. Laminin and laminins containing the $\alpha3B$ or $\alpha4$ subunits that lack the α -subunit short arm (and hence the α LN domain), or that contain the truncated $\gamma2$ subunit, are thought not to polymerize [8,10,18,19]. The rod-like LE domains that constitute much of the mass of the short arms likely provide spacing between LN-domain complexes, facilitating the formation of sheet-like polygonal networks on cell surfaces. However, the α -LN-LEa short arm segments have also been shown to bind to each other [22]. Reinterpreted in the face of evidence that each laminin must possess three different LN domains to polymerize,

this additional short-arm interaction might represent part of a mechanism to explain how polymer layers may in some cases attach to each other to become multilayered.

Netrins comprise a family of extracellular proteins that include axonal guidance factors. They possess N-terminal globular domains that are homologous to the laminin LN domains (reviewed in [23]). While it has been suggested that netrins may interact with basement membranes through their LN domains, this has been demonstrated only for netrin-4 [24].

Anchorage, Cytoskeletal Linkage, and Signaling

Whereas laminins and other basement membrane components are secreted as soluble forms, a cardinal characteristic of basement membranes that they form in association with cell surfaces. A prediction arising from cell surface laminin adhesion (that increases the local laminin concentration) and cooperative polymerization (that prohibits assembly in dilute environments) is that the cell surface provides a nucleation surface for laminin, favoring assembly on that surface. At concentrations below the (solution) critical concentration, laminin would form a molecular monolayer.

Basement membranes are often found on only one of several adjacent cell surfaces. Thus, basement membranes form along the basal side of epithelial cells, but not adjacent fibroblasts; basement membranes selectively form on the abaxonal surfaces of Schwann cells, without incorporating components from the adjacent basement membranes forming in the perineurial sheath; each skeletal muscle fiber forms a single basement membrane over its entire surface that seamlessly incorporates unique isoforms of the principal components at junctional sites with nerve and tendon cells. The specificity with which inherently soluble components form basement membranes on a subset of the adjacent cell surfaces suggests that certain integral cell surface molecules serve as critical receptors in determining which surfaces are competent to form basement membranes.

The identity of the cell surface anchoring molecules that serve to initiate laminin-binding and basement membrane formation has been controversial. Plasma membranes contain multiple binding partners for laminins and other basement membrane components. Moreover, the surface expression and distribution of these receptors is often heavily influenced by basement membrane contact, and the avidity of receptors for their extracellular ligands is regulated by intracellular agents and interactions with the cytoskeleton. Thus, an interplay between receptor expression and activation, accumulation of initial basement membrane components, and co-organization of the subjacent cytoskeleton may cooperatively control sites of basement membrane assembly.

Primary candidates for receptors contributing to basement membrane assembly are those that support the initial accumulation of laminin, the essential component. More specifically, the initial process of laminin deposition appears to substantially depend upon interactions with the LG domains located at the α chain c-terminus. For example, disruption of LG domain interactions through deletion or competition results in a failure of basement membrane assembly in several culture models [10,25–27]. The LG domains contain binding sites for integrins, α -dystroglycan, sulfated glycolipids, and heparan sulfate chains. Integrins including $\alpha 3\beta 1$, $\alpha 6\beta 1$, $\alpha 6\beta 4$ and $\alpha 7\beta 1$ bind principally to epitopes located along the LG1–3 domains of the different laminins; binding also requires participation of the adjacent coiled-coil domains, with a critical contribution from a distal glutamic acid in the $\gamma 1$ subunit [28,29]. Binding to the glycoconjugate-type receptors, including heparan sulfates, galactosylsulfatide (a glycolipid), and α -dystroglycan (for which a dense, mucin-like glycosylation is required to bind laminin), is focused at sites on the LG4 and LG5 domains that bear a high density of surface-exposed lysines and arginines, as revealed by crystallography of the laminin $\alpha 1$ LG4 and laminin $\alpha 2$ LG4–5 domains [30,31]. Further dissection of unique binding site requirements may lead to

mutational studies that define whether specific receptors determine basement membrane assembly, or if several overlap by simply providing anchorage sites. For example, a promising approach to dissecting primary and secondary laminin/receptor interactions in specific tissues involves the transgenic expression of truncated and chimeric laminin α -chain variants in mutant strains of mice lacking the normally expressed laminin α -chain [32].

To date, genetic perturbation studies have failed to support a specific receptor as the sole anchor for basement membrane assembly in animal tissues. Basement membranes form independent of β 1-, β 4- and other of the main basement membrane integrins, α -dystroglycan in most tissues (Reichert's membrane may be an exception), galactosyl-3-sulfate ceramide (the most common sulfatide), and heparin/heparan sulfates. In contrast, studies of cultured cells have implicated the importance of individual anchor candidates. In one study, cultured epithelial cells lost their ability to accumulate surface-bound laminin when rendered null for dystroglycan, and regained this ability when dystroglycan expression was restored by transfection [33]. In another study, β 1 integrin-null and dystroglycan-null embryoid bodies were found to be capable of assembling basement membranes, provided a laminin was present [26]. In a third study, selective removal of sulfatides, but not removal of dystroglycan, β 1-integrins or heparan sulfates, prevented basement membrane assembly in Schwann cells, with the complementary observation that addition of galactosyl sulfatide to the plasma membranes of fibroblasts enabled basement membrane assembly [27]. It may be that redundancy of, and/or compensation by, anchorage molecules obscures their separate contributions in the genetic studies and that different cells utilize different anchors or combinations of anchors. If so, then anchorage may depend on integrated contributions arising from sulfated glycolipids, dystroglycan and integrins with one serving as a principal (but replaceable) anchor for a particular cell by virtue of its preponderance and affinity. Such a hypothesis also suggests a basis for the cell surface binding specificity by different laminins, based upon their different affinities for dystroglycan, sulfatides, integrins, and (in the case of α 5 laminins) the Lutheran (Lu) antigen (see Table 1). This hypothesis may also partially account for differential compensation by alternative isoforms of laminin. For example, α 2-laminins are the principal laminins in muscle and nerve. Mutations that decrease expression of α 2-laminins cause severe muscular dystrophy and defects in nerve myelination, despite a substantial reactive increase in the expression and accumulation of laminins containing the alternative α 4 chain. Clearly α 4-laminins do not functionally compensate for α 2-laminins in muscle or nerve. Interestingly, while α 2-laminins show the highest affinities among laminins for sulfatide and dystroglycan, the laminin α 4 subunit is particularly weak in its ability to bind to α -dystroglycan or almost any integrin. Further, while laminin α 4 exhibits moderate sulfatide-binding, it lacks an aminoterminal LN domain to sustain laminin-laminin interactions or provide additional receptor anchorage. An ability to link the upregulated α 4-laminins to dystroglycan has been the presumptive explanation for the ability of a shortened "mini" form of agrin to compensate loss of α 2-laminins in the muscles of *dystrophic* (*dy*-strain) mice [34].

Integrins and dystroglycan couple the basement membrane to intracellular events through two mechanisms. They serve as signaling receptors by activating receptor-associated components, which regulates such cell functions as proliferation, migration, protection from apoptosis, and differentiated state (reviewed in [35,36]). They also directly link the basement membrane to the underlying cytoskeleton, an attribute that may be particularly important where strong mechanical stresses exist (e.g., skeletal muscle), but which also supports the activity and stability of motile processes. Integrins and dystroglycan form such transmembrane linkages by indirectly binding to the actin cytoskeleton. Integrin-associated adaptor proteins that mediate cytoskeletal linkage include integrin linked kinase (ILK), α - and β -parvins, talin, α -actinin and filamin, whereas dystroglycan-associated proteins that participate in this linkage include dystrophin and utrophin [37–45]. The laminin polymer is thought to directly contribute to basement membrane signaling, in part through the organization of the cytoskeleton, and in

part by mechanosensory transduction mechanisms that are influenced by the viscoelastic properties of an ECM [46–48].

Laminin 3A32 and Junctional Epidermolysis Bullosa

This laminin ($\alpha 3_A\beta 3\gamma 2$) is found in epithelial basement membranes such as that present at the epidermal-dermal junction. The laminin binds to nidogen (which can attach it to the type IV collagen network) but is a non-polymerizing laminin due to its short arm truncations [19]. It binds to the $\alpha 6\beta 4$ and $\alpha 3\beta 1$ integrins and to type VII collagen, enabling it to form strong adhesions between cell hemidesmosomes and anchoring fibrils [49]. Mutations affecting expression of any of its three subunits are causes of *junctional epidermolysis bullosa* (JEB) in which a split develops within the epidermal/dermal basement membrane leading to blister formation [50,51]. Deletions of the $\alpha 6$ integrin subunit, $\beta 4$ integrin subunit, and $\alpha 3$ laminin subunit all result in related epithelial detachment phenotypes while mutations of type VII collagen cause blistering defects within the stroma adjacent to basement membrane [52–55].

Pierson Syndrome

This disease characteristically affects the renal glomerulus, eye, and neuromuscular junction. It results from mutations in the *LAMB2* gene that codes for the laminin $\beta 2$ subunit, present in laminins -221, -421, and -521 [56,57]. In the mouse models of the disease, in which the pathologies of the neuromuscular junction and kidney have been well described, there is a compensatory incorporation of orthologous laminin isoforms containing the $\beta 1$ subunit; however, these forms do not prevent pathogenesis [58–60]. Studies to date have focused on unique activities associated with the laminin $\beta 2$ chain, although a possibility that insufficient expression of the $\beta 1$ chain contributes to pathogenesis in Pierson syndrome has not been excluded.

Laminin $\alpha 2$ -Deficient Congenital Muscular Dystrophy

$\alpha 2$ -laminins are major components of the basement membranes of Schwann cell (endoneurium), skeletal muscle fiber (sarcolemma), neuromuscular junction, and myotendinous junction. Nonsense and missense mutations of the *LAMA2* gene coding for the laminin $\alpha 2$ subunit cause about 1/3 of cases of congenital muscular dystrophy (clinically classified as type MDC1A). The syndrome is characterized by generalized muscle necrosis with variable levels of atrophy and regeneration, in combination with reduced peripheral nerve conduction velocity (mostly motor neuron), and thinning of white matter of the brain as detected by nuclear magnetic resonance imaging [61]. *LAMA2* mutations that eliminate expression of $\alpha 2$ -laminins uniformly result in a severe muscular dystrophy; patients are floppy at birth, fail to ambulate, and typically die of extreme muscle wasting and respiratory failure in the second decade. Dogs, cats, and mice lacking laminin $\alpha 2$ are similarly affected. The series of *dystrophic* (*dy*) mouse *LAMA2* mutations provide a strong experimental model of the disease that accurately reflects the variable extent of muscle and peripheral nerve pathology in the affected human patient population. Specific strains include *dy^{2J}* (in-frame deletion of the $\alpha 2$ LN domain), *dy^{3K}* ($\alpha 2$ knockout), *dy^W* (severe hypomorph of the $\alpha 2$ subunit), and *nmf417* (*dy^{7J}*; point mutation in $\alpha 2$ LN domain) [62–65].

Mutations affecting the laminin $\alpha 2$ subunit, dystroglycan (the core protein itself as well as its O-linked mannosyl carbohydrate required for binding to LG domains in laminin, agrin, and perlecan), the dystrophin-associated sarcoglycans, dystrophin, $\alpha 7$ -integrin, and integrin-linked kinase (ILK) all cause muscular dystrophy [44,61,66–70]. In skeletal muscle, these components and other submembrane-cytoskeletal components vinculin and β -spectrin are co-localized within a transmembrane adhesion complex called the costamere. Co-stameres are organized as an orthogonal surface array across the sarcolemma, and it has been thought that they might mediate the transmission of lateral forces generated by contraction across the sarcolemma

membrane and between neighboring myofibers [71]. Two series of molecular links have been traced between basement membrane and actin cytoskeleton, with a complex of dystroglycan, dystrophin/utrophin, and dystrophin-associated glycoproteins in one case, and integrins, ILK, PINCH, parvins and additional adaptor proteins in the other [45,72]. Thus, deficiency in the key components of the sarcolemmal basement membrane, their sarcolemmal receptor complexes, and their cytoskeletal-anchoring partners within the costamere are thought to represent a common pathogenic pathway to muscular dystrophy by destabilizing the overall linkage between basement membrane and myofiber cytoskeleton. In mouse models, the pathology is typically not appreciated until the affected animal begins to exercise, suggesting that the peri-sarcolemmal structural organization is too unstable to withstand the mechanical stress of muscle contraction against load [73,74].

The sarcolemma basement membrane is a fundamental feature of this model of sarcolemmal pathogenesis in dystrophy. The sarcolemmal lipid bilayer presumably lacks the inherent stability to withstand high levels of tension generated at sarcolemma/cytoskeleton attachment sites when the myofiber contracts. Instead, stresses are transferred across the sarcolemma *via* transmembrane receptor complexes that directly bind to both cytoskeleton and basement membrane. The laminin network within the basement membrane appears ideally suited to withstand the cytoskeletal stresses by virtue of its polygon polymer structure, which serves to evenly redistribute local tension into the surrounding matrix across the fiber surface, as well as into the interstitial matrix between fibers. Consistent with a central role for basement membrane stability, the dystrophy associated with mutations that eliminate expression of $\alpha 2$ -laminins is severe. For comparison, laminin $\alpha 2$ -null mice die from catastrophic myodegeneration between 5 and 8 weeks, while mice lacking dystrophin survive well into adulthood.

Accordingly, therapeutic approaches to ameliorate muscular dystrophy have been directed at repairing the transmembrane bridge between cytoskeleton and basement membrane. Several experimental approaches to inhibiting the dystrophic muscle phenotype and pathology have been evaluated in recent years, using the *LAMA2* mutant *dy*-strains of mice. A direct approach of restoring laminin expression by germline transgenesis is effective [75,76], but faces difficulties in translation to human patients. Thus, there is considerable interest in an alternative strategy which attempts to compensate deficiency for laminin $\alpha 2$ by providing supplementary means to sustain high-affinity of binding of basement membrane to the sarcolemmal surface. One approach has been to upregulate receptor activity through over-expression of the glycosyl-transferase LARGE and $\alpha 7$ -integrins. A second approach has been to supplement the basement membrane with smaller components such as mini-agrin and NtA-perlecan/LG, which are designed to not just bind to the laminin $\alpha 2$ receptors, but restore linkage to the residual components of the basement membrane [41,77,78].

Miniagrin is a shortened version of non-neural agrin, consisting of a fusion of agrin's N-terminal NtA domain and C-terminal cell LG domains [34]. The NtA domain binds to a site in the coiled-coil domain of laminins (primarily through the $\gamma 1$ subunit), and the LG domains bind to α -dystroglycan, $\beta 1$ integrins, and galactosyl sulfatide ([79] and Yurchenco & Capizzi, unpublished observations). Shortening the DNA construct to eliminate unneeded intervening sequences facilitates widespread delivery in postnatal muscles in affected individuals. Transgenic expression of miniagrin, and of a related NtA-perlecan LG fusion protein, substantially ameliorated the muscle pathophysiology of mouse models of laminin $\alpha 2$ deficient muscular dystrophy [34,77,80–82]. Similar benefits occurred when the gene for miniagrin was delivered to the dystrophic mouse tissues using an adeno-associated virus delivery system [83]. The basis for this improvement in muscle structure and function has been attributed to the ability of agrin to simultaneously bind with high affinity to α -dystroglycan and the $\alpha 4$ -laminins that are upregulated in $\alpha 2$ -deficient muscles. This interpretation emphasizes the weak

binding of the laminin $\alpha 4$ LG domains to dystroglycan. Accordingly, miniagrin restores this link to dystroglycan, while the $\alpha 4$ -laminins maintain links to other basement membrane components, including the type IV collagen network.

Nevertheless, there remains some uncertainty regarding the mechanism by which miniagrin stabilizes muscle fibers in *dystrophic* mice. First, miniagrin dramatically rescued the structural integrity of the basement membrane. This result is consistent with the notion that basement membrane stability is critical to muscle fiber survival. However, it is not consistent with the notion that mini-agrin acts primarily through interactions with $\alpha 4$ -laminins, which entirely lack the ability to polymerize, organize, and maintain a structurally integrated sheet of basement membrane. In principle, this result could be taken as evidence that there are alternative mechanisms to form and maintain basement membrane architectures, apart from the tripartite LN-domain interactions between laminins. However, miniagrin expression also resulted in high level incorporation of laminin-511 into the sarcolemma basement membrane [34]. Laminin-511 (laminin-10) contains the $\alpha 5$ subunit, which contains an LN polymerization domain. The $\alpha 5$ -laminins are fully competent to form and maintain basement membranes; a synapse-specific isoform (laminin-521) maintains the synaptic cleft in *dy*-strain mouse muscles [84,85]. Thus, an alternative possibility is that miniagrin restores the integrity of the sarcolemma basement membrane by increasing the incorporation of $\alpha 5$ -laminins. The basis for this increase is not certain, but could include effects on laminin assembly, secretion, transcription, or surface “capturing” of a laminin that otherwise would dissociate from the cell surface.

While the principal contributors to muscle stabilization studied to date have focused on receptor-associated linkages, the state of the matrix structure is important as well. The *dy^{2J}* mice, which lack most of the $\alpha 2$ -LN domain due to a splice donor defect, are characterized by a moderate skeletal muscle dystrophy that includes a disrupted organization of the sarcolemmal costameric array [86]. An analysis of laminin extracted from the skeletal muscle of these mice and of a recombinant laminin fragment containing the same deletion revealed that the *dy^{2J}* $\alpha 2$ -laminins have increased proteolytic instability of the $\alpha 2$ LN domain and defective polymerization [62,87]. The *dy^{2J}* phenotype therefore estimates the contribution of the LN-domain-mediated laminin network to muscle fiber stability, and most directly implicates a loss of basement membrane strength in the increased fragility of muscle fibers in *dy^{2J}* mice. However, even a modest (~three-fold) decrease of otherwise normal laminins cause dystrophic muscle changes (Yang, McKee, Patton, Chen, Strickland and Yurchenco, unpublished observations), suggesting that a certain density of laminin interactions are necessary to stabilize basement membrane structure. In contrast, a point-mutation disruption of the $\alpha 2$ -LN domain was recently discovered, which causes dystrophy with subtle defects in basement membranes and no decrease in laminin levels [65]. The muscle basement membranes were not interrupted, as with other $\alpha 2$ mutations, but were significantly thicker in some regions, possibly destabilizing myofibers through a lack of flexibility under contraction. Viewed in broad strokes, mutations that fully or nearly eliminate $\alpha 2$ -laminins from the sarcolemma greatly destabilize myofibers, while mutations that preserve moderate levels of $\alpha 2$ -laminins but disrupt the organization of the basement membrane cause moderate or mild forms of dystrophy.

Laminins and Peripheral Myelination

Laminins play critical roles in the developmental myelination of peripheral nerves. Embryonic nerves consist of naked axons tightly fasciculated into several distinct bundles; each bundle is covered by the flat, wide processes of immature Schwann cells. During myelination, each axon becomes isolated by a dedicated cohort of Schwann cells. Large axons are myelinated (wrapped dozens of times by their Schwann cells), but many small sensory axons are ensheathed without becoming myelinated. The developmental process by which the population and type of

Schwann cells is matched to the population and type of axon is called axonal sorting, or “radial sorting”, for the processive nature by which the embryonic axon fascicles are reduced to separate nerve fibers. The ability of Schwann cells to participate in axonal sorting is dependent on the laminin concentrated in the endoneurial basement membrane that Schwann cells assemble along their outer (abaxonal) surface. This matrix is composed principally of laminins-211 and -411, collagen IV ($\alpha1\alpha2$), nidogen-1, and perlecan. In mutant mice in which Schwann cells lack both isoforms of laminin, through targeted disruption of the $\alpha2+\alpha4$ genes or cell-specific down-regulation of the $\gamma1$ gene, the basement membrane fails to properly assemble, and axonal sorting fails completely [88,89]. While the mutant mice survive postnatal development, their nerves remain essentially embryonic: nearly all axons remain tightly bundled in their original fascicles, and each bundle remains surrounded by the processes of immature Schwann cells (e.g., lacking nuclear localization of Krox-20). Thus, complete loss of Schwann cell laminins arrests axonal sorting and results in complete “amyelination”.

These findings established a baseline for assessing the individual contributions of the two laminin isoforms, 211 and 411, to myelination. In brief, the discovery that deficiency for each individual laminin isoform causes a partial amyelination implicates distinct, non-redundant roles for each. Moreover, combined with patent evidence that only one of the laminins (211) supports basement membrane stability, the comparative analysis of phenotypes unexpectedly challenges long-held views that axonal sorting depends on the ability of promyelinating Schwann cells to assemble basement membranes, and that the critical role of laminin during axonal sorting is mediated by its special role in organizing and/or stabilizing basement membranes.

The original idea that Schwann cell basement membrane structure is essential to promyelinating differentiation of immature Schwann cells began with observations of the nerves in laminin $\alpha2$ -mutant *dy* mice [90–93]. Of the two laminins expressed by developing Schwann cells, only laminin-211 possesses three intact LN domains (in α , β , and γ) and is therefore competent to scaffold basement membrane assembly. Thus, loss or mutation of the $\alpha2$ subunit in the *dy*-strains of mice prevent Schwann cells from forming or maintaining stable basement membranes [93] despite high levels of laminin-411, which lack the α -subunit LN domain [94,95]. Each mutation of laminin $\alpha2$ causes a characteristic “partial” amyelination: spinal roots are severely amyelinated, and half the axons in the sciatic nerve are amyelinated. Of particular interest, mutations targeted to the LN domain of the $\alpha2$ (*dy2J* and *nmf417*) almost completely inactivate laminin-211 for axonal sorting. For example, the LN-domain point mutation in *nmf417* causes the same severe amyelination in mouse nerve spinal roots as the $\alpha2$ -null allele (*dy3K*). Further, a combination of the *dy2J* $\alpha2$ -mutation with the laminin $\alpha4$ -null mutation results in severe amyelination, revealing that when the *dy2J*-variant of laminin-211 is isolated on Schwann cells it has almost no inherent activity. In short, the ability of laminin-211 to promote axonal sorting is fully dependent on the LN-domain interactions that underlie the laminin network and basement membrane assembly. Complementing these findings, cell culture studies found that Schwann cells require media conditions which stimulate basement membrane assembly in order to begin myelinating axons [96–99]. Thus, the integrity of the Schwann cell basement membrane appears linked to the onset of myelination both *in vivo* and *in vitro*. The combination led to a proposal extrapolated from epithelial differentiation, that the endoneurial basement membrane polarizes the immature Schwann cells in preparation for differentiation into mature myelinating Schwann cells [96].

However, there is considerable evidence which is not neatly accommodated by a simple basement membrane model of axonal sorting. First, while loss of laminin $\alpha2$ disrupts endoneurial basement membranes throughout the mouse PNS, many nerves are normally myelinated, including the large brachial nerves supplying the arms. Second, non-mammals complete myelination before endoneurial basement membranes form. Thus, a more

comprehensive assessment suggests that basement membrane formation (as assessed by conventional fixed EM preparations) is not tightly correlated with the ability of Schwann cells to undergo promyelinating differentiation and complete axonal sorting.

Third, perhaps the strongest contradictions to the initial basement membrane hypothesis are provided by the discovery that laminin-411, containing the $\alpha 4$ subunit, has an independent and indispensable role in axonal sorting. A null mutation in the mouse laminin- $\alpha 4$ gene causes partial amyelination of peripheral nerves very similar to that caused by loss of $\alpha 2$ -laminins, including nearly identical deficits in myelinated axons and distributions of naked axon bundles [89,100]. Loss of non-polymerizing $\alpha 4$ -laminins and polymerizing $\alpha 2$ -laminins are equally disruptive. Importantly, loss of $\alpha 4$ laminin had no effect on levels of $\alpha 2$ -laminins, and no apparent effect on the ultrastructural appearance of the Schwann cell basement membrane. That is, $\alpha 4$ -laminins appear to be required for Schwann cells to complete axonal sorting and myelination in sciatic nerves, even though they provide no added value to the appearance of the basement membranes. Conversely, by reference to the complete lack of sorting in $\alpha 2/\alpha 4$ -double null mutant mice, it is apparent that laminin-411 is responsible for all of the sorting and myelination that occurs in $\alpha 2$ -mutant *dy*-strains of mice, in the absence of laminin-211 and without the support of well-formed basement membranes. Finally, observations in the spinal roots of *dy2J/\alpha 4*-null double mutant mice reveal that $\alpha 2$ -laminins also activate axonal sorting and myelination without ultrastructurally-detectable basement membrane [89]. Together, the results reveal little correlation exists between the appearance of the endoneurial basement membrane and the ability of Schwann cells to proceed with axonal sorting.

The discovery that both laminins-211 and -411 are required to complete sorting in mouse sciatic nerves implies they have non-redundant activities, either mediated by distinct receptors or mediated by different structures that result in different types of information transmitted to the Schwann cell. Because $\alpha 4$ -laminins lack the ability to support basement membrane assembly, they most likely serve to activate signaling receptors on the surface of immature Schwann cells. $\beta 1$ integrins would logically represent a candidate receptor class for $\alpha 4$ -laminins in axonal sorting given the strong overlap in the characteristic distribution of amyelination that results from Schwann cell specific knockdown of the integrin $\beta 1$ subunit [89,100–102]. Since the loss of $\beta 1$ -integrins caused a more extensive amyelination than that seen in the absence of the laminin $\alpha 4$ subunit, this suggests a non-exclusive interaction between $\alpha 4$ -laminin and $\beta 1$ -integrins. Receptors which selectively mediate laminin-211 in axonal sorting have not been strongly implicated. Schwann cell specific knockdown of dystroglycan caused little or no defect in axonal sorting or basement membrane structure [103]. Laminin-211 activity relies heavily on LN-domain function, but appears less dependent on the broader superstructure of the basement membrane that results from laminin-polymerization. The LN domain might have direct signaling activities. Alternatively, laminin-211 might stabilize multi-receptor/cytoskeletal signaling complexes through short-range LN-domain polymer interactions.

A question that arises is whether laminins represent the principal integrin ligand involved. Given laminin-integrin binding specificities, this may be unlikely. First, the principal integrin receptor for $\alpha 2$ -laminins is the $\alpha 7\text{X}1\beta 1$ integrin, and not $\alpha 6\beta 1$ (enriched in the endoneurium), $\alpha 6\beta 4$ or $\alpha 3\beta 1$ as once thought, whereas $\alpha 4$ -laminins can support Schwann cell attachment through the $\alpha 6\beta 1$ integrin even though they at best bind only weakly to integrins in general [28,89,104]. The $\alpha 7\beta 1$ integrin is expressed quite late [28] in peripheral nerve development and knockout of the $\alpha 7$ subunit is not associated with a developmental nerve phenotype [105]. One can question whether the laminin-specific $\alpha 6\beta 1$ integrin is the key receptor (analysis of the null phenotype in nerve will require a conditional knockout approach) given that knockout of its cognate laminin $\alpha 4$ ligand produces a more restricted peripheral nerve myelination defect compared to that seen with knockout of the laminin $\alpha 2$ subunit [89]. It should be emphasized, however, that loss of laminin in a basement membrane is generally

associated with a loss of the other BM components as well [11,102]. A simple explanation of the apparent conundrum may be that it is the composite binding between β 1-integrin and most or all basement membrane components (i.e. α 2- and α 4-laminins through α 7 β 1 and α 6 β 1 integrins respectively, collagen-IV through α 1 β 1 and α 2 β 1, nidogens and agrin through α 3 β 1, and perlecan through α 2 β 1) that provide the relevant set of interactions rather than selective interactions with laminins. A related issue concerns the role of dystroglycan in basement membrane – Schwann cell interactions. Dystroglycan, similar to integrin α 7 β 1, is expressed late in peripheral nerve development just prior to myelination [106] and knockout of DG produced milder defects of slowed nerve conduction with abnormal Schwann sheath folding and nodal architecture [103].

In summary, our review of the current data provides an alternative model with the view that laminins promote axonal sorting and myelination as autocrine signaling factors that act independent of the basement membrane structure. However, no study in other tissues provides a similar example of laminin function divorced from the context of basement membrane integrity. It therefore seems wise to consider whether a proxied view of basement membrane structure limits our ability to discern more essential matrix characteristics on which laminin function and axonal sorting does depend. We offer a primary caveat to the above consideration of the relationship of basement membrane assembly and receptor interactions to radial sorting, i.e. the absence or presence of a basement membrane has been generally referred primarily to its ultrastructural appearance. It should be noted that basement membrane-like extracellular matrices can be detected by immunofluorescence in the absence of a linear *lamina densa* (Yurchenco, unpublished observations) while modifications of staining techniques (e.g. tannic acid or permanganate treatment) can affect the sensitivity of detection of a *lamina densa* by electron microscopy. Furthermore, it can be argued that the presence of a continuous *lamina densa* does not guarantee the stability or functionality of the basement membrane. Proper functionality may depend upon the integration of molecular architecture with sufficient cellular anchorage and correct coding of receptor-ligand interactions in which defects of one can affect the other. An alternative hypothesis to be considered, then, is as follows: The structural contributions of α 2-, α 4- and α 5-laminins to the endoneurial basement membrane differ such as α 2 assembly leads to a prominent *lamina densa*, α 4 assembly leads to an alternate architecture in which type IV collagen and nidogen are tethered to non-polymerized laminin, and α 5 assembly, which can provide partial functional compensation, leads to formation of an attenuated laminin/collagen network structure (the nature of which remains to be determined). Each laminin contributes different signaling to the Schwann cell by virtue of its receptor specificities and different architectural characteristics. A combination of the separate α 2 and α 4 contributions are required to enable Schwann cells to undergo proper axonal sorting. Resolving these different models is an important goal, necessary to developing therapeutic modalities as much as understanding mechanism, and requiring new insights gained from the integration of genetic, cellular and molecular approaches.

TYPE IV COLLAGENS

Type IV collagen self-assembles into the only known covalently-stabilized network polymer. The first evidence for the molecular nature of this network was provided by an elegant biochemical and electron microscopic analysis of type IV collagen (α 1 $_2$ α 2[IV] composition) extracted from the EHS tumor matrix. Both N-terminal (7S domain) and C-terminal (NC1) domains that contributed to the network were characterized [107]. The 7S domains interact to form a multimeric complex, named for the sedimentation constant of the collagenase resistant fragment. The 7S complex forms spontaneously in solution through dimeric and trimeric intermediates, combining the N-terminal segments from four separate collagen chains to create a ~30 nm region of overlap that becomes further stabilized through disulfide and lysyl-oxidase derived covalent crosslinks [108–114]. At the C-terminal end of the collagen chain, the NC1

globular domain undergoes self-interactions that extend the collagen IV network indefinitely [115]. Since each NC1 domain consists of three C-terminal segments, the dimeric complex has also been described as a hexamer. The NC1 domain crystal structure revealed a flat interacting face for dimerization [116,117]. Subsequent analysis of the domain structure revealed an unusual covalent crosslink between $\alpha 1$ Met93 and Hy1211 that stabilizes the domain complex [118,119]. The NC1 domains of the less common $\alpha 3\alpha 4\alpha 5$ [IV] undergo a similar self-assembly, and it has been shown that the NC1 subunits can define the specificity of intramolecular collagenous chain association [120–122].

A third contributing interaction to the formation of the collagenous network is that of lateral (side-by-side) associations. These were first identified morphologically, as segmental associations of parallel collagenous filaments with frequent branchings in low angle rotary shadowed Pt/C replicas of extracted collagen, and supported biochemically as part of the heat of gelation [109]. These networks were later visualized in high angle Pt/C replicas of freeze-dried amniotic, EHS, and corneal basement membranes [4,6,7]. The lateral associations were not detected in the absence of the N- and C-terminal interactions, and it is likely they represent low affinity associations that drive network complexity.

During development of the renal glomerular basement membrane (GBM), the type IV collagen $\alpha 1$ and $\alpha 2$ chains become replaced by type IV collagen $\alpha 3$, $\alpha 4$ and $\alpha 5$ chains. Analysis of collagen containing these alternative chains revealed a collagen network with disulfide crosslinks not found with the $\alpha 1$ and $\alpha 2$ chain type IV collagen, which renders portions of the network resistant to dissociation [123]. These covalent links likely stabilize the GBM network, and may help explain the GBM defect seen in Alport's disease and in the Alport's-like phenotype of the $\alpha 3$ -collagen-IV null mouse [124].

Type IV collagen does not organize basement membrane assembly. The time-line of laminin and type IV collagen expression during development of the genetic model *C. elegans*, a nematode, provided early evidence for layered functions of the two types of basement membrane components [125–127]. Nematodes possess two laminins (differing in their α -subunit) that appear in basement membranes at the time of gastrulation, while type IV collagens does not appear until embryonic elongation. Inhibiting the expression of both laminin α subunits was found to result in loss of basement membranes and early embryonic lethality, providing evidence for an early requirement for laminins [127]. In contrast, mutations preventing type IV collagen expression did not prevent the initial assembly of basement membranes, but resulted in late embryonic lethality as muscle contractions commenced [125, 126]. The correlation of degeneration with the onset of activity suggests that type IV collagen is needed to enable the basement membrane to withstand mechanical stress. Primary evidence for a similar stabilizing role in vertebrates for type IV collagen comes from generation of a mouse null allele of the *Col4 $\alpha 1/\alpha 2$* locus (where the genes are paired) and analysis of the phenotype resulting from the loss of the two principal collagen chains [13]. Homozygous mutant embryos developed up to E9.5 with basement membrane proteins deposited at the expected sites. However, similar to events in *C. elegans*, lethality occurred between E10.5–E11.5 with structural deficiencies clearly evident in the basement membranes, including failure of integrity of Reichert's membrane. Further evidence that basement membranes in early embryogenesis do not rely on collagens comes from analysis of knockout of the Hsp47 chaperone, which is required for procollagen (including type IV collagen) triple-helical folding [128,129]. Embryos progress until ~E11.5, when they terminate with basement membrane disruptions accompanied by abnormal epithelial orientation and ruptured blood vessels.

Alport's Syndrome

Mutations of the X-linked *COL4A5* gene cause *Alport's syndrome* in 85% of cases [130]. Less commonly mutations of the *COL4A3*, *COL4A4* genes that encode the other chains of the

$\alpha3\alpha4\alpha5$ [IV] collagen produce the disease. Classically, the disease presents in males as progressive renal failure with hematuria and proteinuria accompanied by hearing loss. The GBM contains the embryonic collagen isoform ($\alpha1_2\alpha2$ [IV]) rather than the mature $\alpha3\alpha4\alpha5$ [IV] isoforms, and it is thought that this collagen degrades more readily due to the absence of stabilizing disulfide bonds. Histologically, the Alport's GBM is thin and contains splits or laminations. Patients are often treated with dialysis, sometimes followed by transplantation. A small fraction of the transplanted patients develop anti-GBM autoantibodies.

Hereditary Porencephaly and Hemorrhagic Stroke

While null mutations of the *COL4A1* and *COL4A2* genes cause embryonic lethality, mutations in the *COL4A1* gene that lead to only a reduction of the widely expressed $\alpha1$ [IV] subunit through faulty chain assembly are a cause of hereditary porencephaly and have been linked to hemorrhagic stroke in humans [131,132A]. Thus while null mutations of type IV collagen and laminin $\gamma1$, $\beta1$, and $\alpha5$ may cause embryonic lethality, more subtle mutations in these components may be a cause of more common human diseases.

NIDOGENS

Nidogen-1 and nidogen-2, also known as entactins, are homologous glycoproteins (150 kDa) found widely distributed among basement membranes. Each consists of three globular domains (G1, G2, G3) with intervening rod-like regions [133–138]. G1 and G2 are located in the N-terminal moiety, separated by a small linkage region. EGF-like repeats dominate the central region. Domain G3 is located at the carboxyl terminal region. The nidogen-1 G3 domain binds strongly to laminins, through a specific sequence element residing in domain LEB3 of the laminin $\gamma1$ short arm, confirmed through solution of the crystal structure of a nidogen-laminin complex [139]. The nidogen G2 domain binds to type IV collagen (as well as G3), as well as perlecan and fibulins [140–142]. Nidogen thereby directly tethers the laminin and collagen IV networks. A recent analysis of basement membrane assembly on Schwann cells using various recombinant laminins confirmed this bridging role [10]. The laminin-nidogen-collagen linkage is thought to provide stability to basement membranes, particularly important in those tissues exposed to mechanical stress, such as skeletal muscle [13,15]. While tissue/organ culture studies have supported a preeminent role for nidogen as the key bridge between laminins and type IV collagen, genetic studies in both mice and nematodes demonstrate that the absence of nidogen(s) or the nidogen-binding site in laminin $\gamma1$ does not adversely affect the accumulation of either laminin or type IV collagen into developing basement membranes [15,143–147]. The findings argue there must be additional interactions that incorporate type IV collagen into assembling basement membranes. Possibilities include direct collagen-laminin interactions and binding through perlecan. However, although laminin isoforms may vary, collagen IV binds weakly to laminin-111, and perlecan interactions are dominated by heparan sulfates, which typically lack specificity and high affinity. Thus, an alternative possibility is that integrin-mediated binding suffices to concentrate the assembly of the collagen IV network to the cell surface.

AGRIN

Agrin is a large (>400 kDa) multi-domain heparan sulfate proteoglycan of basement membranes that exists in several variant forms generated through alternative splicing and transcription initiation sites [148]. The predominant form of agrin in basement membranes contains an N-terminal NtA domain, which binds to the laminin $\gamma1$ subunit in the coiled-coil of the laminin heterotrimer. In tissues lacking basement membranes, such as the central nervous system, the NtA domain of agrin is often replaced by an alternative aminoterminal sequence that provides a direct GPI-mediated linkage to the cell surface. All agrins contain C-terminal LG domains, which bind to α -dystroglycan and integrins [79,149–152]. Agrin LG binding to

dystroglycan has the highest affinity of all of the LG domains tested. The affinity of NtA-agrin for both laminin and dystroglycan may serve to increase laminin anchorage to cell surfaces in some tissues. As noted above, this role is generally consistent with the improvement in sarcolemmal basement membrane structure and function seen by over-expression of a shortened version of agrin in laminin $\alpha 2$ -deficient mice [34]. Motor neurons secrete a unique set of agrin splice variants that regulate the organization and maintenance of the postsynaptic apparatus at the neuromuscular junction. The neuronal splice variants of agrin contain a short loop of residues inserted at the fringe of the beta-sheet sandwich of the LG domain. Interestingly, a conserved pattern of splice-generated sequence variations occurs at similar fringe sites in the LG domains of neuexins, which participate in synaptic maturation at interneuronal synapses.

PERLECAN

Perlecan is a heparan sulfate proteoglycan in basement membranes and a chondroitin sulfate proteoglycan in cartilaginous tissues [153–155]. Its core protein is large (~400 kDa) and consists of a tandem arrangement of five “domains” (each really a distinct region composed of one or more structural domains). The fourth and fifth “domains” (immunoglobulin repeats; and, LG domains separated by LE repeats, respectively) bind to a host of basement membrane components and BMZ receptors. These associations include nidogen-1 (domain IV), integrin $\alpha 2\beta 1$, and α -dystroglycan (domain V). Perlecan heparan sulfate chains, located primarily in the N-terminal domain I, have the potential to bind to laminins and to type IV collagen, and to tether FGF2 and other growth factors. Iozzo and co-workers have found that domain V, renamed endorepellin, is a potent inhibitor of angiogenesis through an activity dependent upon its binding to the $\alpha 2\beta 1$ integrin [156,157].

Perlecan may have a critical role in basement membrane maintenance in some tissues, directly or through support to the underlying cells, but does not appear to have a principal role in basement membrane assembly. Basement membranes were found to develop normally in mutant mouse embryos homozygous for a null-allele for perlecan gene, which survived to between ~E10.5 and E19 (i.e., birth) [158,159]. Embryos that died early a cardiac defect in which basement membranes developed disruptions through which blood exited to the developing pericardial space. Specific mutations of perlecan in humans have been found to cause *Schwartz-Jampel syndrome*, a disorder combining chondrodysplasia and myotonia. The latter is thought to occur because perlecan is required for binding to acetylcholinesterase at the neuromuscular junction [160–162]. The former may result because perlecan carries an oversulfated form of chondroitin sulfate (chondroitin 2,6 disulfate) in cartilage that accelerates the rate of assembly and increases the diameter of type II collagen fibers [163].

TYPE VI, VII, XV, AND XVIII COLLAGENS AND THE STROMAL INTERFACE

Basement membranes commonly lie adjacent to an extracellular matrix stroma that contains banded interstitial collagens. Extracellular matrix components, in particular several collagens, are thought to stabilize the interface by binding to both basement membrane and stromal components. However, no single collagen has been found to mediate this function.

In skin, anchoring fibrils reinforce attachment of the epidermal basement membrane to the dermal stroma. Type VII collagen, a major component of the fibrils that assembles into anti-parallel dimers, has been reported to bind to laminin-332 through its $\beta 3$ subunit short arm, and to bind to both type IV collagen and to interstitial banded collagen fibrils [164–166]. Dominant and recessive mutations of this collagen are a cause of *dystrophic epidermolysis bullosa*, a blistering skin disease in which the split occurs in the dermis at the level of anchoring fibrils (reviewed in [167]).

In skeletal muscle, type VI collagen is thought to anchor basement membrane to adjacent connective tissue (reviewed in [168]). The collagen triple helical monomer has been shown to self-assemble into secreted disulfide-stabilized anti-parallel tetramers and to form beaded microfibrils and filamentous networks in tissues. The collagen has been found to interact with type IV collagen, type I collagen, type II collagen, type XIV collagen, perlecan, and the microfibril-associated glycoprotein MAGP1. Mutations of the collagen are a cause of both *Betham myopathy* and *Ullrich congenital muscular dystrophy*.

Type XV and XVIII collagens are also candidates for providing basement membrane-stromal linkage. Type XV collagen contains many interruptions of its gly-x-y sequence and is modified by chondroitin sulfate and heparan sulfate chains. It is present in most basement membrane zones where it is associated with large collagen fibers [169]. The function of type XV collagen is not well understood, in part because inactivation of the gene for this collagen was not found to be associated with basement membrane defects or a relevant phenotype [170]. Type XVIII collagen is also a heparan sulfate proteoglycan [171]. Proteolytic cleavage of its NC1 domain releases a fragment called endostatin that has been reported to inhibit angiogenesis and that has been studied for its ability to inhibit tumor growth. However, a general physiological vascular role for the collagen was not established from investigation of the phenotype generated by inactivation of the type XVIII collagen gene [172]. On the other hand, that study supported the concept that the collagen is involved in connecting the basement membrane to stromal collagen in a subset of tissues, especially vitreous vessels. Mutations of the gene for this collagen are associated with *Knobloch syndrome* that is characterized by age-dependent vitreoretinal degeneration and occipital encephalocele.

CONCLUSIONS

One can consider the basement membrane as a cross-linked heteropolymer that on the one hand provides lateral stability to the cell surface receptors and adjacent cytoskeleton, and that on the other that provides a series of stabilizing chain links that extend from the basement membrane to the cell cytoskeleton. Disruptions of these interconnecting components along either axis are destabilizing to tissues, affecting structure and signaling. While the diseases that result from such defects can theoretically be repaired by replacing the missing component, more workable solutions might be to provide simpler and smaller linking components derived from existing basement membrane components that are easier to express in tissues or to affect the expression of redundant/compensatory pathways. Whatever therapies ultimately emerge, their development will likely require detailed knowledge of the mechanisms of assembly, structure and function.

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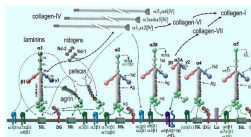


Fig. (1). Basement Membrane Components and Interactions

Examples are shown for major laminins. Each possesses three subunits and possesses globular, rod-like and coiled-coil domains (these are indicated for laminin-111). Various proteolytic cleavages (jagged lines) generate additional diversity. Each type IV collagen is a heterotrimer with three different chain compositions (most common is $\alpha1\alpha2[\text{IV}]$). In addition are found two nidogens (Nd1, Nd2), and the heparan sulfate proteoglycans agrin (Ag) and perlecan (perl). Major binding and minor (heavy and thin dashed arrows) binding interactions are indicated among the structural components (black lines) and with cell surface components (green lines) to integrins, α -dystroglycan (α DG), hemidesmosomal (HD) BP180, sulfated glycolipids (SGL) and the Lutheran (Lu) antigen. In squamous epithelia, laminin 3A32 is processed by several proteases. It binds to the $\alpha6\beta4$ integrin and BP180 and binds to type VII collagen.

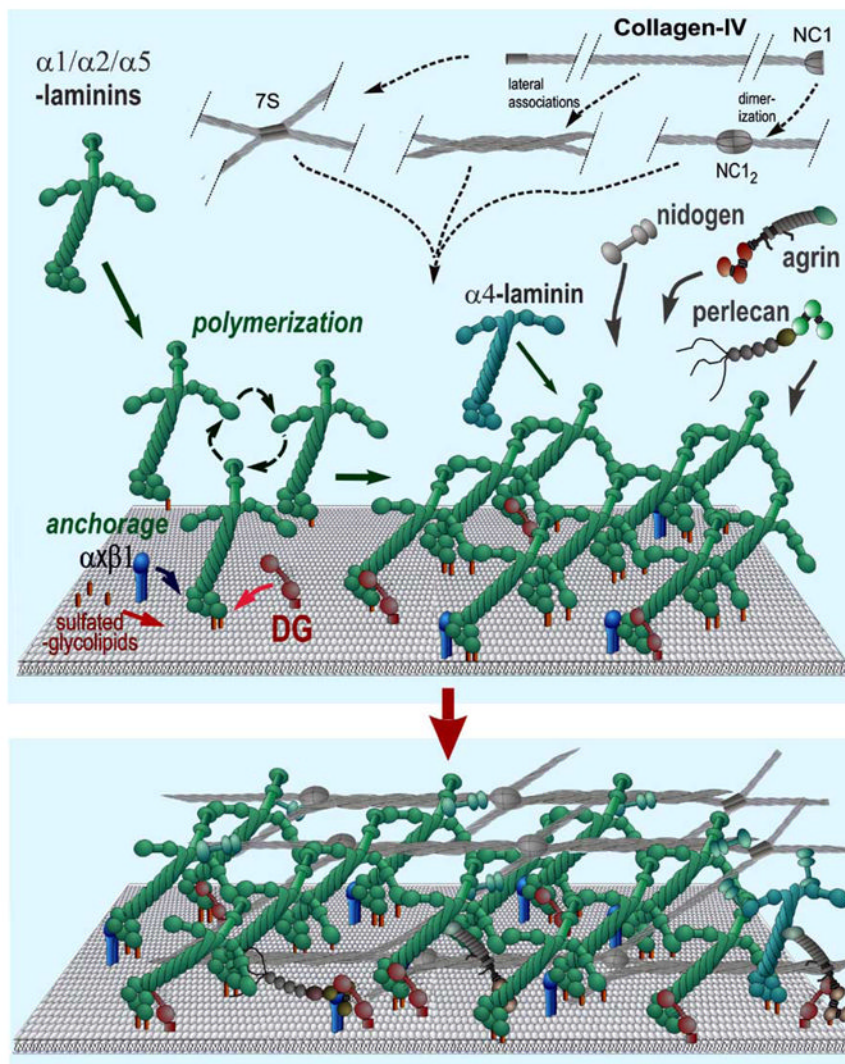


Fig. (2). Basement Membrane Assembly

In this simplified model, laminins become anchored to the cell surface through their LG domains. Anchorage is further enhanced through binding of the α -LN domain to sulfated glycolipids. If the laminin has three LN domains it polymerizes, creating a “nascent” scaffolding. Nidogens, type IV collagens, perlecan, and agrin, are incorporated into this initial matrix by binding to laminin (or by binding through a nidogen bridge). The type IV collagen self-assembles into a covalently-crosslinked network. The non-laminin components provide crucial stability and increase ligand complexity. The basement membrane ligands interact with integrins and dystroglycan and the heparan sulfates of agrin and perlecan enable the tethering of tissue-specific growth factors.

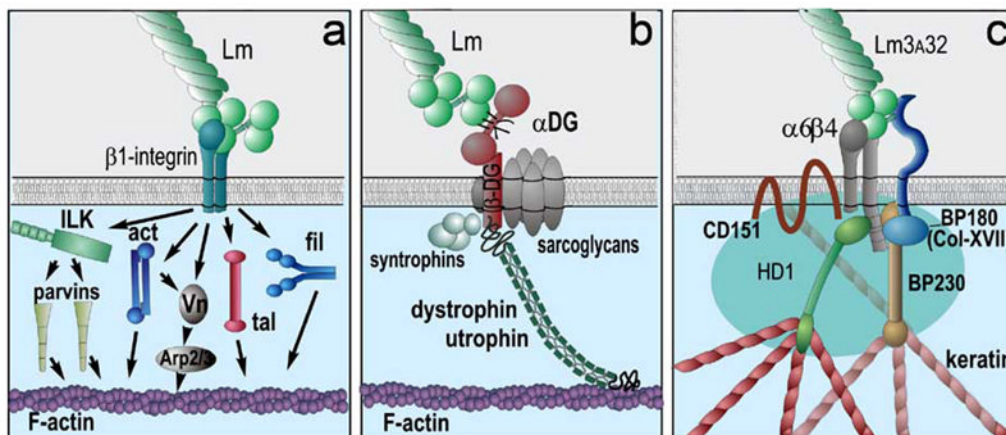


Fig. (3). Cytoskeletal Linkages

Basement membrane ligands can establish links to the actin and keratin cytoskeletons by binding to integrins and dystroglycan. **(a)** β 1-integrins: Nearly all basement membrane components bind to β 1-integrins. These integrins bind to cytoskeletal intermediates that bind to F-actin. The intermediates shown are integrin linked kinase (ILK) and α - and β -parvin, α -actinin (act), talin (tal) vinculin (Vn) and Arp2/3, and filamin (fil) (drawing after [45]). **(b)** Dystroglycan: The LG domains of laminins, agrin and perlecan bind to α -dystroglycan (α DG). α DG binds to β -dystroglycan, a transmembrane protein that binds to F-actin through dystrophin and utrophin. In muscle, dystroglycan is part of a complex that includes the sarcoglycans and other proteins. Homologues of dystrophin and utrophin exist in other tissues. **(c)** α 6 β 4-integrin: Laminin 332 binds to this unique integrin that forms part of hemidesmosome complexes and that forms a linkage with keratin filaments [14].

Table 1

Basement Membrane Anchoring and Receptor Interactions

Receptor/Anchor	Ligands	Nature of Interaction	References
Integrin $\alpha 1\beta 1$	Col-I, Col-IV, $\alpha 1$ -laminins ($\alpha 1LN$) and $\alpha 2$ -laminins ($\alpha 2LN$).		[173,174]
Integrin $\alpha 2\beta 1$	Col-I, Col-IV, $\alpha 2$ -laminin LN domains, Perlecan LG domains (endorepellin)		[156,173,175]
Integrin $\alpha 3\beta 1$	$\alpha 3$ - and $\alpha 5$ -laminins, nidogens.	K_D : $\alpha 5$ -laminin (3.4 nM^*) and $\alpha 3$ -Lm (14 nM^*); Nidogen-2 > Nidogen-1.	[28,138]
Integrin $\alpha 6\beta 1$	$\alpha 1$ -, $\alpha 3$ -, $\alpha 5$ -laminins. The integrin interacts with LG domains 1-3 & the distal coiled-coil.	K_D : $\alpha 5$ -Lms (0.73 nM^*) > $\alpha 3$ -Lms (7.5 nM^*) \approx $\alpha 1$ -Lms (9.5 nM^*) \gg $\alpha 4$ -Lms; $\alpha 2$ - and $\alpha 4$ -laminin interaction detected by cell adhesion only.	[28,176]
Integrin $\alpha 6\beta 4$	$\alpha 3$ - and $\alpha 5$ -laminins. Mediates attachment to hemidesmosomes.	$\alpha 3$ -Lms (12 nM^*) > $\alpha 5$ -Lms (25 nM^*) (LG1-3 domains).	[28]
Integrin $\alpha 7X1\beta 1$	$\alpha 2$ -laminins (0.6 nM) > $\alpha 5$ -Lms (1.2 nM^*) (LG1-3/distal coiled-coil)	Integrin expressed in muscle, peripheral nerve and other tissues.	[28]
Integrin $\alpha 7X2\beta 1$	$\alpha 1$ - and $\alpha 2$ -laminins.	K_D : $\alpha 1$ -Lms (1 nM^*) > $\alpha 2$ -Lms (2.6 nM^*)	[28]
Integrin $\alpha v\beta 3/\beta 1$	$\alpha 5$ -Lms (domain L4b)	Possible role in vasculature	[177]
α -dystroglycan (αDG)	$\alpha 1$ -, $\alpha 2$ -, $\alpha 5$ -laminins, agrin, perlecan. αDG binding is dependent upon O-linked carbohydrate. Reduced/absent DG binding adversely affects only some (e.g. muscle and brain) tissues.	K_D (muscle agrin) = 2 nM; perlecan-LG = 16 nM; $\alpha 1$ -Lm \approx 20 nM; $\alpha 2$ -Lm = 43 nM; $\alpha 5$ -Lm = 150 nM; $\alpha 4$ -Lm \geq 1 μM (activity resides in LG domains).	[152,178-184]
heparin/heparan sulfates of various cellular proteoglycans	Laminins (LG and αLN domains), collagen-IV, agrin (LG), perlecan (LG)	Heparan sulfate chains tether growth factors (e.g. FGF-2, TGF β family members).	[30,31,141,185, 186]
galactosyl sulfatide (and related sulfated glycolipids)	Laminin-, agrin-, perlecan-LG domains; laminin-LN domains. These glycolipids, expressed in Schwann cell, kidney and other tissues, are thought to mediate and/or enhance anchorage of laminins. The LN interactions may enable the short arms to bind the cell surface in addition to the LG domains.	<p>a. <i>LG domains</i>: K_D agrin (15 nM), perlecan (20-30 nM); $\alpha 2$-Lms (LG4-5 = 6 nM); $\alpha 1$-Lms (LG4-5 = 65 nM); $\alpha 4$-Lms (LG1-3 & 4-5 = 50 & 100 nM); $\alpha 5$-Lms (LG1-3 & 4-5 = 80 & 20 nM).</p> <p>b. <i>LN domains</i>: Lm$\alpha 2$ (20 nM); Lm$\alpha 1$ (40 nM) Lm$\alpha 3B$ (50 nM); Lm$\alpha 5$ (70 nM).</p>	[17,27,30,31,181, 182,184,187-192]; Capizzi & Yurchenco, unpublished data (agrin and charge-density effects)
galectins	Laminin-111 and other ECM molecules	May contribute to cell adhesion and signaling.	[193]
receptor tyrosine kinase discoidin-domain receptor-1 (DDR1)	Collagens (including collagen-IV)	Absence of receptor associated with GBM thickening and hearing loss.	[194,195,196]
CD44 (chondroitin sulfate PG)	Collagen-IV	May contribute to cell adhesion.	[197]
Extracellular $\beta 1,4$ -gal-transferase	Laminin-111 and other ligands	May contribute to cell adhesion and signaling.	[198]

* detected in solid-phase binding assay with soluble clasped integrins in 1 mM Mn⁺⁺.

Table 2

Basement Membrane Scaffold Inter-Component Binding Interactions

Bond	Ligand Domain(s)	Nature of Interaction(s)	References
laminin - laminin	LN domains of the α , β and γ short arms interact with corresponding LN domains.	Polymerization (crit. conc. $\approx 0.1 \mu\text{M}$) requiring three short arm LN domains. Individual LN+LEa	[10,16–19]
nidogen - laminin	Nidogen-G3 to Lm γ 1 (LEb3), γ 2, γ 3	<p>a. Nd1-Lm111, $K_D \leq 1 \text{ nM}$</p> <p>b. Nd2-Lm111, $K_D \leq 1 \text{ nM}$</p>	[134,138]
agrin - laminin	Agrip NtA to Lm- γ 1 coiled-coil	Lm-111: $K_D \approx 2 \text{ nM}$	[79,151]
laminins - heparin/ heparan sulfate	<p>a. LG domains; α1, unprocessed α2, α4 >processed α2,</p> <p>b. LN domains of Lmα1>Lmα2>Lmα3B (Lmα5 does not bind to heparin)</p>	<p>a1) Lmα1LG4–5 binds to heparin with $K_D = 22 \text{ nM}$ (elutes $\sim 0.3 \text{ M NaCl}$), 38 nM for α1LN in E1'. (a2) rLm-211 (LG+LN) elutes from heparin column at 0.16 and 0.33 M NaCl while rLm211ΔLG1–5 (LN) elutes at 0.09 M.</p> <p>b) LN protein fragment elution off heparin column with 0.27 (α1), 0.24 (α2), 0.14 (α3B) and 0.0 (α5) M NaCl in 50 mM Tris, pH 7.4.</p>	[17,179,199, 200]
nidogen - collagen IV	Nd (G3>G2 domains) to collagenous chain	<p>a. Nd1-CollIV, $K_D < 1 \text{ nM} < 1 \text{ nM}$</p> <p>b. Nd2-CollIV, K_D</p>	[134,138]
nidogen - perlecan	Nd1-G2 and Nd2 to perlecan-domain IV	$K_D \approx 10 \text{ nM}$	[142,191]
perlecan - fibronectin	Perlecan domain IV	$K_D \approx 10 \text{ nM}$	[142]
perlecan - laminin	Perlecan domain-I (likely heparan sulfate chains) to laminin LG and LN domains	Likely relatively low affinity and specificity.	[142]
perlecan - collagen IV	Perlecan domain-I (likely heparan sulfate chains) to collagen-IV	Likely relatively low affinity and specificity.	[142]
perlecan - heparin	Perlecan domain IV	see above	[142]
nidogen - fibulin 2		$K_D \leq 0.5 \text{ nM}$	[138]
collagen IV - collagen IV	<p>a. NC1 - NC1</p> <p>b. 7S</p> <p>c. lateral (collagenous)</p>	<p>a. high affinity dimerization of collagen trimeric NC1 domains (forms "hexamer")</p> <p>b. overlap of four triple helical ends covalently stabilized by disulfides</p> <p>c. side-by-side associations that help form a network</p>	[6,107,109, 112,120,201]