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PROSPECTS

Domains of Laminin

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Abstract Extracellular matrix molecules are often very large and made up of several independent domains, frequently with autonomous activities. Laminin is no exception. A number of globular and rod-like domains can be identified in laminin and its isoforms by sequence analysis as well as by electron microscopy. Here we present the structure-function relations in laminins by examination of their individual domains. This approach to viewing laminin is based on recent results from several laboratories. First, some mutations in laminin genes that cause disease have affected single laminin domains, and some laminin isoforms lack particular domains. These mutants and isoforms are informative with regard to the activities of the mutated and missing domains. Second, laminin-like domains have now been found in a number of other proteins, and data on these proteins may be informative in terms of structure-function relationships in laminin. Finally, a large body of data has accumulated on the structure and activities of proteolytic fragments, recombinant fragments, and synthetic peptides from laminin. The proposed activities of these domains can now be confirmed and extended by in vivo experiments.

Key words: basement membrane, cell binding, epidermolysis bullosa, extracellular matrix, gene knock-out, integrin, laminin, muscular dystrophy

Laminin has the shape of a cross as examined under the electron microscope after rotary shadowing. The basic domain structure of a laminin molecule as derived from protein chemistry and cDNA sequence information is illustrated in Figure 1 [Sasaki et al., 1988]. Laminin is a heterotrimer of three subunits, α, β, and γ. The subunits are homologous but represent different gene products. The α subunit distinguishes itself by having a large extra domain at the C-terminus, the G domain. This makes the α subunit the heavy or dominant polypeptide of laminin. This and the fact that laminin assembly and secretion seem to be controlled by the synthesis of the α subunits may make it practical to classify laminins according to their α subunits.

A number of isoforms of each subunit are known. These are designated as α1, α2, α3, etc. The expression of these subunits is differentially regulated so that the subunits are characteristically present only in certain tissues and at particular times of development. The subunits assemble into a number of different laminin variants (Table I). Some of these are well documented, others are as yet anticipated. The relative abundance of these variants may be determined by the level of synthesis of particular subunits in the cell or by other subunit selection mechanisms, which are not yet known. [For reviews on laminins, see Timpl, 1989; Engel, 1992; Tryggvason, 1993; Burgeson et al., 1994; Wewer and Engvall, 1994. For information on the latest laminin subunits, α3, α4, and α5, see Iivanainen et al., 1995; Galliano et al., 1995; Miner et al., 1995.]

THE SHORT ARMS OF LAMININ

The short arms are the amino-terminal parts of the three laminin polypeptides. In contrast to the long arm, each short arm is constructed from a single subunit. The short arms contain two types of globular domains, designated domains VI and IV, which are separated by rod-like segments consisting of cysteine-rich repeats (Figs. 1 and 2). These rod-like segments are designated domains V and III. Some subunits lack certain short arm domains, for example α3

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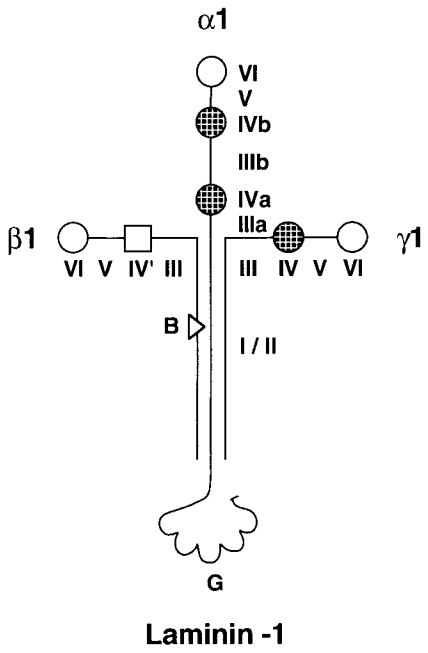

Fig. 1. Schematic representation of a laminin molecule. Roman numerals designate general domains in the α, β and γ subunits. G and B designate domains unique for the α and β subunits. [Modified from Sasaki et al., 1988].

TABLE I. The Laminins*

Full size laminins	*laminin-10*	*α5-β1-γ1*	
	laminin-11	*α5-β2-γ1*	
Mediums	laminin-1	α1-β1-γ1	classical (EHS) laminin
	laminin-2	α2-β1-γ1	merosin
	laminin-3	α1-β2-γ1	S-laminin
	laminin-4	α2-β2-γ1	S-merosin
Petites	laminin-5	α3-β3-γ2	kalinin/nicein/epiligrin
	laminin-6	α3-β1-γ1	K-laminin
	laminin-7	α3-β2-γ1	KS-laminin
	laminin-8	*α4-β1-γ1*	
	laminin-9	*α4-β2-γ1*	

*Laminins organized by the nature and size of their α subunits. *Laminins in italics are anticipated but await confirmation as proteins.*

and α4, β3 and γ2 (Fig. 2). Thus, laminin-5 (α3, β3, γ2) has three very short arms, and laminin-6 (α3, β1, γ1) has two regular sized arms and one truncated short arm (Fig. 2).

Domain VI; Essential for Laminin Polymerization

Domain VI is approximately 250 amino acid residues long and corresponds to the most amino-terminal globule in the α1, α2, α5, β1, β2, β3, and γ1 chains. It is not present in the processed forms of the α3 subunit or in the γ2 subunit. The sequence of domain VI predicts a mixture of α-helix, β sheet, and random coil, and a globular conformation. The sequence is the most highly conserved among the various laminin subunits as well as among the same subunits from different species. Several conserved cysteine residues and the sequence WWQS are characteristic of all the domains VI in laminin subunits sequenced so far.

In nematode and chicken, laminin-like proteins termed netrins are involved in cell migration and axon movement [Ishii et al., 1992; Serafini et al., 1994]. Netrins contain a motif similar to that of the laminin N-terminal domains VI and V (Fig. 3). Netrins have an additional motif similar to domains found in complement components. Netrins interact with cells via cell surface receptors of which *unc*-5 in C. elegans is believed to be one. It will be important to determine which of the three types of netrin domains bind to extracellular matrix and which bind to cells. A cell binding activity in one of the laminin-like domains would suggest similar binding in the homologous domain in mammalian laminin.

The domains VI in each laminin subunit are critically involved in the intermolecular assembly of laminin [Yurchenco and Cheng, 1993]. The polymerization of laminin, which is Ca^{2+}-dependent, results in a laminin network that is formed independently of other basement membrane components. Laminin polymerization can be quantitatively inhibited by fragments containing domain VI of the β1 or α1 subunit [Colognato-Pyke et al., 1995]. A fragment containing the β1 domain VI binds to laminin but is not able to polymerize by itself. Extrapolation of these data on laminin-1 suggests that laminin-5/kalinin with only one domain VI, which is in its truncated β3 subunit, may not be capable of self aggregating and therefore not be directly crosslinked into a basement membrane. Instead, laminin-5 in the anchoring filaments of the skin may form a separate laminin network or become linked to the basement membrane via binding to laminin-6.

A crucial role for domain VI in the in vivo function of laminin is indicated by the observation that mutations in this domain may cause disease. α2-Containing laminins are the predominant laminins in striated muscle and peripheral nerve and mutations leading to reduction or absence of laminin α2 in mouse and humans may result in muscular dystrophy [Hayashi et al., 1993; Xu et al., 1994a,b; Sunada et al., 1994;

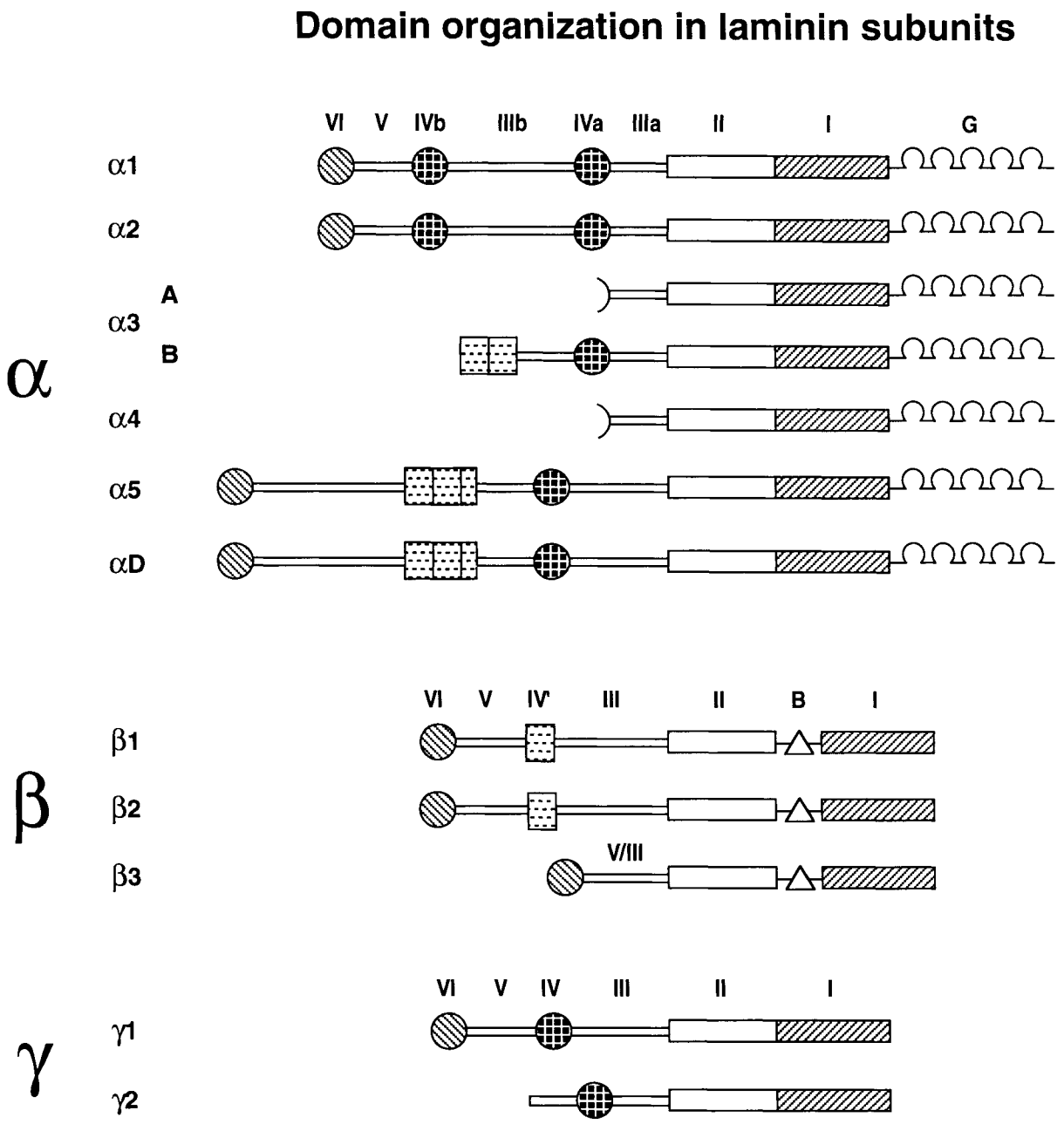

Fig. 2. Schematic representation of domain organization in the various laminin subunits, similar to Figure 1.

Tomé et al., 1994; Helbling-Leclerc et al., 1995; Wewer et al., 1995]. The dy^{2J} dystrophic mouse has a point mutation in a mRNA splicing consensus sequence, which results in exon skipping and expression of polypeptide with a small deletion in the domain VI [Xu et al., 1994b]. The truncation in domain VI of the α2 chain in the dy^{2J} mice does not seem to affect distant domains of the α2-containing laminins, as relatively normal levels of the mutant laminin α2 are synthesized, secreted, and deposited extracellularly in the skeletal muscle. Nevertheless, the defect in these mice is severe enough so that a structurally identifiable basement membrane can not be detected in peripheral nerves in these mice. This result may be surprising because laminin-6/K-laminin (α3, β1, γ1), which lacks domain VI in its α3 subunit but has intact domains VI in its β1 and γ1 subunits, is incorporated into the skin basement membrane. Why does the defect in the domain VI of laminin α2 then cause such a severe phenotype? To answer

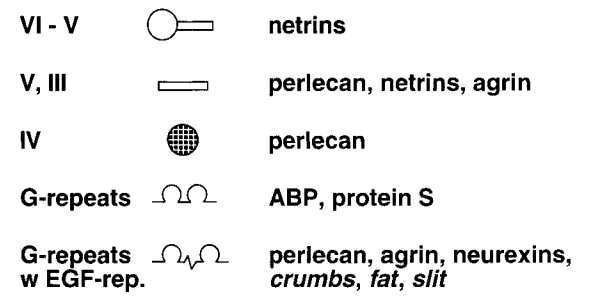

Fig. 3. Examples of domain motifs in laminin and other proteins in which they occur. Netrins, extracellular proteins involved in cell migration; perlecan, the basement membrane proteoglycan; agrin, a protein involved in the aggregation of the acetylcholine receptor at the neuromuscular junction; ABP (androgen binding protein) and protein S, serum proteins; neurexins, neuronal cell surface receptors; *crumbs, fat, slit,* Drosophila mutants.

this question it will be important to determine if the domain VI in the α2 subunit contains binding sites for heparin and cell surface receptors as shown for the domain VI in the α1 subunit [Colognato-Pyke et al., 1995] and if these binding sites are affected in the dy^{2J} mutant mouse. Recently, a point mutation in domain VI of laminin β3 was identified in a family with benign epidermolysis bullosa [McGrath et al., 1995].

Domain IV: Two Different Motifs, Unknown Functions

The inner globules of the short arms have been designated domain IV. However, this is not to be interpreted that they are all homologous. There are two apparently unrelated domains IV, which have no obvious sequence similarity and which presumably have different functions. One domain, designated IV′, is characteristic of the β subunits, the other, designated IV, is characteristic of most α and γ subunits. Exceptions include the α5 subunit and the Drosophila α subunit, which interestingly have both IV and IV′ domains (Fig. 2).

Domain IV of laminin α and γ subunits. Domain IV consists of approximately 200 amino acids. The sequence predicts a mixture of α-helix, β sheet, and random coil. There are no cysteines to form disulfide bonds in this domain. The sequence of domain IV is well conserved among laminin subunits and among laminin subunits from different species. Perlecan, the basement membrane proteoglycan, has three domains with homology to domain IV (Fig. 3) [Noonan et al., 1991]. Domain IV is absent from one form of laminin α3 and from α4, but the functional significance of absence of domain IV in a laminin subunit is not known.

Epidermolysis bullosa is a group of skin blistering diseases. One form of this disease, known as the lethal junctional form, is caused by absence of laminin-5 (α3, β3, γ2). One patient with junctional epidermolysis bullosa has been shown to have a mutation and a predicted small deletion in the γ2 subunit affecting domains IV and III [Pulkkinen et al., 1994]. Whether the deletion might have changed the conformation of distant domains in the γ2 chain and whether the mutated γ2 chain was assembled into laminin molecules was not analyzed. Therefore, at this point it is not easy to draw any conclusions about domain function from the phenotype of this patient.

Domain IV′, a domain unique for laminin β. Domain IV′ is similar in size (about 220 amino acid recidues) to domain IV but shows no obvious sequence similarity to domain IV. In contrast to domain IV, domain IV′ has a number of cysteine residues and therefore presumably contains intradomain disulfide bonds. Domain IV′ is present in one copy in the β1 and β2 subunits in mammalian laminins and is absent from β3 (Fig. 3). Human laminin α5 and the Drosophila laminin αD have two and a half copies of domain IV′. No other known proteins contain structural motifs similar to domain IV′. Mutations in domain IV′ have not yet been detected or created.

Domains V and III, Some Repetition

Domains V and III of the rod-like regions in the short arms of laminin-1 contain about 40 cysteine-rich repeats that are arranged in groups of 3 to 9. Each repeat is about 50 amino acids long and contains eight cysteine residues with a conserved pattern.

The cysteine-rich repeat sequences in the laminin subunits show similarity to EGF-like repeats but contain eight cysteine residues per repeat; the "true" EGF-like repeats contain only six cysteine residues per repeat and are usually encoded by a single exon [Engel, 1989]. The first six cysteine residues in the laminin-type repeats are thought to be arranged as in EGF, while a bond between cysteine residues 7 and 8 could form an extra loop not present in the EGF repeats. The 8-cysteine, EGF-like repeats in laminin are termed "the laminin-type cysteine-rich repeats" to distinguish them from the 6-cys-

teine EGF repeats. EGF repeats are present in many extracellular proteins [Baron et al., 1991], while the laminin-type cysteine-rich repeats have been found only in laminins, perlecan [Noonan et al., 1991], agrin [Rupp et al., 1991], and netrins [Serafini et al., 1994]. The EGF repeats of several proteins have been shown to mediate protein-protein interactions [Baron et al., 1991]. The laminin-type cysteine repeats may also be engaged in specific binding functions, although some might serve as spacers [Engel, 1989]. Only one such repeat in laminin has a function which is understood in some detail. A repeat in domain III of the mouse γ1 subunit contains the binding site for entactin/nidogen, a protein that is thought to link the laminin and type IV collagen networks in basement membranes [Mayer et al., 1993]. Moreover, putative cell adhesion sequences have been mapped to domain III of the α1 and β1 chains; these sequences include RGD in mouse α1 and YIGSR in mouse β1 [Yamada and Kleinman, 1992].

THE LONG ARM OF LAMININ

The long arm of laminin consists of an approximately 75 nm long rod-like structure and a globular domain with a diameter of about 20 nm. The rod comprises the carboxy-terminal one third of the β and γ subunits and the homologous part of the α subunit. This heterotrimeric portion has two domains designated domains I and II. The β subunits are distinguished from the α and γ subunits in this region by the presence of a small structure that separates domains II and I. This additional structure has previously been called α domain. Considering the new laminin nomenclature [Burgeson et al., 1994] this designation may cause confusion and we propose to rename this domain B since it is characteristic of the β subunits. The globular G-domain is composed only of sequences from the α subunit; it contains the C-terminal 950 or so amino acids and has a molecular weight of about 100 kDa.

Domains II and I; Mainly Helical

Domains II and I guide the intramolecular assembly of laminin and determine the subunit composition of the laminin. These domains contain multiple heptad repeats characteristic of fibrous proteins. The extended rod of the long arm is formed through associations of these domains in the three subunits. The heterotrimeric assembly is then stabilized by disulfide bonds located near the center of the laminin cross and near the G domain in the region that corresponds to the C-termini of the β and γ subunits.

The sequence of the approximately 600 amino acids that comprise domains II and I is poorly conserved among the laminin subunits (20–40% identity). Nevertheless, the general structural features of the domains are well conserved. Experimental data including circular dichroism spectroscopy have confirmed that the long arm has a high α-helical content. Each heptad repeat, "abcdefg," contains a hydrophobic residue at positions "a" and "d" and a charged residue at positions "e" and "g." Approximately 95% of the long arm segment of the laminin β1 subunit (excluding the B domain) and the other subunits is made up of these heptad repeats. Domain II has more interruptions in the heptad repeats than domain I. Proteins with extended α-helices often form dimers or trimers with a hydrophobic core and charged periphery. In laminin the coiled-coil region is more stable as a trimer that as a dimer. This region appears to represent one of the longest coiled-coils now known.

Because laminins are heterotrimers, one focus of interest in the laminin field has been to determine what interactions are responsible for the formation of a heterotrimer, as opposed to dimer or homotrimer. Additional questions relate to the mechanisms of assembly of particular combinations of laminin subunits when more than three subunits are produced by the same cell. Biosynthetic studies of laminin-1 in cell culture have shown that the cells first produce a β1-γ1 dimer as an intracellular intermediate, and that the mature secreted laminin molecule is composed of one each of the three types of subunits (α, β, and γ) [Peters et al., 1985].

The two-step model for laminin biosynthesis and assembly is supported by theoretical calculations [Beck et al., 1993] and by in vitro assembly studies using purified fragments of EHS laminin (E8), recombinant proteins, and synthetic peptides [Hunter et al., 1992; Utani et al., 1994, Nomizu et al., 1994]. Using proteolytic fragments of laminin-1, Hunter et al. [1992] found that homotrimers were less stable than a β1-γ1 dimer and that the α1-β1-γ1 trimer was preferentially formed when the α1 subunit was present. Thus, the stability of the coiled-coil structure with different trimeric compositions determines the nature of the product assembled. More recent studies employing recombinant fragments and synthetic peptides have shown that

the 100 C-terminal amino acids of domain I contain the information for chain selection and assembly [Utani et al., 1994; Nomizu et al., 1994]. In the mouse β1 and γ1 subunits the amino acid sequences necessary for dimer and trimer formation have been determined. The α1 and α2 subunits contain only sites for trimerization. In the same study, the importance of both charged and hydrophobic amino acids for the formation and stabilization of the heterotrimer were also documented.

Both theoretical analyses and experimental assembly studies have indicated that the β1 and β2 subunits are equally capable of participating in heterotrimer formation [Utani et al., 1994], suggesting that the relative amount of the different subunits could regulate the composition of the laminin variants secreted by a cell. However, the situation may be different in vivo as evidenced from cell culture studies [Wewer et al., 1994a]. The folding, oligomerization, transport, and secretion of laminin are likely to be complex processes regulated by cellular factors [Hurtley and Helenius, 1989] and may also contribute to laminin assembly.

Domain I has been reported to contain several cell adhesion recognition sites. In rat laminin β2, the tripeptide LRE has been found to be a motor neuron-specific attachment site [Porter et al., 1995]; however, this sequence is not found in this position in the human β2 [Wewer et al., 1994b] but is found in other positions in other laminin subunits. That the β2 subunit is essential for synaptic function was demonstrated by the phenotype of the β2 knockout mouse [Noakes et al., 1995]. Another sequence, IKVAV in the α1 subunit, has been reported to promote cell attachment and neurite outgrowth [Yamada and Kleinman, 1992]. This sequence is unique to and highly conserved in the α subunits.

A mutation in domain I in the laminin α2 subunit was the cause of congenital muscular dystrophy in two patients [Helbling-Leclerc et al., 1995]. The mutation would lead to premature termination of the α2 subunit. This would likely preclude the assembly of the mutated α2 with β and γ subunits, thus resulting in absence of laminin-2. As the sequences of domains II and I are among the least well conserved in laminin, other types of mutations within these domains may exist and be tolerated without serious effects on laminin structure and function.

The B Domain (Formerly α Domain)

Domains II and I of the β subunits are separated by a 33–35 amino acid residue domain that contains six cysteines and eight glycines, previously called the α domain but here renamed the B domain. This domain does not adopt an α-helical conformation but rather may loop out of the long arm, although it has not been possible to confirm this by rotary shadowing electron microscopy, perhaps because of the small size of the loop. No specific function has yet been attributed to the B domain. As it is present only in the β subunits and is unique to laminin, one may speculate that it may play a role in chain selectivity and assembly, or in the binding of laminin to other basement membrane proteins.

The G Domain

The G (globular) domain at the end of the long arm is unique to the laminin α chains. It is composed of 5 repeats of about 180–200 amino acids. These repeats are characterized by the consensus sequence YVGGLP and contain a number of conserved cysteine and glycine residues. The predicted structure includes predominantly β sheet and little α-helix. G-type repeats are present in a number of other proteins, including the basement membrane proteins perlecan and agrin, the serum proteins androgen-binding protein (ABP) and protein S, and cell surface proteins such as neurexins and Drosophila *crumbs, fat,* and *slit* [Noonan et al., 1991; Rupp et al., 1991; Ushkaryov et al., 1992; Patthy, 1992; Joseph and Baker, 1992]; (Fig. 3). The G type repeats are directly adjacent to one another in laminin, ABP and protein S; in the other proteins, the G-type repeats are separated by EGF-like repeats.

The number of G-type repeats may vary in the mature, processed forms of the different α chains. The domain remains intact with five repeats in the α1 chain. In the α2 chain, repeats 3–5 are found in tissues as a separate fragment even under apparently physiological conditions. This may be the result of physiological processing of α2-containing laminins. The last two repeats are easily cleaved off by protease treatment in vitro in both the α1 and α2 chains, generating the well characterized E3 fragment from the α1 chain and a homologous fragment from the α2 chain [Leivo and Engvall, 1988].

Although the short arms have important activities in being involved in the intermolecular assembly of laminin, many look upon the G domain as the "business end" of the molecule. A number of molecules have been shown to bind to or near the G domain in laminin. These include extracellular matrix molecules such as heparin and heparan sulfate proteoglycans, and the basement membrane-associated glycoprotein fibulin [Pan et al., 1993]. Most importantly, a number of cell surface receptors [Mecham, 1991] bind to the area of the G domain in laminin.

Laminin is the basement membrane component with the most striking effects on cells in vitro. Binding of cells to laminin promotes their migration or neurite outgrowth, and changes their pattern of gene expression and state of differentiation. The G domain and/or adjacent portion of laminin is responsible for most of the cell binding activity in laminin. However, it has been extremely difficult to identify the cell binding sites in the G domain, since cell and receptor binding to this region of laminin require a native conformation, which can only be maintained with contributions from the β and γ subunits. The E8 fragment of the EHS laminin consists of the lower portion of the long arm and G repeats 1–3. This fragment contains most of the cell adhesion and spreading activity in laminin. Attempts to reduce the size of the E8 fragment while retaining activity have been only partially successful [Deutzmann et al., 1990]. Work with recombinant G domain has confirmed the cell binding activity of the G domain [Sung et al., 1993]. However, the cell spreading activity corresponding to that of the intact molecule requires contribution from the β and γ subunits. Apparently the lighter subunits induce a particular conformation in the G domain.

After a decade of intense research a large number of potential cellular receptors for laminin has been identified. Two types of laminin receptors have captured most of the recent interest. One is dystroglycan, a member of the dystrophin-associated membrane complex, which is believed to link the dystrophin-containing cytoskeleton in muscle and nerve to the extracellular matrix [Campbell, 1995]. The other type of laminin receptors are found among the integrins [Kramer, 1994; Giancotti and Mainieri, 1994; Mercurio, 1995].

Dystroglycan is a widely expressed transmembrane glycoprotein. The sequence of dystroglycan shows many potential N-linked and O-linked carbohydrate attachment sites in its extracellular portion and it may be a proteoglycan. The cytoplasmic domain of dystroglycan has several features of a signaling receptor. Dystroglycan was independently identified by a number of laboratories as a major laminin-binding component in various tissues and cells. Although laminin specifically recognizes dystroglycan in complex mixtures of proteins, dystroglycan binds to other proteins in addition to laminin. A major dystroglycan-binding protein is agrin [Fallon and Hall, 1994], which shares with laminins the motif of G-type domains (Fig. 3).

Integrins are the major class of cellular receptors for extracellular matrix components. Integrins are heterodimeters of α and β subunits which are both membrane spanning polypeptides. At least eight integrins bind laminin. Many of the laminin binding integrins also bind other proteins. It is beyond the scope of this article to try to give a complete picture of this large and complicated research field. We would like to close with the note that receptors for laminin and other extracellular matrix proteins, integrins and others, are not necessarily designed to be specific. The interactions of these receptors with laminin are merely a part of a more complex set of interactions between the cell and the extracellular matrix. The sum of these interactions will determine the fate of the cell.

CONCLUSIONS

Two avenues of laminin research have evolved over the last couple of years. First, the realization that the laminin family is composed of many separate gene products. The latest discovered laminin subunit, α5, is most similar to the Drosophila α subunit and may represent the most ancient of the α subunits. The other subunits are variations on the α5 theme. Interestingly, they all vary in the N-terminal parts of the polypeptides; the subunits α3, α4, β3, and γ2 in particular all lack domains in the short arms, whereas the domains of the long arm are conserved and comparable with the other laminin subunits. Perhaps these variations in the short arms confer functional specificities among the laminin subunits that we have yet to understand. The full meaning of the alternatively spliced variants of the γ2 chain and of the two α3 variants remains to be explored. Are all these variants present in basement membranes? Do cells respond to changes by regulating the expression of one variant versus another? We expect to

see many studies on the regulation of transcription and translation of different laminin subunits in the near future.

Second, laminins have been implicated in two types of genetic disease: 1) primary defects in laminin α2 in congenital muscular dystrophy in humans and mice and secondary defects in laminin α2, β1, or β2 in other forms of human muscular dystrophy; 2) defects in laminin α3, γ2 and β3 in the skin blistering disease junctional epidermolysis bullosa. The first man-made mutation in a laminin gene has been reported, the knock-out of the laminin β2 gene in mice. The β2-deficient mice died within 4 weeks after birth and had abnormal motor-nerve terminals. The mice also had defects in kidney function similar to those found in the human congenital minimal change nephrotic syndrome. Certainly more knock-out mice, each with distinct phenotype, are likely to be reported in the near future. Advances in gene technology have made tissue specific gene knock-outs possible and exciting experiments will bring us closer to the understanding of the role of laminin as a key molecule in the basement membrane in normal and diseased tissues. It is intriguing to realize that all these laminin subunits, although conserved in domain organization, apparently may not be functionally redundant at all.

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