Molecular Dissection of the α-Dystroglycan- and Integrin binding Sites within the Globular Domain of Human Laminin-10

(ラミニン-10の球状ドメインに存在する
α-ジストログリカンおよび
インテグリン結合部位の解析)

A Thesis Presented to Osaka University
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Abbreviations

ECM: extracellular matrix

EHS: Engelbreth-Holm-Swarm

G domain: globular domain

ELISA: enzyme-linked immunosorbent assay

TBS: tris-buffered saline

BSA: bovine serum albumin

SDS-PAGE: SDS-polyacrylamide gel electrophoresis

FN70K: N-terminal 70-kDa region of human fibronectin

GST: glutathione *S*-transferase

LG: laminin G-like

mAb: monoclonal antibody

rLN10: recombinant laminin-10

General Introduction

The extracellular matrix (ECM) is the material underlies all epithelia and endothelia, and surrounds all connective tissue cells providing physical strength and mechanical support to tissues and organs. Although the ECM should not be viewed as simply providing physical support and strength for tissues. It is now quite apparent that ECM affects profound influences on both the behavior and pattern of gene expression of the cells in contact with it. In simple multicellular animals such as hydra, the physical support for the whole organism may consist solely of a basement membrane. In more complex animals such as human, different types of ECM have evolved in connective tissues such as cartilage, bone, and skin in addition to the ubiquitous basement membrane.

Basement membranes are highly specialized ECM with unique functions in tissue development, function, and stability. They are largely composed of a characteristic set of abundant and widespread glycoproteins, which include laminin, collagen IV, nidogen, and perlecan (Timpl and Brown, 1996). Basement membranes form thin coats around mesenchymal cells or sheets underneath epithelial and endothelial cell layers (Fig. 1). Basement membranes play several biological roles. First, they supply mechanical support for cell layers. Second, they form barriers between tissue compartments that inhibit the transmigration of cells and passively regulate the exchange of macromolecules. Third, they serve as interactive surfaces for cells, providing adhesion, cell shape, migratory signals, and modulating the behaviour of the cells with which it interacts but also communicating information for the regeneration and differentiation of cells.

In general, laminins are a family of glycoproteins present in the ECM and the major components of basement membranes. It is widely accepted that laminins contribute to basement membrane architecture and influence cell adhesion, spreading, and migration (Ryan et al., 1996). However, laminins are more than mere glue and a surface over which cells move. There is emerging evidence that laminins can regulate cell proliferation and have the capability of specifying cell and tissue development, differentiation, and function, through their interaction with cell surface receptors (Belkin and Stepp, 2000). It is now considered that laminins have an outstanding impact on the cells with which they interact and are the conduit of signals from the matrix to cells and vice versa.

All laminins are large (400-600 kDa) heterotrimeric glycoproteins composed of an α, β, and γ chain, which associate to form a cross-like structure (Fig. 2A). Thus far, five different α chains

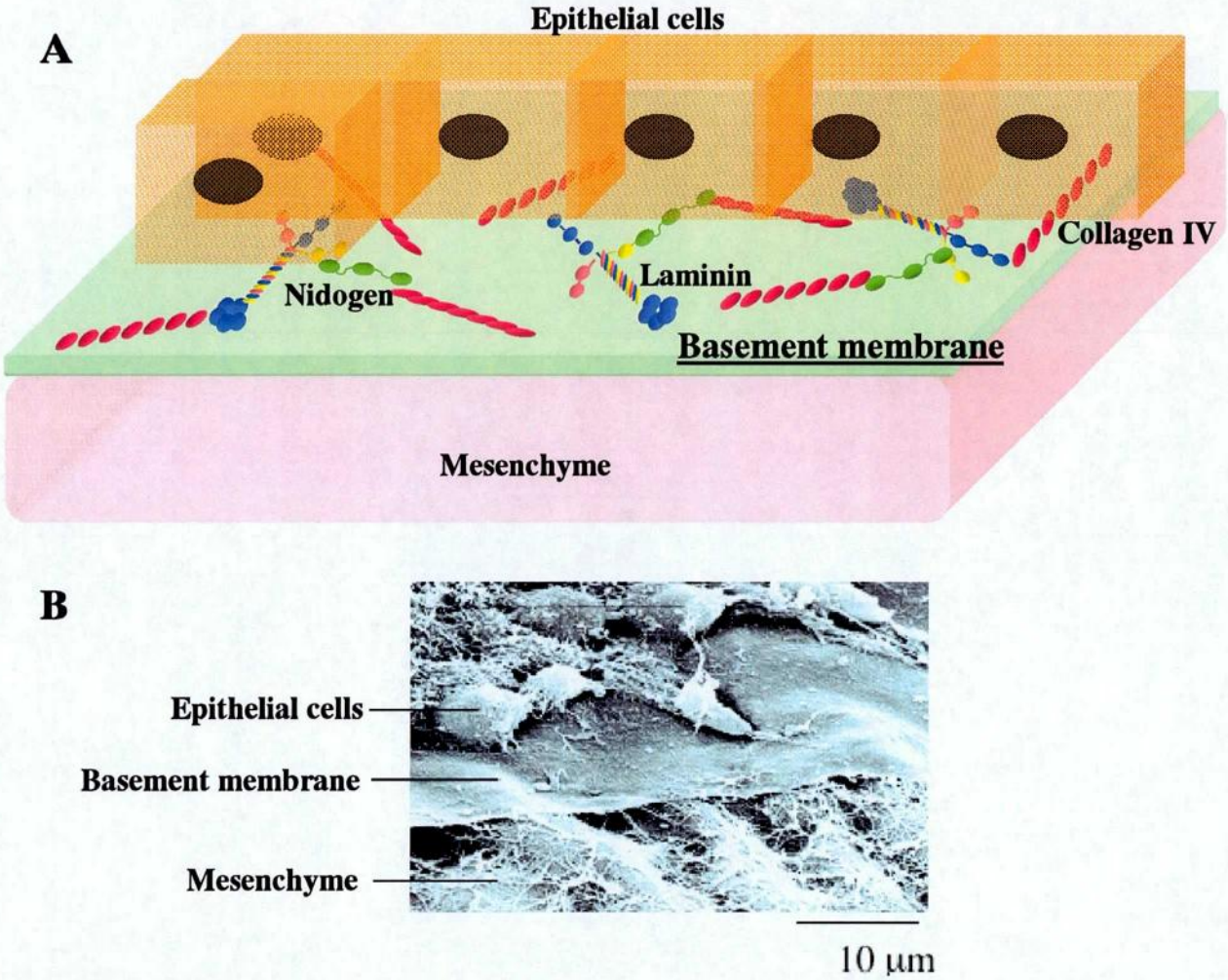

Fig. 1. Anatomical view of the basement membrane in tissue architecture.

(*A*) Schematic diagram of basement membrane. Basement membranes (*light green*) underlie the epithelial cells and separate these cells from mesenchyme (*pink*). Basement membranes also function as bioactive architectures promoting cell polarity and migration, regulating cell proliferation and differentiation, and preventing apoptosis of epithelial cells. (*B*) The basement membrane in the cornea of a chick embryo. In this scanning electron micrograph, some of epithelial cells have been removed to expose the sheet-like basement membrane. A network of collagen fibrils in the underlying mesenchyme is seen. (B is cited from Molecular Biology of the Cell, Forth Edition, Garland Science, with minor modifications)

termed α1 to α5, three different β chains (β1-β3), and three different γ chains (γ1-γ3) have been identified, theoretically allowing the formation of 45 different trimeric laminin isoforms. However there are limitations in which trimer combinations are actually expressed. So far, the natural

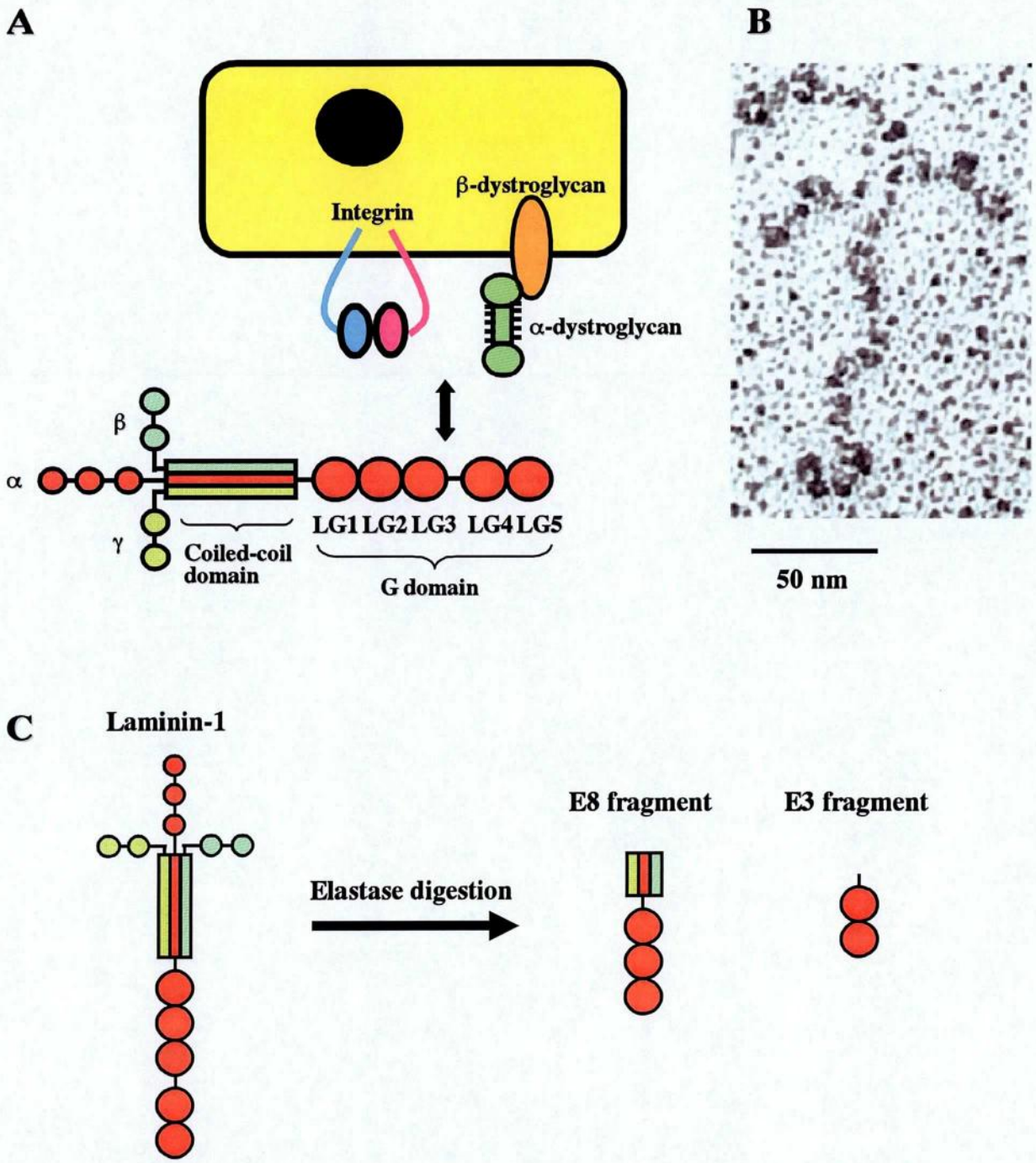

Fig. 2. The structure of laminin and its receptors.

(*A*) All laminin isoforms are composed of three disulfide-linked subunits, α, β and γ chains. These chains are self-assembled into coiled-coil domain. The C-terminal region of α chain is called "G domain", which consists of five distinct modules (LG1-LG5). Cell surface receptors including integrins and dystroglycan are thought to recognize the G domain. (*B*) Rotary shadow images of laminin-1 (Photograph B was cited from Yurchenco et al., (Yurchenco et al., 1997)). (*C*) Schematic diagram showing the major proteolytic fragments of laminin-1. Laminin-1 can be cleaved by limited elastase digestion to give rise to two biologically active fragments. E8 fragment possesses the integrin-binding activity, and E3 fragment possesses the α-dystroglycan binding activity.

α chain	Subunit composition	Tissue distribution
α1	α1β1γ1 (Laminin-1) α1β2γ1 (Laminin-3)	Embryo
α2	α2β1γ1 (Laminin-2) α2β2γ1 (Laminin-4) α2β1γ3 (Laminin-12)	Muscle Peripheral nerve
α3	α3β3γ2 (Laminin-5) α3β1γ1 (Laminin-6) α3β2γ1 (Laminin-7)	Skin Bladder Lung
α4	α4β1γ1 (Laminin-8) α4β2γ1 (Laminin-9)	Blood vessel Nerve
α5	α5β1γ1 (Laminin-10) α5β2γ1 (Laminin-11)	Diverse epithelia Blood vessel Nerve

Table 1. Subunit composition and tissue distribution of laminin isoform.

occurrence of 12 trimeric laminin isoforms has been clearly demonstrated (Table. 1).

Laminin containing α1 chain (laminin-1/3) is expressed in epithelial basement membrane early in development but it appears to be lost at many sites during maturation (Miner et al., 1997). The α2-chain laminin (laminin-2, -4, and -12) is found in the basement membranes surrounding skeletal and cardiac muscles (Leivo and Engvall, 1988; Paulsson et al., 1991). It also found in the basement membranes produced by Schwann cells surrounding peripheral nerves (Paulsson et al., 1991). The α3-chain laminin (laminin-5/6) is expressed in the basement membranes surrounding the skin, bladder, and lung. The α4-chain laminin (laminin-8/9) is present in the endothelial basement membranes of blood vessels. In most of epithelial tissues, the α5-chain laminin (laminn-10/11) appears to be the major α chain occurring in later embryonic development and with some exceptions it is retained in the adult (Ekblom et al., 1998; Miner et al., 1995; Sorokin et al., 1997).

The expression of each isoform shows precise tissue and cell regulation. Further, each isoform is expressed in a different spatial and temporal manner. The essential importance of the laminins for development and correct function of the organism has been demonstrated by gene targeting studies

in mice as well as by the characterization of naturally occurring mutations within the laminin genes.

Due to disruption of the skeletal muscle basement membrane, natural or experimental mutations of the LAMA2 gene which encodes the laminin α2 chain result in congenital muscular dystrophy in man (Helbling-Leclerc et al., 1995; Tome et al., 1994) and in mice (Sunada et al., 1994; Xu et al., 1994). Similarly, laminin-2/4 (α2β1/β2γ1) knockout mice showed congenital muscular dystrophy and postnatal lethal phenotype (Kuang et al., 1998; Miyagoe et al., 1997). Muscular dystrophy is a group of genetic disorders which primarily affects skeletal muscle and is clinically characterized by progressive muscle wasting and weakness. Mutations in the genes that encode for the subunits of laminin-5 (α3β3γ2) lead to a blistering skin disease termed junctional epidermolysis bullosa (Aberdam et al., 1994; Kivirikko et al., 1995; McGrath et al., 1995). Also, targeted disruption of LAMA3 gene which encodes the laminin α3 chain results in epidermolysis bullosa and die at birth (Ryan et al., 1999). Disruption of the LAMA5 gene which encodes the laminin α5 chain results in a complex phenotype with deficient separation of digits, protrusion of mesenchymal tissues through the developing epithelium at the tip of the digits, and a failure in the closure of the neural tube resulting in exencephaly (Miner et al., 1998). Many of these events could be due to a primary mechanical weakness in the basement membrane.

The different phenotypes observed in the knockout mice indicated that each laminin isoform is expressed in tissue-specific patterns and selectively affected. All these functions are exerted through the interaction with the cell surface receptors. It is well-known that integrins are the major receptors for laminins.

Integrins are αβ transmembrane receptors that play critical roles in both cell-matrix and cell-cell adhesion in multicellular organisms (Fig. 2A). In addition to their ability to link cells to their ECM, integrins possess cytoplasmic domains that function in cellular signaling via their ability to associate with and activate signal transduction pathways. The cytoplasmic domains also play essential structural roles; they interact with and affect the assembly of the cytoskeleton and thus regulate cell shape and migration (Hynes, 1999). To date, 18 α subunits and 8 β subunits have been characterized at the molecular level in vertebrates. Several members of the integrin family, including α3β1, α6β1, α6β4, and α7β1 heterodimers serve as laminin receptors on a variety of cell types. The specificity of integrins for different laminin isoforms has been determined for a few of the laminin-integrin interactions. It has been shown that α3β1 and α6β1 binds more strongly to laminin-5 (α3β3γ2) and laminin-10/11 (α5β1/β2γ1). Also, α7β1 binds more efficiently to

laminin-2/4 ($\alpha2\beta1/\beta2\gamma1$).

There are non-integrin receptors that also mediate cellular attachment to laminin. Dystroglycan is cell surface-associated receptor shown to interact directly with laminins (Fig. 2A). The single gene product of the dystroglycan gene gives rise to two proteins after posttranslational cleavage (Durbeej et al., 1998). α-dystroglycan is a highly glycosylated peripheral membrane protein associated with the membrane spanning protein β-dystroglycan. The laminin-binding activity of the dystroglycan resides exclusively in α-dystroglycan. α-dystroglycan has been shown to associate with the laminin-1 and laminin-2 (Andac et al., 1999; Talts et al., 1999). In skeletal muscle, dystroglycan links the cytoskeletal protein dystrophin and the basement membrane protein laminin. The integrity of this linkage seems critical for maintaining normal muscle function. In various types of muscular dystrophy, the disruption of this linkage leads to muscle cell necrosis. Thus, dystroglycan is believed to act as a transmembrane link between the laminins and the cytoskeleton and this linkage seems to be important for maintaining normal muscle function. Furthermore recent studies have also indicated other roles for dystroglycan's interactions with laminins. The phenotype of the dystroglycan-null mouse indicated that dystroglycan is required for the formation of a basement membrane during early development. Subsequently, Henry and Campbell (1998) showed that dystroglycan is required for the formation of the basement membrane in embryoid bodies. In the same study, they employed embryoid stem cells to show that dystroglycan is required for the organization of laminin-1 on the cell surface, indicating that this cellular activity of dystroglycan might underlie its requirement for basement membrane assembly at the tissue level of organization. Dystroglycan might simply serve to increase the local concentration of laminin at the cell surface, thus facilitating the laminin self-assembly reaction.

In 1979, first laminin had been isolated from mouse Engelbreth-Holm-Swarm (EHS) tumor and it was later named laminin-1(Timpl et al., 1979). Laminin-1 was overexpressed in EHS tumor and purification of laminin-1 was comparatively easy. Therefore functional analyses have been primarily focused on laminin-1, and laminin-1 is the most extensively studied within laminin isoforms. Rotary shadowing electron microscopy and proteolytic fragmentation studies have demonstrated that laminin-1 is the heterotrimeric molecule, and consists of α1 (400 kDa), β1 (200 kDa), and γ1 chain (200 kDa). The structure of laminin-1, as seen by electron microscopy after rotary shadowing, resembles a cross with three short arms and one long arm (Fig. 2B). The long arm ends in a globular structure called "G domain". G domain is formed exclusively by the α1

chain (Sasaki et al., 1988) and consists of five distinct modules. Studies employing amino acid sequence comparisons and electron microscopy suggest that the overall domain structures are well conserved within other laminin isoforms.

A combined approach using limited proteolysis and antibody mapping has proven to be useful for the study of laminin. In addition, isolation of proteolytic fragments obtained through controlled enzymatic digestion of laminin has been widely utilized to elucidate the influence of individual laminin domains or groups of domains on cellular behavior. Limited elastase digestion of laminin-1 generates two major fragments E3 (50 kDa) and E8 (280 kDa), as well as a number of small fragments (Paulsson et al., 1985) (Fig. 2C). Detailed studies based on amino terminal sequence analysis, electron microscopy, and antibody mapping localized the E8 fragment to the long arm of laminin-1, indicating that these elastase products contain portions of all three subunits. In contrast, E3 is comprised of the carboxy terminal fragment of the α1 chain containing the fourth and fifth globular module of the α1 G domain.

The E3 fragment has heparin binding activity similar to intact laminin-1, and may thus contribute to interaction of laminin-1 with heparin-containing proteoglycans in the extracellular matrix or on the cell surface (Ott et al., 1982; Yurchenco et al., 1990; Yurchenco et al., 1993). Cellular adhesion to fragment E3 is likely mediated by non-integrin cell surface receptors such as α-dystroglycan (Gee et al., 1993).

The E8 fragment of laminin-1 has been shown to promote cellular attachment, spreading, and migration. The E8 fragment can mediate cellular attachment via β1 integrins on the cell surface, making it the major cell binding domain of laminin (Ziober et al., 1996). Furthermore the mapping of cell-adhesive sites using trypsin digested proteolytic fragments and inhibitory antibody assays strongly supports the idea that most of the cell-adhesive sites are near or within the G domain.

All of the known laminins have G domains at the C-terminal region of the α chain. The G domain can be resolved into five tandemly arrayed modules, designated LG1 to LG5. Although G domain represents about one third (1000 residues) of the amino acid sequence of the α chains, it appears folded into a rather compact structure. The independent folding of each LG domain was confirmed by solving the crystal structure of the α2LG5 module (Hohenester et al., 1999).

It is assumed that the G domain contains important sites via which laminins interact with integrins. Many studies have attempted to identify specific sequences that mediate integrin-binding to laminin. As described above, the use of limited proteolysis to generate laminin fragments for

searching the integrin-binding sites is as common strategy. However neither fragments nor peptides derived from protease digested E8 fragment has exhibited the cell-adhesive activity compared with the intact laminin-1. Moreover, recombinant fragment of G domain or synthetic peptides of the portion of the G domain also showed only weak cell-adhesive activity. Therefore, integrin-binding sites within laminins have not been determined.

The purpose of this study is to elucidate the integrin- and α-dystroglycan-binding sites within laminin G domain in detail. Especially laminin-10 (α5β1γ1) is the best ligand for integrin α3β1 and α6β1, thus I selected the laminin-10 to analyze the integrin- and α-dystroglycan binding sites. Due to the high molecular weight of laminin-10 (approximately 800 kDa), amount of purified laminin-10 from cell culture medium is often very small. Therefore, I established the recombinant laminin-10 expression system using 293F cells by triple transfection of α5, β1, and γ1 expression vectors and secreted recombinant laminin-10 was purified from the conditioned medium by immunoaffinity chromatography. Next I produced the series of recombinant laminin-10 with deletions of the LG modules within the G domain. Using these deletion mutants, I performed the integrin and α-dystroglycan binding assays. Finally I revealed that the LG4 module is the binding site of α-dystroglycan, and the LG3 module is essential for integrin-binding. And I found the several amino acid residues which play important roles in integrins and α-dystroglycan-binding to laminin-10.

Abstract

The adhesive interactions of cells with laminins are mediated by integrins and non-integrin type receptors such as α-dystroglycan and syndecans. Laminins bind to these receptors at the C-terminal globular domain (G domain) of their α chains, but the regions recognized by these receptors have not been precisely mapped. In this study, we sought to locate the binding sites of laminin-10 (α5β1γ1) for α3β1 and α6β1 integrins and α-dystroglycan through the production of a series of recombinant laminin-10 proteins with deletions of the LG modules within the G domain. We found that deletion of the LG4-5 modules did not compromise the binding of laminin-10 to α3β1 and α6β1 integrins, but completely abrogated its binding to α-dystroglycan. Further deletion up to the LG3 module resulted in loss of its binding to the integrins, underlining the importance of LG3 for integrin binding by laminin-10. When individually expressed as fusion proteins with glutathione *S*-transferase or the N-terminal 70 kD region of fibronectin, only LG4 was capable of binding to α-dystroglycan, while neither LG3 nor any of the other LG modules retained the ability to bind to the integrins. Site-directed mutagenesis of the LG3 and LG4 modules indicated that Asp-3198 in the LG3 module is involved in the integrin binding by laminin-10, while multiple basic amino acid residues in the putative loop regions are synergistically involved in the α-dystroglycan binding by the LG4 module.

Introduction

Laminins are the major basement membrane proteins ubiquitously expressed throughout the metazoa. Laminins are heterotrimers of three subunits, termed α, β, and γ chains, which assemble into cross-shaped molecules with three short arms and one long rod-like arm. To date, five α chains, three β chains and three γ chains have been identified, combinations of which yield at least 12 isoforms with distinct subunit compositions (Colognato and Yurchenco, 2000). These isoforms have been shown to be involved in many biological processes, including cell adhesion, proliferation, migration and differentiation (Colognato and Yurchenco, 2000; Timpl, 1996).

The interaction of cells with laminins is mediated by a variety of cell surface receptors including integrins and non-integrin type receptors such as α-dystroglycan and syndecans (Colognato and Yurchenco, 2000; Henry and Campbell, 1999). Integrins are of crucial importance among these receptors with respect to controlling the growth and differentiation of cells. There are more than 20 integrins with distinct subunit compositions, of which α3β1 and α6β1 integrins have been shown to be the major laminin receptors expressed in many cell types (Kikkawa et al., 2000; Kikkawa et al., 1998; Nishiuchi et al., 2003). α-dystroglycan is a highly glycosylated protein containing novel *O*-mannosyl-type oligosaccharides (Chiba et al., 1997), and forms a complex with a single-pass transmembrane protein called β-dystroglycan (Henry and Campbell, 1999). α-dystroglycan binds to various types of laminin isoforms including laminin-1 (α1β1γ1) and laminin-2 (α2β1γ1) in a Ca^{2+}-dependent manner (Ervasti and Campbell, 1993; Gee et al., 1993; McDearmon et al., 1998; Pall et al., 1996). Binding sites for integrins and α-dystroglycan have been mapped to the G domain, the C-terminal globular domain of the laminin α chain (Andac et al., 1999; Talts et al., 1999; Talts et al., 2000). The G domain consists of five tandem repeats of LG modules of ~200 amino acid residues, designated LG1 through LG5. However, the binding sites within the G domain for integrins and α-dystroglycan remain to be defined.

Laminin-10 (α5β1γ1) is a major laminin isoform widely expressed in adult tissues (Miner et al., 1995). Mice lacking the laminin α5 gene exhibit embryonic lethality due to severe developmental abnormalities, such as syndactyly, exencephaly, and placental dysmorphogenesis (Miner et al., 1998). Laminin-10 also seems to be essential for hair morphogenesis, since ablation of laminin-10 results in arrest of hair follicle development at the hair germ elongation phase (Li et al., 2003). Previously, we purified laminin-10/11 from the conditioned medium of human lung

carcinoma cells and demonstrated that adhesion of epithelial cells to laminin-10/11 was mainly mediated by α3β1 integrin (Kikkawa et al., 1998), although adhesion of fibroblastic cells was mediated through both α3β1 and α6β1 integrins (Kikkawa et al., 2000). The roles of α3β1 and α6β1 integrins as major receptors for laminin-10/11 were further confirmed by direct binding of laminin-10/11 to α3β1 and α6β1 integrins (Nishiuchi et al., 2003). Recently, Yu and Talts (2003) produced recombinant fragments modeled after the G domain of the mouse α5 chain and demonstrated that the fragment consisting of the LG1-3 modules had cell-adhesive activity dependent on α3β1 and α6β1 integrins, while the fragment consisting of the LG4-5 modules was capable of mediating cell adhesion via interaction with α-dystroglycan (Yu and Talts, 2003). However, precise mapping of the binding sites for integrins and α-dystroglycan within the G domain of the α5 chain remains undefined.

In the present study, we produced a panel of recombinant laminin-10 mutants with serial deletions of the LG1-5 modules, and examined their binding activities to purified α3β1 and α6β1 integrins, α-dystroglycan and heparin. Our results show that the LG3 module is indispensable for binding to α3β1 and α6β1 integrins, although the LG3 module alone is not sufficient to recapitulate the integrin-binding activity. In contrast, the binding site(s) for α-dystroglycan and heparin have been mapped to the LG4 module, which alone can exhibit potent binding activities toward α-dystroglycan and heparin. We also attempted to identify the amino acid residues involved in integrin and α-dystroglycan binding by site-directed mutagenesis of the LG3 and LG4 modules, respectively.

Experimental Procedures

cDNA Cloning and Construction of Expression Vectors—A full-length cDNA encoding the human laminin α5 subunit (GenBank Accession Number AF443072) was amplified by RT-PCR as a series of ~1.2 kb fragments, and each fragment was subcloned into pGEM-T (Promega, Madison, WI) or pCRscript (Stratagene, La Jolla, CA) according to manufacturer's instructions. RNA was extracted from A549 human lung adenocarcinoma cells and used as a template for RT-PCR. The list of primer sequences is available upon request. After sequence verification, error-free cDNA fragments were ligated in tandem to construct a cDNA encompassing the whole open reading frame. The α5 cDNA was inserted into the *Nhe*I/*Pme*I sites of pcDNA3.1 (Invitrogen, Carlsbad, CA), yielding the α5 chain expression vector pcDNA-α5. Expression vectors for laminin β1 (pCEP-β1) and γ1 (pcDNA3.1-γ1) were prepared as described previously (Hayashi et al., 2002). Expression vectors for laminin α5 chains lacking LG5 (nucleotides 1-10554; pcDNA-α5ΔLG5), LG4-5 (nucleotides 1-9891; pcDNA-α5ΔLG4-5), LG3-5 (nucleotides 1-9360; pcDNA-α5ΔLG3-5), LG2-5 (nucleotides 1-8802; pcDNA-α5ΔLG2-5) and LG1-5 (nucleotides 1-8241; pcDNA-α5ΔLG1-5) were constructed as follows. cDNA fragments encompassing nucleotides 5795-8241 (for the deletion of LG1-5), 5795-8802 (for the deletion of LG2-5), 5795-9360 (for the deletion of LG3-5), 9365-9891 (for the deletion of LG4-5) and 9365-10554 (for the deletion of LG5) were amplified by PCR using KOD DNA polymerase (TOYOBO, Osaka, Japan) with a *Not*I (for the deletions of LG1-5, LG2-5 and LG3-5) or *Asc*I (for the deletions of LG4-5 and LG5) site at the 5' end and a stop codon and a *Pme*I site at the 3' end. The PCR products were digested with *Not*I/*Pme*I or *Asc*I/*Pme*I, and the resultant cDNA fragments were recloned into the pcDNA-α5 vector cleaved with the same restriction enzymes.

Expression vectors for individual LG modules of the laminin α5 chain as GST fusion proteins were prepared as follows. cDNAs encoding the individual modules were amplified by PCR using pcDNA-α5 as a template. The PCR products were digested with *Eco*RI and *Xho*I and inserted into the corresponding restriction sites of the pGEX4T-1 expression vector (Amersham Bioscience, Piscataway, NJ). The PCR primers used were:

5'-TTCAATGAATTCTCAGGGGTGCAGC-3'and

5'-CGGGTCCTCGAGCTACTTGGAGCGGGC-3' (for LG1);

5'-GCCCGCGAATTCTCGACCGGGGACCCG-3' and

5'-GGCGCGCTCGAGCTACAGGTCGGCGGTGC-3' (for LG2);

5'-ACCGCCGAATTCCTGGTGGGGCGCGCC-3' and

5'-CGGGGTCTCGAGCTACAGGGCGGGTGC-3' (for LG3);

5'-AATGAATTCAGGACCACCCGAGAC-3' and

5'- TATCTCGAGCTAGGGGCCCAAGATGCAG-3' (for LG4);

5'-GTCACAGAATTCATCTTGGGCCCCCTG-3' and

5'-CTGCAGGCTCGAGACTAGGCGGCTGG-3' (for LG5).

Expression vectors for fusion proteins of the LG3 and LG4 modules with the N-terminal 70 kDa domain of human fibronectin (FN70K), designated FN70K-LG3 and FN70K-LG4, respectively, were prepared as follows. The expression vector pMTX-1 for the truncated form of human fibronectin (Matsuyama et al., 1994) was digested with *Hin*dIII and *Not*I and inserted into the corresponding restriction sites of pFLAG-CMV-5c (Sigma-Aldrich, St. Louis, MO), yielding the expression vector pFLAG-FN70K for FN70K with a FLAG tag. cDNA fragments encoding the LG3 and LG4 modules were amplified by PCR using pcDNA-α5 as a template with a *Not*I site at the 5' end and an *Eco*RV site at the 3' end. The PCR products were digested with *Not*I/*Eco*RV and inserted into the corresponding restriction sites of the expression vector pFLAG-FN70K. The PCR primers used were:

5'- ATATGCGGCCGCACCGCCGACCTGCTG-3' and

5'- TATGATATCTTGCAGGGCGGGTGC-3' (for LG3);

5'- ATATGCGGCCGCAGGACCACCCGAGAC-3' and

5'- ATAGATATCGGGGCCCAAGATGCAG-3' (for LG4).

Site-directed Mutagenesis—Site-directed mutagenesis of the LG4 module was accomplished by overlap extension PCR with KOD polymerase using pGEX-LG4 encoding the GST-LG4 fusion protein as a template. The list of primer sequences used for the site-directed mutagenesis is available upon request. For site-directed mutagenesis of the LG3 module, the cDNA fragment encoding LG3 was excised from pcDNA-α5ΔLG4-5 with *Asc*I and *Pme*I and recloned into pSecTag2A (Invitrogen) at the *Asc*I/*Pme*I sites. The resulting plasmid was used as a template for site-directed mutagenesis of the LG3 module by overlap extension PCR as described above. The list of primer sequences used for the site-directed mutagenesis is available upon request. The purified PCR products containing the mutations were digested with *Asc*I and *Pme*I and inserted into the

corresponding restriction sites of the expression vector pcDNA-α5ΔLG4-5.

Expression and Purification of Recombinant Proteins—Recombinant laminin-10 (rLN10) and its mutants were produced using the FreeStyle™ 293 Expression system (Invitrogen). Briefly, 293-F cells were simultaneously transfected with expression vectors for α5, β1, and γ1 using 293fectin (Invitrogen), and grown in serum-free FreeStyle 293 Expression medium for 72 h. For the expressions of rLN10 and rLN10 lacking LG5, heparin (200 μg/ml) was included in the medium to inhibit the proteolytic cleavage between the LG3 and LG4 modules (Amano et al., 2000). The conditioned media were clarified by centrifugation, and passed through immunoaffinity columns conjugated with an anti-human laminin α5 mAb 5D6 (Fujiwara et al., 2001). The columns were washed with 50 mM Tris-HCl (pH 7.4) containing 500 mM NaCl to remove bound heparin, and then bound laminins were eluted with 0.1 M triethylamine, neutralized and dialyzed against TBS (50 mM Tris-HCl, pH 7.4, 150 mM NaCl).

Individual LG modules of the α5 chain produced as GST fusions were induced in *E. coli* with 0.1 mM isopropyl-β-D-thiogalactopyranoside, and purified on glutathione-Sepharose 4B columns (Amersham Bioscience) after lysis of the cells by sonication. Bound proteins were eluted with 50 mM Tris-HCl (pH 8.0) containing 10 mM glutathione. Purified proteins were dialyzed against TBS. GST-LG3 was recovered as insoluble aggregates upon sonication, and therefore solubilized in TBS containing 8 M urea and dialyzed against TBS before passing through a glutathione-Sepharose 4B column. FN70K, FN70K-LG3, and FN70K-LG4 were produced using the FreeStyle™ 293 Expression system as described above. The conditioned media were applied to anti-FLAG M2 columns (Sigma-Aldrich), and the columns were washed with TBS. Bound proteins were competitively eluted from the columns with 100 μg/ml FLAG peptide (Sigma-Aldrich), and dialyzed against TBS.

Proteins and Antibodies—α3β1 and α6β1 integrins were purified from human placenta and reconstituted into ^{3}H-labeled phosphatidylcholine liposomes as previously described (Nishiuchi et al., 2003). α-dystroglycan was purified from rabbit skeletal muscle according to Brancaccio et al. (Brancaccio et al., 1995) with the following modifications: α-dystroglycan partially purified using DEAE Sephacel and wheat germ agglutinin-agarose chromatography was further purified by laminin-1 affinity chromatography, followed by CsCl gradient centrifugation (Pall et al., 1996).

Heparin-BSA was purchased from Sigma-Aldrich. mAbs against the human laminin α5 chain (15H5 and 5D6) were produced in our laboratory (Fujiwara et al., 2001). A mAb against human laminin γ1 (Mab1920) was purchased from Chemicon. A mAb against human fibronectin (FN9-1) was obtained from Takara Biomedicals (Kyoto, Japan). A mAb against human β1 integrin (AIIB2) developed by Dr. Caroline Damsky (University of California, San Francisco) was obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA). A polyclonal anti-GST antibody was produced by immunizing rabbits with purified GST and affinity-purified on a GST-conjugated Sepharose column.

Binding Assays for Integrins, α-Dystroglycan and Heparin— Integrin binding assays were performed using α3β1 and α6β1 integrins reconstituted into ^{3}H-labeled phosphatidylcholine liposomes as described previously (Nishiuchi et al., 2003). Briefly, 96-well microtiter plates were coated with the proteins to be tested at 20 nM, blocked with 1% BSA, and incubated with integrin-liposomes in the presence of 1 mM Mn^{2+} at room temperature for 6 h. Plates were washed with TBS containing 1 mM Mn^{2+}, and the bound integrin-liposomes were recovered with 1% SDS and quantified with a Packard TRI-CARB 1500 liquid scintillation analyzer. For binding assays for α-dystroglycan and heparin, 96-well microtiter plates were coated with α-dystroglycan (5 μg/ml) or heparin-BSA (5 μg/ml), blocked with 1% BSA, and incubated with rLN10, its mutant forms or individual LG modules expressed as GST- or FN70K-fusion proteins in TBS containing either 1 mM $CaCl_2$/$MgCl_2$ or 1 mM EDTA at room temperature for 1 h. After washing with TBS, bound proteins were quantified with the anti-laminin γ1 mAb (rLN10 and its mutants), anti-GST antibody (GST fusion proteins), or anti-fibronectin mAb FN9-1 (FN70K fusion proteins), followed by incubation with HRP-conjugated rabbit anti-mouse or goat anti-rabbit IgG antibodies.

Cell Adhesion Assay—Cell adhesion assays were performed using HT1080 human fibrosarcoma cells (Kikkawa et al., 2000). Briefly, 96-well microtiter plates were coated with 5 nM rLN10 or its mutants at 4°C overnight and blocked with 1% BSA for 1 h at room temperature. HT1080 cells were harvested with PBS containing 1 mM EDTA, suspended in serum-free DMEM at a density of 3 x 10^5 cells/ml, and then plated on the wells coated with rLN10 or its mutants at 3 x 10^4 cells/well. After incubation in a CO_2 incubator at 37°C for 30 min, the attached cells were fixed and stained with Diff-Quik (International Reagents Corp., Japan), washed with distilled water, and

extracted with 1% SDS for colorimetric quantification at 590 nm.

Cell adhesion inhibition assays were performed based on the cell adhesion assays. HT1080 cells were preincubated with a function-blocking anti-β1 integrin mAb (AIIB2) or control mouse IgG for 20 min at room temperature and then added to the precoated wells. After 30 min incubation at 37°C, the cells attached to the substrates were quantified as described above.

Results

Production of Wild-type Laminin-10 and its Deletion Mutants—To map the binding sites for integrin and α-dystroglycan within the G domain of the laminin α5 chain, we expressed recombinant laminin-10 (rLN10) and a series of its deletion mutants lacking LG modules (Fig. 3) by triple transfection of cDNAs encoding the laminin α5, β1, and γ1 subunits into 293-F cells. Secretion of endogenous laminins containing β1 and/or γ1 chains was undetectable in 293-F cells (data not shown). Both wild-type and mutant proteins were purified from conditioned media using immunoaffinity columns conjugated with an anti-α5 chain mAb. To minimize the cleavage at the spacer segment between the LG3 and LG4 modules, wild-type rLN10 and its mutant lacking LG5 (rLN10ΔLG5) were expressed in cells grown in medium containing heparin, since heparin has been shown to partially inhibit the proteolytic processing of laminin-5 at the spacer region (Amano et al., 2000).

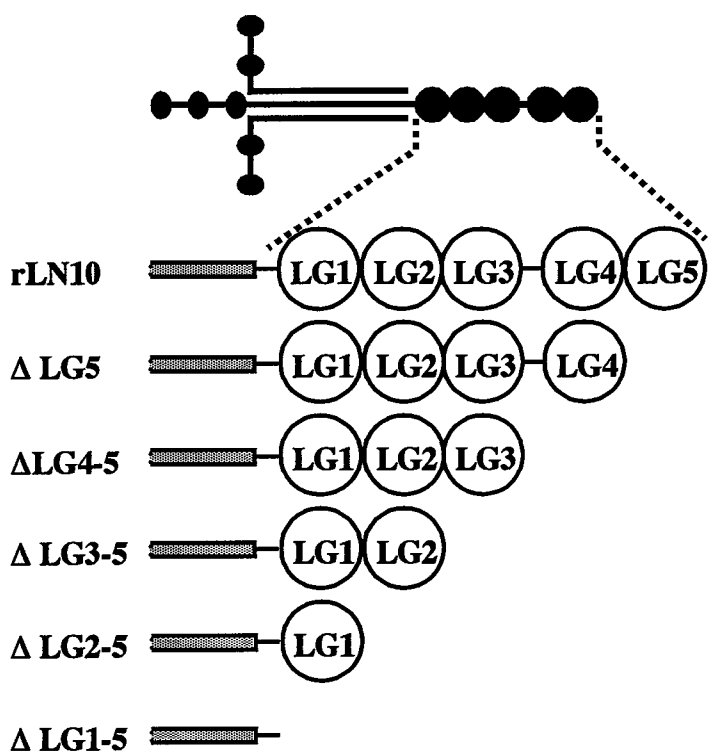

Fig. 3. Schematic diagrams of rLN10 and its deletion mutants.

rLN10, recombinant laminin-10; ΔLG5, rLN10 lacking the LG5 module; ΔLG4-5, rLN10 lacking the LG4-5 modules; ΔLG3-5, rLN10 lacking the LG3-5 modules; ΔLG2-5, rLN10 lacking the LG2-5 modules; ΔLG1-5, rLN10 lacking the LG1-5 modules.

The authenticity of the recombinant proteins was verified by SDS-PAGE and immunoblotting with a mAb against the α5 chain. Under reducing conditions, each recombinant protein gave three bands upon silver staining, one corresponding to the α5 chain, with a molecular mass of 300,000-400,000 depending on the extent of the deletion, and two lower bands corresponding to the β1 and γ1 chains (Fig. 4). When wild-type rLN10 and rLN10ΔLG5 were expressed in 293-F cells grown in medium without heparin, the majority of the recombinant proteins were processed at the spacer region, yielding bands that comigrated with rLN10 lacking the LG4-5 modules (rLN10ΔLG4-5; data not shown). Under non-reducing conditions, rLN10 and its deletion mutants barely entered the gel, confirming that they were purified as trimers of the α5, β1 and γ1 chains.

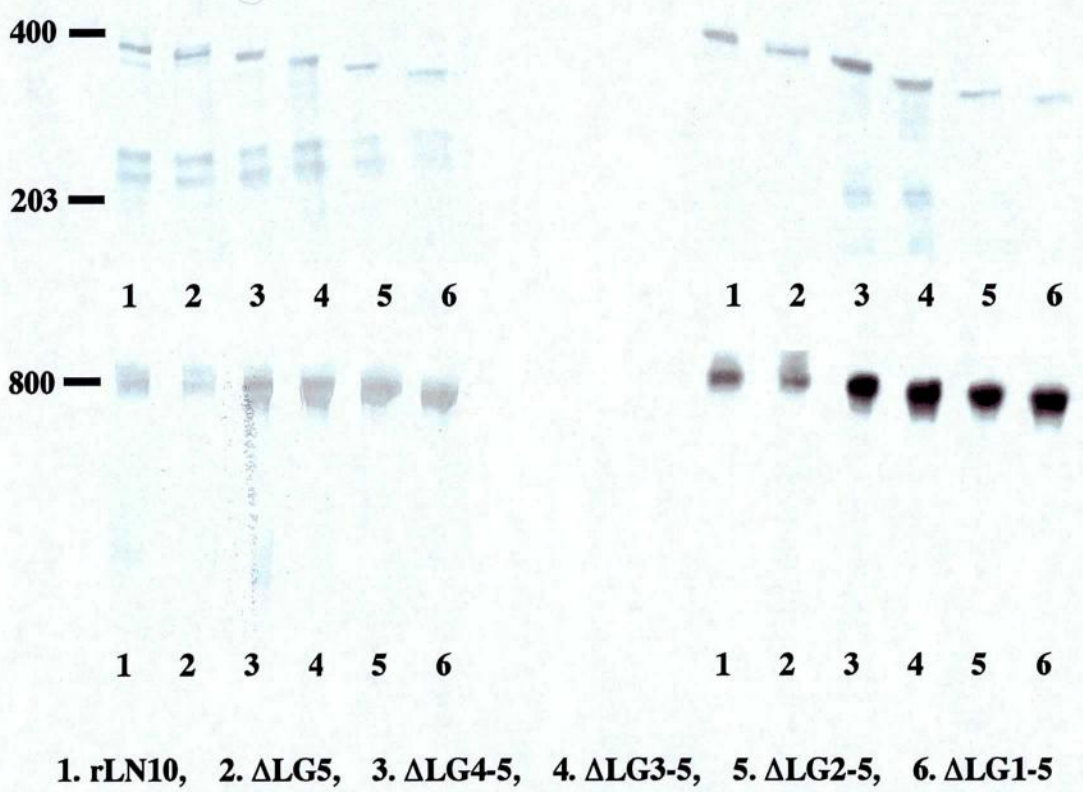

Fig. 4. SDS-PAGE and immunoblotting analyses of purified rLN10 and its deletion mutants.
Purified rLN10 and its deletion mutants were analyzed by SDS-PAGE on 4% gels under reducing (*upper panels*) or non-reducing (*lower panels*) conditions, followed by silver staining of the gels (*left panels*) or immunoblotting with a mAb against the laminin α5 chain (*right panels*). The positions of molecular size markers are shown in the *left margin*.

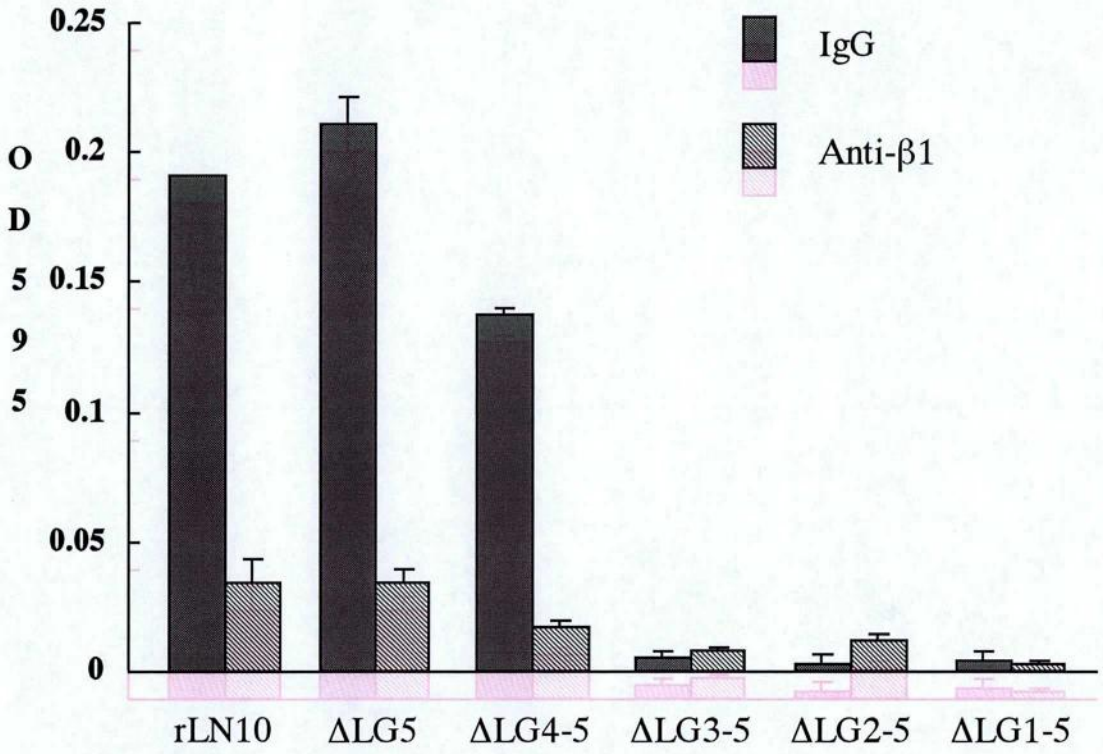

Fig. 5. Cell-adhesive activities of rLN10 and its deletion mutants.

HT1080 cells were preincubated with either a function-blocking mAb against β1 integrin (20 μg/ml, *hatched bars*) or control IgG (20 μg/ml, *shaded bars*) for 20 min at room temperature and then added to 96-well microtiter plates coated with rLN10 or its deletion mutants at 5 nM. After 30 min incubation at 37°C, the cells attached to the substrates were fixed and quantified as described in the Experimental Procedures. The deletion mutants are abbreviated as described in the legend for Figure 1. Each *column* and *bar* represents the mean of triplicate assays and the standard deviation, respectively. Pretreatment with control IgG did not affect the cell-adhesive activity of rLN10.

Cell-adhesive and Integrin-binding Activities of Wild-type Laminin-10 and its Deletion Mutants —The purified rLN10 and its deletion mutants were assayed for their cell-adhesive activities using HT1080 cells. rLN10ΔLG5 and rLN10ΔLG4-5 exhibited potent cell-adhesive activities equivalent to that of wild-type rLN10, but those lacking the LG3-5, LG2-5 and LG1-5 modules were barely able to mediate cell adhesion to substrates (Fig. 5). Cell adhesion to wild-type rLN10 and the mutants lacking LG5 and LG4-5 was strongly inhibited by a function-blocking mAb against β1 integrin, confirming the role of β1 integrins as major cell surface receptors for laminin-10 (Kikkawa et al., 2000; Nishiuchi et al., 2003). Cell adhesion to wild-type rLN10 and the

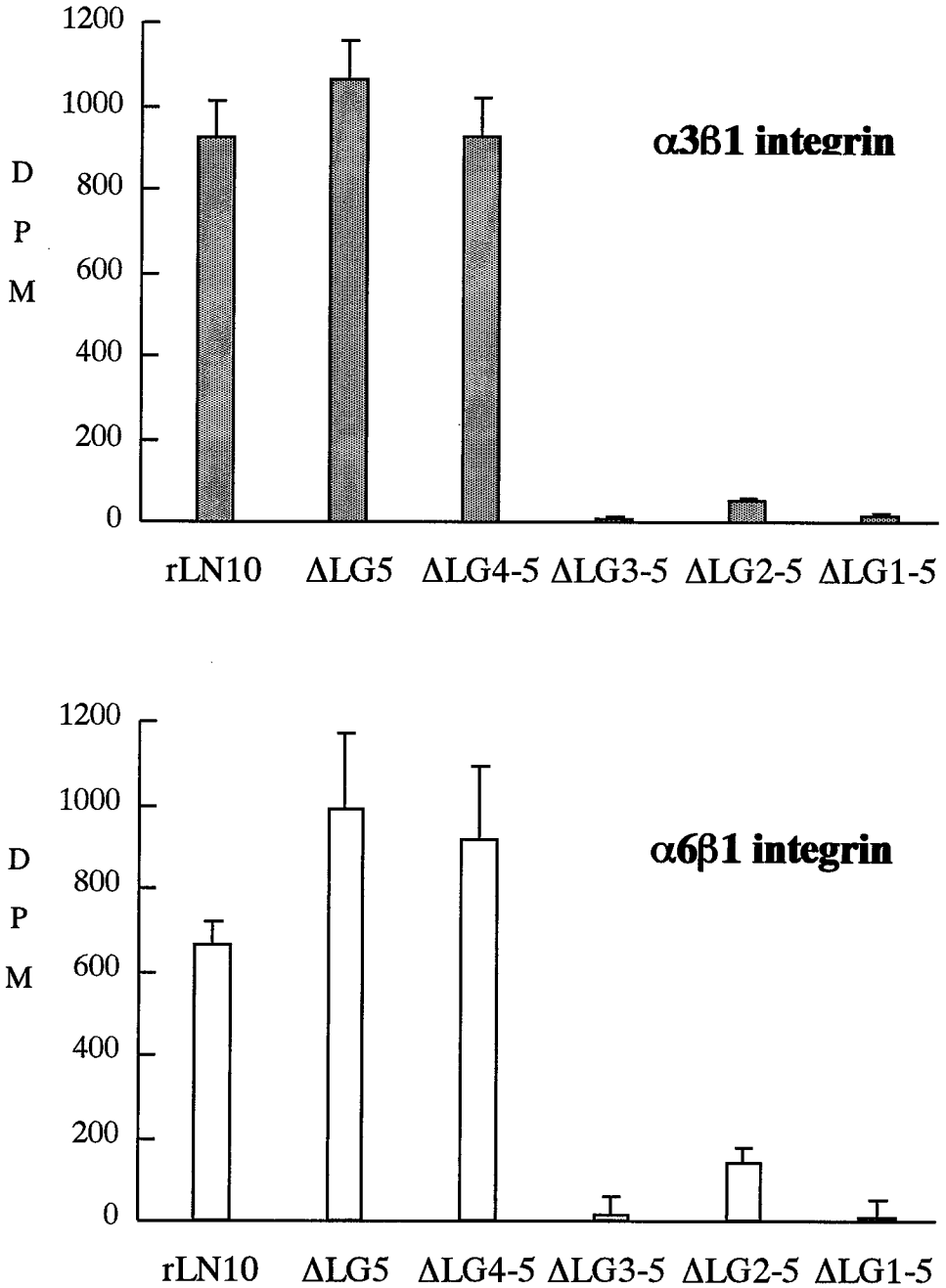

Fig. 6. Integrin-binding activities of rLN10 and its deletion mutants.

96 well microtiter plates were coated with rLN10 or its deletion mutants at 20 nM. After blocking with BSA, the wells were incubated with ^{3}H-labeled phosphatidylcholine liposomes containing α3β1 integrin (*upper panel*) or α6β1 integrin (*lower panel*) in the presence of 1 mM Mn^{2+} at room temperature for 6 h. Bound integrin-containing liposomes were quantified with a Packard TRI-CARB 1500 liquid scintillation analyzer. Each *column* and *bar* represents the mean of triplicate assays and the standard deviation, respectively.

mutants lacking LG4-5 and LG5 was also inhibited by a combination of anti-α3 integrin and anti-α6 integrin mAbs, but not by either mAb alone (data not shown), consistent with previous observations (Kikkawa et al., 2000). A dramatic loss of the cell-adhesive activity upon deletion of LG3 indicated that the LG3 module is indispensable for the potent cell-adhesive activity of laminin-10 and that LG3-dependent cell adhesion is mainly mediated by α3β1 and/or α6β1 integrins.

To confirm the importance of the LG3 module in the binding of laminin-10 to α3β1 and α6β1 integrins, we examined the binding of rLN10 and its mutants to these integrins. We purified α3β1 and α6β1 integrins from placenta, and examined their binding to laminin-10 and its mutants after reconstitution into ^{3}H-labeled phosphatidylcholine liposomes (Fig. 6). As expected, the mutants lacking the LG5 and LG4-5 modules were capable of binding to both α3β1 and α6β1 integrins with potencies that were comparable to that of wild-type rLN10, although mutants lacking LG3-5, LG2-5 or LG1-5 were almost devoid of any activity. The binding activities of mutants lacking LG5 and LG4-5 to these integrins were completely abrogated in the presence of EDTA (data not shown), confirming that the mutants lacking LG5 and LG4-5 retained the same integrin-binding properties as intact laminin-10.

α-Dystroglycan- and Heparin-binding Activities of Wild-type Laminin-10 and its Deletion Mutants—We also examined the binding of wild-type laiminin-10 and its deletion mutants to α-dystroglycan and heparin by solid-phase binding assays using microtiter plates coated with α-dystroglycan or heparin-BSA. Wild-type rLN10 was capable of binding to α-dystroglycan in the presence of Ca^{2+} ions, but not in the presence of EDTA (Fig. 7), consistent with previous reports that the binding of laminin-1/2 to α-dystroglycan is Ca^{2+}-dependent (Ervasti and Campbell, 1993; Gee et al., 1993; McDearmon et al., 1998; Pall et al., 1996). In contrast, wild-type rLN10 bound to heparin irrespective of the presence of Ca^{2+} or EDTA, confirming that heparin binding to laminin-10 does not require divalent cations, e.g., Ca^{2+} (Andac et al., 1999; Nielsen et al., 2000; Talts et al., 1999). Among the deletion mutants tested, rLN10ΔLG5 retained full binding activities toward α-dystroglycan and heparin, but other mutants including rLN10ΔLG4-5 were only marginally active at binding to either α-dystroglycan or heparin (Fig. 7). These results provide loss-of-function evidence that both α-dystroglycan and heparin bind to the LG4 module of laminin-10.

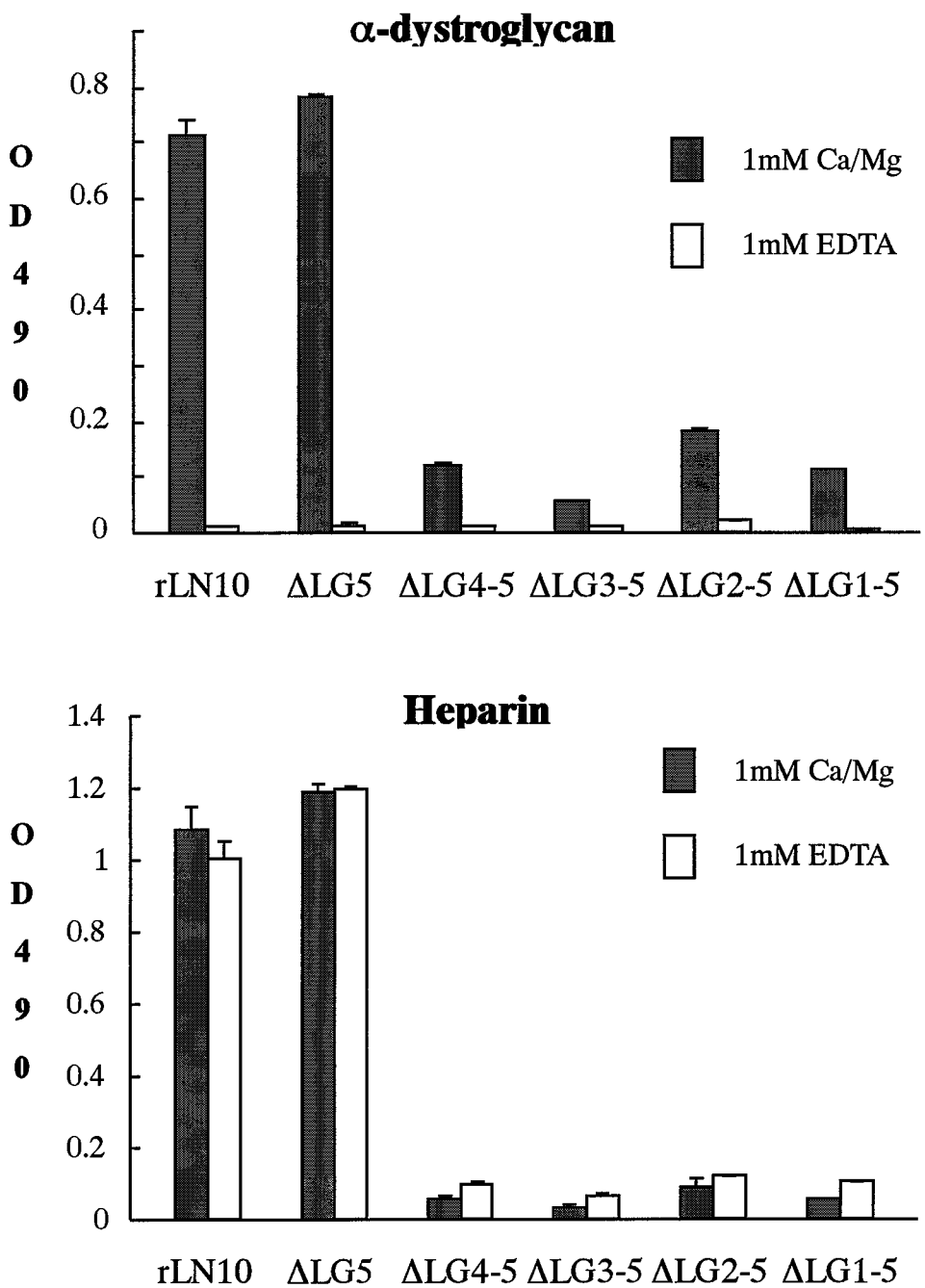

Fig. 7. α-dystroglycan- and heparin-binding activities of rLN10 and its deletion mutants.

96-well microtiter plates were coated with α-dystroglycan (*upper panel*) or heparin-BSA (*lower panel*), and incubated with rLN10 or its mutant forms in the presence of 1 mM $CaCl_2/MgCl_2$ (*closed bars*) or 1 mM EDTA (*open bars*) at room temperature for 1 h. Bound proteins were detected with an anti-γ1 mAb. Each *column* and *bar* represents the mean of triplicate assays and the standard deviation, respectively.

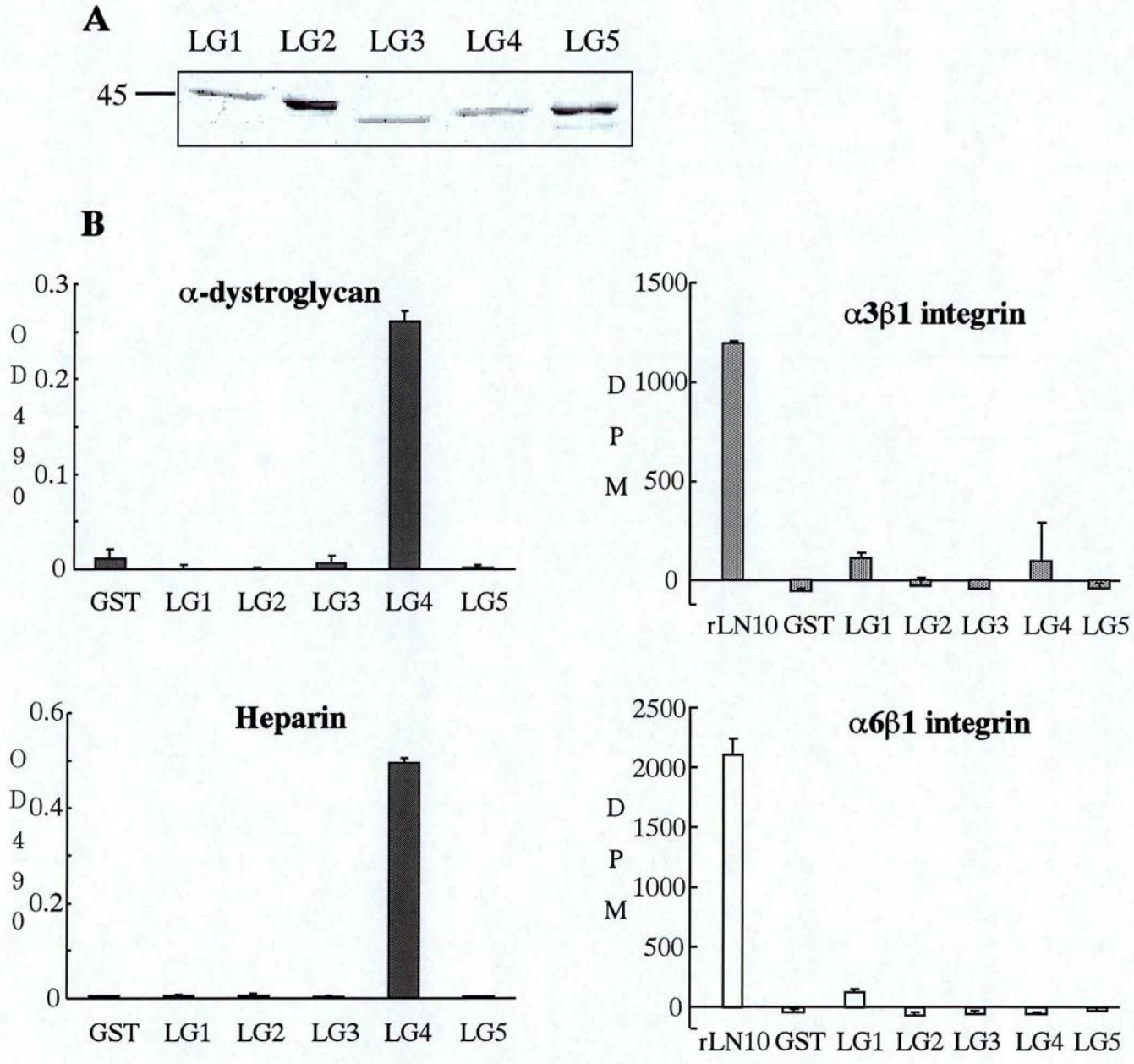

Fig. 8. α-dystroglycan-, heparin- and integrin-binding activities of individual LG modules.

(*A*) SDS-PAGE profiles of individual LG modules of the α5 chain expressed and purified as GST fusion proteins. Proteins were stained with CBB. The position of the 45 kDa molecular size marker is shown in the *left margin*. (*B*) α-dystroglycan- and heparin-binding activities of GST-LG modules. Bound proteins were detected with an anti-GST polyclonal antibody. (*C*) Integrin-binding activities of individual GST-LG modules. Microtiter plates were coated with individual GST-LG modules (20 nM) and then incubated with ^{3}H-labeled phosphatidylcholine liposomes containing α3β1 integrin (*upper panel*) or α6β1 integrin (*lower panel*) in the presence of 1 mM Mn^{2+} at room temperature for 6 h. Each *column* and *bar* represents the mean of triplicate assays and the standard deviation, respectively.

Receptor Binding Activities of Individual LG Modules—The binding profiles of the deletion mutants of laminin-10 toward α3β1/α6β1 integrins and α-dystroglycan/heparin suggested that the LG3 module was the likely binding site for α3β1 and α6β1 integrins, while the LG4 module was the likely binding site for α-dystroglycan and heparin. To explore these possibilities further, we expressed the individual LG modules of the α5 chain in bacteria as GST fusion proteins (Fig. 8A) and assayed their abilities to bind α3β1/α6β1 integrins as well as α-dystroglycan/heparin. Only GST-LG4 was capable of binding to α-dystroglycan and heparin among the five GST-LG modules (Fig. 8B), confirming that LG4 is the major binding site for both α-dystroglycan and heparin. In contrast, neither LG3 nor any of the other LG modules showed any significant binding to α3β1 or α6β1 integrins (Fig. 8C), except that GST-LG1 exhibited a very weak integrin-binding activity. Since the failure of LG3 and the other LG modules to bind to these integrins could result from misfolding and/or the absence of glycosylation due to their expression in bacteria, we expressed the LG3 and LG4 modules in 293-F cells as fusion proteins with FN70K, which serves as a vehicle for the secretion of recombinant proteins in mammalian expression systems (Matsuyama et al., 1994). FN70K-LG4 retained the ability to bind α-dystroglycan (Fig. 9A) and heparin (data not shown), but FN70K-LG3 did not show any significant binding to either α3β1 or α6β1 integrin (Fig. 9B). These results raise the possibility that LG3 is necessary, but not sufficient, for the integrin-binding activity of laminin-10 (*see* Discussion).

Mapping of α-Dystroglycan- and Heparin-binding Sites within the LG4 Module by Site-directed Mutagenesis—Previous studies have demonstrated that the basic amino acid residues within the LG4 module of the α1 chain and the LG5 module of the α2 chain participate in binding to α-dystroglycan and heparin (Andac et al., 1999; Talts et al., 1999). These basic amino acid residues are predicted to be within the loops connecting adjacent β strands, based on the crystal structure of the LG4-5 modules of the α2 chain (Hohenester et al., 1999; Timpl et al., 2000). To specify the amino acid residues within LG4 involved in binding to α-dystroglycan and heparin, we produced a series of alanine substitution mutants of GST-LG4 for the basic amino acid residues predicted within the loop regions of the LG4 module (designated L1 through L11; Fig. 10A). All these GST-LG4 mutants showed identical electrophoretic mobilities and were indistinguishable from wild-type GST-LG4 (data not shown). Partial reductions in the binding activities to

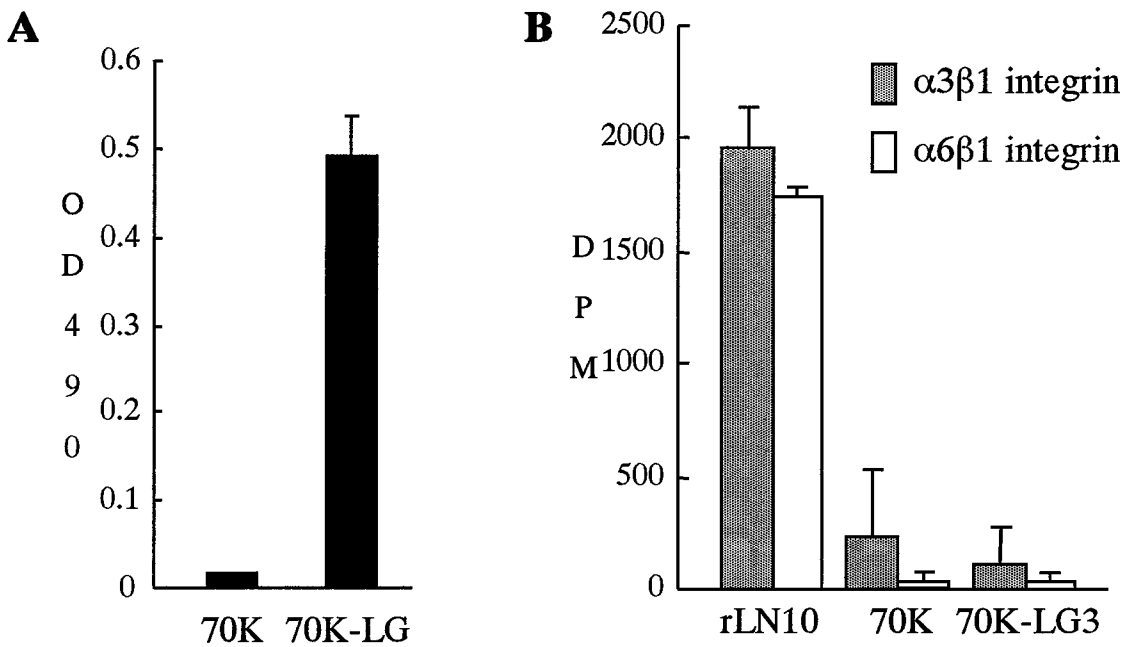

Fig. 9. Receptor binding activities of the LG3 and LG4 modules expressed in mammalian cells. (*A*) α-dystroglycan-binding activities of FN70K (70K) and FN70K-LG4 (70K-LG4). Bound proteins were detected with a mAb against human fibronectin. (*B*) Binding activities of rLN10, FN70K (70K) and FN70K-LG3 (70K-LG3) to α3β1 (*shaded columns*) and α6β1 (*open columns*) integrins. Each *column* and *bar* represents the mean of triplicate assays and the standard deviation, respectively.

α-dystroglycan and heparin were observed with the L2, L3, L5, L6 and L9 mutants (Fig. 10B), suggesting that the basic amino acid residues in these loops are involved in the binding of the LG4 module to α-dystroglycan and heparin. Since the overall binding profiles of the LG4 mutants toward α-dystroglycan and heparin were very similar, it is likely that the amino acid residues involved in binding to α-dystroglycan and heparin mostly overlap each other. To explore this possibility, we examined whether heparin could compete with α-dystroglycan for binding to GST-LG4 (Fig. 10C). As expected, GST-LG4 binding to α-dystroglycan was inhibited by increasing concentrations of heparin, supporting the possibility that laminin-10 binds to both α-dystroglycan and heparin through closely overlapping, if not identical, basic amino acid residues within LG4.

Production of Laminin-10 Mutants with Amino Acid Substitutions within the LG3 Module and Their Integrin-binding Activities—We also produced a series of rLN10 mutants with single amino acid substitutions within the LG3 module to explore the amino acid residue(s) involved in its

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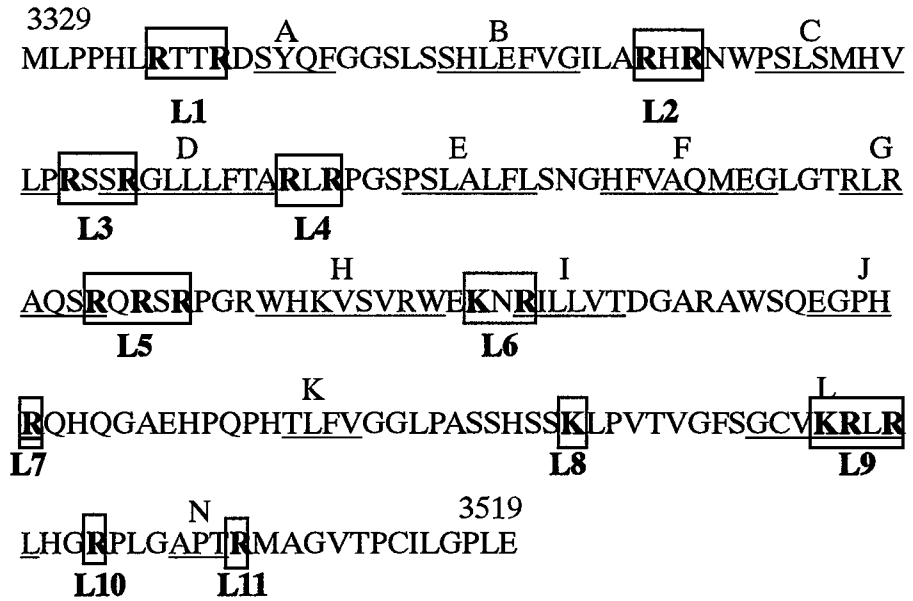
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MLPPHLRTTRDSYQFGGSLSSHLEFVGILARHRNWPSLSMHV
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AQSRQRSRPGRWHKVSVRWEKNRILLVTDGARAWSQEGPH
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RQHQGAEHPQPHTLFVGGLPASSHSSKLPVTVGFSGCVKRLR
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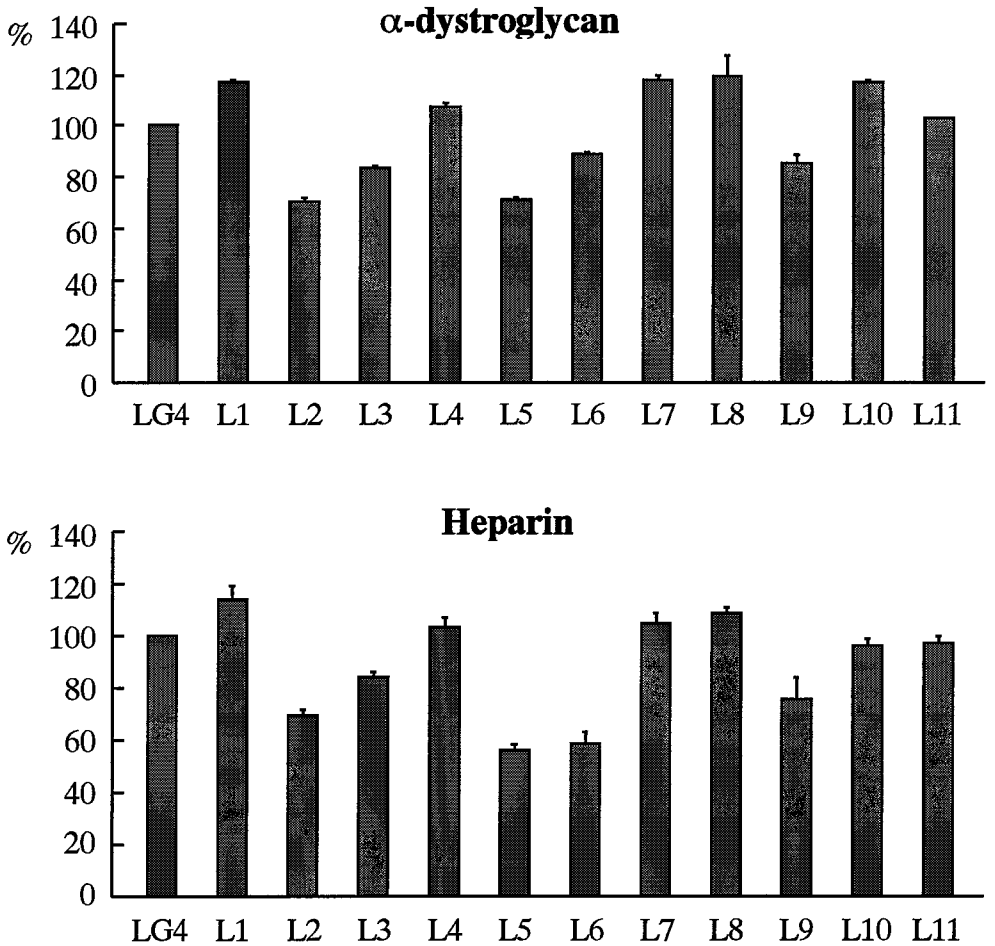
α-dystroglycan
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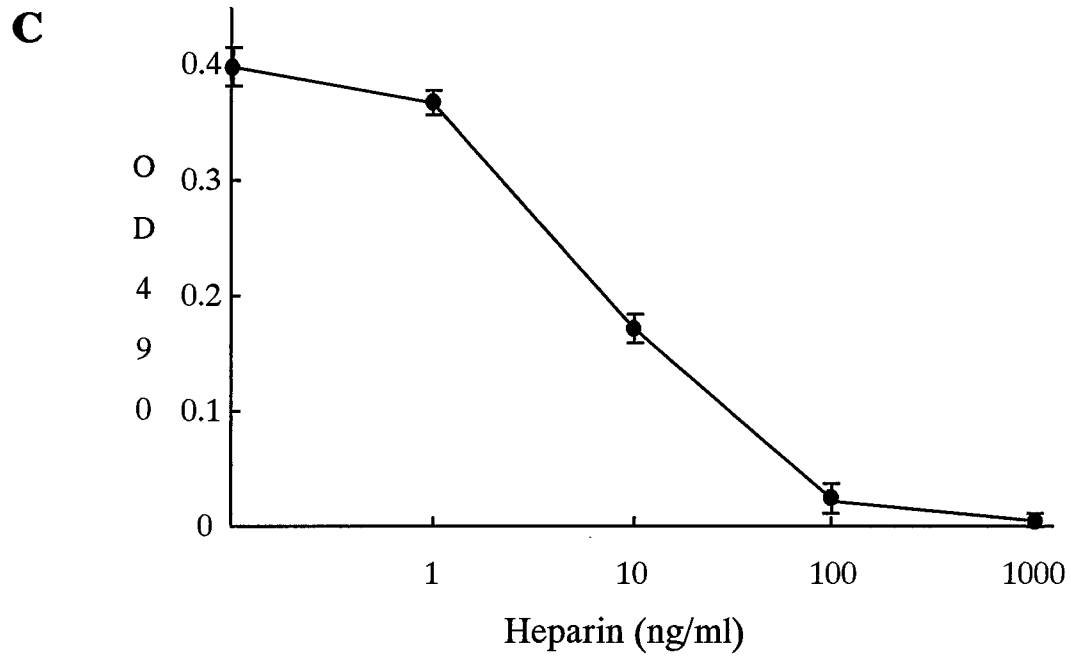


Fig. 10. α-dystroglycan- and heparin-binding activities of LG4 mutants with alanine substitutions of basic amino acid residues.

(*A*) Amino acid sequence of the LG4 module of the α5 chain. Putative β-sheet structures were deduced from the crystal structure of the LG4 module of the laminin α2 chain (Hohenester et al., 1999; Timpl et al., 2000). β-sheets are indicated by underlined regions and alphabetical letters (A to N). The Arg and Lys residues indicated by *bold* letters in the boxed regions were substituted to alanine (designated L1 through L11). (*B*) Binding activities of individual LG4 mutants for α-dystroglycan (*upper panel*) and heparin-BSA (*lower panel*). The averaged α-dystroglycan-binding activity of GST-LG4 was taken as 100%. (*C*) Inhibition of α-dystroglycan binding of GST-LG4 by heparin. GST-LG4 (10 nM) was incubated with microtiter plates coated with α-dystroglycan (5 μg/ml) in the presence of increasing concentrations of heparin for 1 h. Bound GST-LG4 was quantified with an anti-GST polyclonal antibody. Each *point* and *bar* represents the mean of triplicate assays and the standard deviation, respectively.

binding to α3β1 and α6β1 integrins. Since Asp and/or Glu residues have been shown to be the key residues within the known integrin recognition motifs (Humphries, 1990; Hynes, 1992), we focused on the Asp and Glu residues within the predicted loop regions of the LG3 module of the α5 chain, and replaced them with Ala (Fig. 11A). Due to the failure to detect integrin-binding activity of GST-LG3, we expressed rLN10ΔLG4-5, which retained the full integrin-binding activity of rLN10,

and its mutants with alanine substitutions in 293-F cells and purified them on immunoaffinity columns. The integrin binding assays with these rLN10ΔLG4-5 mutants demonstrated that alanine substitution of Asp-3198 resulted in significant reductions in the binding activities toward both α3β1 and α6β1 integrins (Fig. 11B). Other mutants exhibited only marginal decreases in the integrin-binding activities, suggesting that Asp-3198 is involved in integrin recognition by the G domain of laminin-10, although other residues within the LG3 and/or LG1-2 modules may also serve as part of integrin recognition sites.

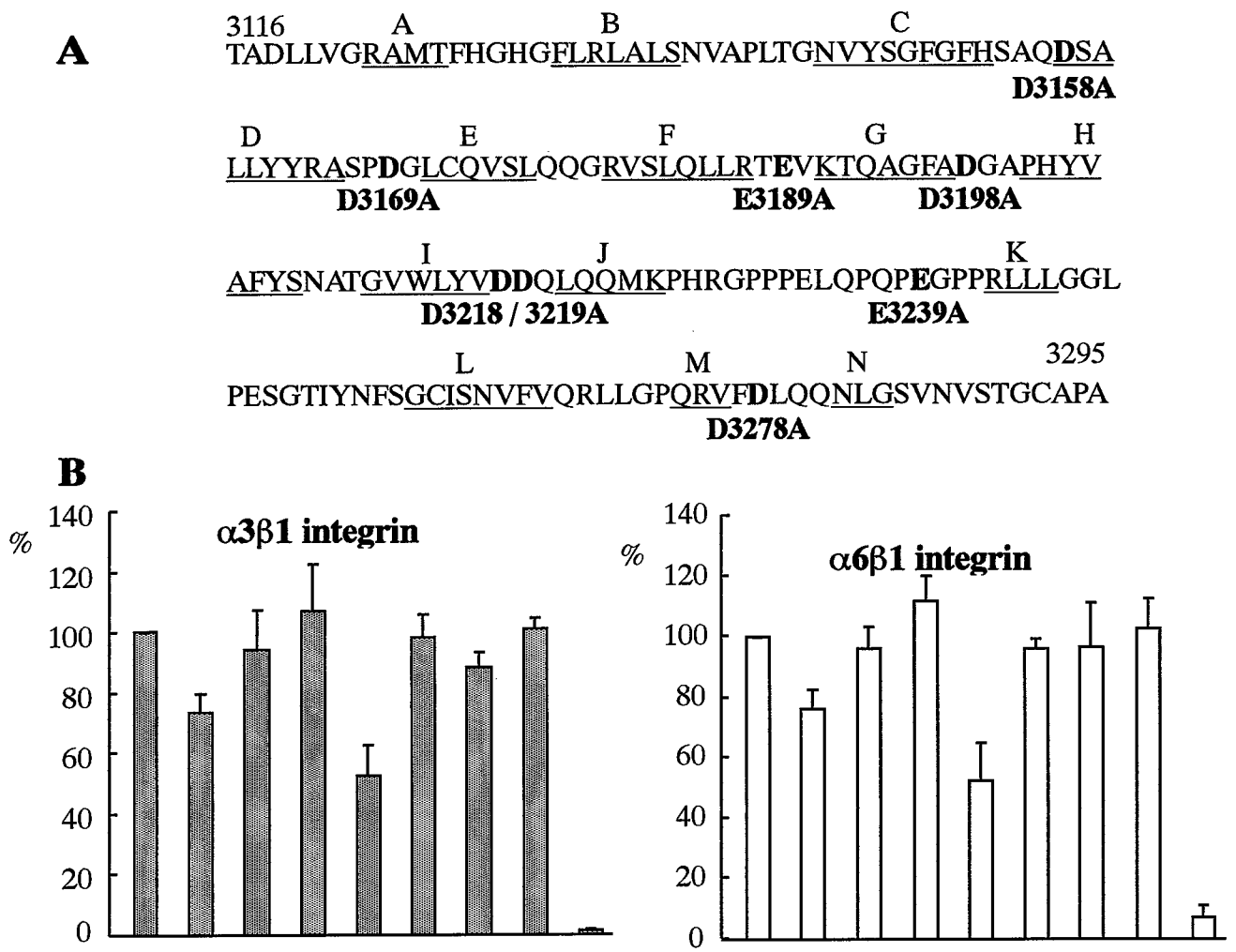


Fig. 11. Mapping the amino acid residues within the LG3 module involved in integrin binding.

(*A*) Amino acid sequence of the LG3 module of the α5 chain. Putative β-sheets are indicated by underlined regions and alphabetical letters (A to N). The Asp and Glu residues indicated by *bold* letters were substituted to alanine. (*B*) Integrin-binding activities of rLN10ΔLG4-5 and its alanine substitution mutants. Binding assays with ^{3}H-labeled phosphatidylcholine liposomes containing α3β1 (*upper panel*) or α6β1 (*lower panel*) integrins were performed as described in the Experimental Procedures. The averaged integrin-binding activity of rLN10ΔLG4-5 was taken as 100%. Each *column* and *bar* represents the mean of triplicate assays and the standard deviation, respectively.

Discussion

In the present study, we attempted to locate the binding sites for α3β1/α6β1 integrins and α-dystroglycan within the G domain of laminin-10 by producing a series of deletion and substitution mutants of rLN10 as well as individual LG modules expressed as GST or FN70K fusion proteins. We employed the FreeStyle™ 293 Expression system for the production of rLN10 to maximize the yields of the transiently expressed rLN10 and its mutants upon triple transfection of cDNAs encoding α, β and γ chains. We also improved the yields of intact rLN10 by blocking the proteolytic processing at the linker segment between the LG3 and LG4 modules with heparin included in the medium (Amano et al., 2000). Our data clearly show that LG4 harbors the binding site(s) for α-dystroglycan and heparin, while LG3 is necessary, but not sufficient, for the binding to α3β1/α6β1 integrins.

There is accumulating evidence that the LG4-5 modules are involved in laminin binding to α-dystroglycan. Thus, the LG4-5 modules of the α1 (Andac et al., 1999), α2 (Smirnov et al., 2002; Talts et al., 1999), α4 (Talts et al., 2000) and α5 chains (Yu and Talts, 2003) have been shown to bind to α-dystroglycan, although the LG1-3 modules of the α2 and α4 chains were also active in binding to α-dystroglycan to variable extents (Talts et al., 1999; Talts et al., 2000). Further dissection of LG4-5 into individual modules identified LG4 as the major α-dystroglycan-binding site within the α1 chain (Andac et al., 1999), but often failed to detect any significant activities with either the LG4 or LG5 modules of other α chains (Talts et al., 1999; Yu and Talts, 2003). Yu and Talts (2003) argued that the α-dystroglycan-binding site within the α5 chain spans at least two LG modules in a manner analogous to the interaction of the LG1-3 and LG4-5 modules of the α2 chain, based on their results that neither LG4 nor LG5 exhibited any significant binding to α-dystroglycan. Our results that the LG4 module alone, expressed as a GST or FN70K fusion protein, was active in binding to α-dystroglycan are apparently controversial to these previous observations. The importance of LG4 in the binding of laminin-10 to α-dystroglycan was further supported by the observation that rLN10 lacking LG5 retained the full binding activity to α-dystroglycan, but rLN10 lacking both LG4 and LG5 was barely active. The reason for this discrepancy remains to be clarified, although it may be possible that the α-dystroglycan binding activity of LG4 is conformation-dependent and that the putative active conformation is stabilized by LG5 when connected in tandem. The expression of LG4 as fusion proteins with GST or FN70K could mimic

the neighboring effect of LG5, allowing us to detect the α-dystroglycan-binding activity of LG4 even in the absence of LG5. Alternatively, the apparent discrepancy might reflect the specificity of the antibodies used in the α-dystroglycan binding assays of LG4 and/or LG5. Yu and Talts (Yu and Talts, 2003) used a polyclonal antibody raised against LG4-5 tandem modules, which may not be able to recognize LG4 when it is bound to α-dystroglycan, due to masking of the epitope(s) by the bound α-dystroglycan.

Binding of laminin-1 and -2 to α-dystroglycan has been shown to be strictly Ca^{2+}-dependent. The crystal structure of the LG4-5 tandem modules of the α2 chain revealed that two aspartic acid residues conserved in the LG modules of the α1 and α2 chains are involved in the Ca^{2+} binding, and therefore considered to be important in the Ca^{2+}-dependent α-dystroglycan binding (Hohenester et al., 1999; Timpl et al., 2000). However, these two aspartic acid residues do not seem to be conserved in the LG4 modules of other laminin α chains including α5 (Hohenester et al., 1999). Nevertheless, our results clearly show that binding of rLN10 to α-dystroglycan is strictly Ca^{2+}-dependent, making it likely that the Ca^{2+} binding site(s) involved in the Ca^{2+}-dependent α-dystroglycan binding of laminin-10 are different from those in laminin-1 and -2. Consistent with this possibility, binding of laminin-8 to α-dystroglycan is also Ca^{2+}-dependent (H.I., unpublished observation), although laminin-8 lacks the two aspartic acid residues equivalent to those conserved in the LG4-5 modules of the α2 chain.

The LG4 module has been shown to be the major heparin-binding region within the G domain of most laminin α chains, except for α2 (Andac et al., 1999; Nielsen et al., 2000; Talts et al., 1999; Utani et al., 2001; Yamaguchi et al., 2000; Yu and Talts, 2003). Consistent with previous observations, only the LG4 module was capable of binding to heparin among the five LG modules of the α5 chain when they were expressed as GST fusion proteins. The critical role of LG4 in heparin binding of laminin-10 was further confirmed by the absence of heparin binding activity in rLN10 lacking the LG4-5 modules. Although heparin and α-dystroglycan differ in their dependence on Ca^{2+} for binding to laminin-10, site-directed mutagenesis of the LG4 module indicated that both heparin and α-dystroglycan bind to overlapping sites in LG4. In support of this view, heparin competed with α-dystroglycan for the binding sites within LG4. Overlapping of the binding sites for heparin and α-dystroglycan has also been documented for laminin-1 and -2 (Andac et al., 1999; Hohenester et al., 1999; Talts et al., 1999). Since alanine substitution of multiple basic amino acid residues within any single stretch of the oligopeptide sequences predicted to form loops resulted in

only moderate reduction in the α-dystroglycan and heparin binding activities, it seems likely that the binding activities of LG4 towards α-dystroglycan and heparin are elicited by a cooperative interplay of multiple basic amino acid residues situated discretely over a broad range of the LG4 module, consistent with previous studies using site-directed mutagenesis of the LG4-5 modules of the α2 chain (Talts et al., 1999).

Integrin-mediated cell adhesion is the hallmark of the biological functions of laminin G domains. Many studies on the functional dissection of G domains have addressed the regions of various laminin isoforms that are responsible for the integrin-mediated cell adhesion. One approach to map the integrin-binding sites within the G domain is to express individual LG modules separately or in tandem arrays and examine their cell-adhesive activities. This approach has been successful in defining the binding sites for non-integrin-type receptors such as α-dystroglycan (Andac et al., 1999; Talts et al., 1999) and syndecans (Okamoto et al., 2003; Utani et al., 2001) as described above, but has suffered from difficulties in reproducing the full cell-adhesive activities of intact laminins characterized by their specific binding to integrins (Mizushima et al., 1997; Shang et al., 2001; Yu and Talts, 2003). Similar difficulties have also been encountered in reproducing the activities of G domains with vast arrays of oligopeptides which together cover the entire G domains (Nomizu et al., 1995; Okazaki et al., 2002). An alternative approach to circumvent these drawbacks is to produce mutant laminins with deletions or amino acid substitutions within the G domain in their trimeric configuration. This approach has been successful in narrowing down the major cell-adhesive activities to the LG1-3 modules in laminin-2 (Smirnov et al., 2002) and laminin-5 (Hirosaki et al., 2000), consistent with the previous observation that the E8 fragment of laminin-1 consisting of the α1 chain fragment containing LG1-3, but not LG4-5, exhibited a potent integrin-mediated cell-adhesive activity upon heterotrimer formation with a truncated version of the β-γ dimer (Deutzmann et al., 1990). Hirosaki et al. (Hirosaki et al., 2000) demonstrated that further deletion of LG3 from recombinant laminin-5 resulted in an almost complete loss of cell-adhesive activity, underscoring the importance of LG3 in the integrin-mediated cell-adhesive activity of laminin-5, although they did not examine the direct integrin binding of their recombinant laminin-5. Our results are consistent with their report in that the LG3 module is indispensable for reproducing the potent cell-adhesive activity of laminin-10, but provide further direct evidence that LG3 is required for the binding of laminin-10 to α3β1 and α6β1 integrins, the major adhesion receptors for laminin-10 (Nishiuchi et al., 2003). Given that cell adhesion to laminin-10 is mediated not only by

these integrins but also by other non-integrin-type receptors such as α-dystroglycan and syndecans, cell-adhesive activities per se may not be a reliable measure for defining the region(s) involved in integrin recognition by laminins.

Recently, Shang et al. (Shang et al., 2001) demonstrated that a bacterially-expressed LG3 module of rat laminin-5 was active in promoting cell adhesion and migration in an α3β1 integrin-dependent manner, and capable of directly binding to α3β1 integrin from cell lysates when immobilized on a column. The cell-adhesive activity of the LG3 module was, however, significantly lower than that of intact laminin-5, requiring 250-fold more LG3 than intact laminin-5 to attain half-maximal cell adhesion. We carefully examined the cell-adhesive as well as integrin-binding activities of the LG3 module expressed in bacteria as a GST fusion protein or in mammalian cells as a fusion protein with FN70K, but failed to detect any significant activities, except that a very weak integrin-binding activity was reproducibly detected with LG1, but not with LG3. Given that the activity detected with the rat LG3 module was significantly lower than that of intact laminin-5 and that heterotrimerization with a truncated β-γ dimer was required for integrin-binding activity of the α chain-derived fragment containing the LG1-3 modules (Deutzmann et al., 1990; Kunneken et al., 2004), the integrin-binding activity of the LG3 module of laminin-10 per se may be very low and beyond the technical limits for detection. Shang et al. (Shang et al., 2001) proposed the possibility that the LG1 and/or LG2 modules function as synergy sites for LG3 to produce fully active integrin-binding sites, as has been demonstrated for the integrin-binding domain of fibronectin, in which the III-10 module containing the RGD cell-adhesive motif needs to be connected with its preceding III-9 module to exert its full integrin-binding activity (Aota et al., 1994). The role of LG1-2 modules may possibly be more conformational, stabilizing the active conformation of LG3 when connected adjacent to LG3 and assembled with the β-γ dimer.

The importance of the LG3 module in integrin binding of rLN10 was further underscored by the significant reduction in the integrin-binding activity upon alanine substitution for Asp-3198 in LG3. Among the six Asp residues within the LG3 module that were substituted with alanine, only Asp-3198 resulted in a reduction in the integrin-binding activity of rLN10ΔLG4-5 upon alanine substitution, making it unlikely that the effect of Asp-3198 substitution was nonspecific, e.g., due to the reduced negative charge of LG3. Recently, Kariya et al. (Kariya et al., 2003) reported that the substitution of three consecutive alanines for the Lys-Arg-Asp sequence within the LG3 module of laminin-5 strongly compromised the cell-adhesive activity of recombinant laminin-5, which was

mainly dependent on α3β1 and α6β1 integrins. Despite the similarity between laminin-5 and laminin-10 in their integrin-binding specificities, i.e., both are high-affinity ligands for α3β1 and α6β1 integrins (Nishiuchi et al., 2003), none of the single amino acid residues of the Lys-Arg-Asp sequence are conserved in the corresponding region of the LG3 module of the α5 chain, making it unlikely that the Lys-Arg-Asp sequence serves as part of the common recognition sites for α3β1/α6β1 integrins. In contrast, the Asp residue corresponding to Asp-3198 in the α5 chain is conserved in the LG3 module of the α3 chain. Since the reduction in the integrin-binding activity of rLN10ΔLG4-5 by alanine substitution for Asp-3198 was still partial, it is unlikely that Asp-3198 is the sole recognition site for α3β1/α6β1 integrins, but rather comprises part of the recognition site for α3β1/α6β1 integrins, possibly with putative synergy sites within the LG1-2 modules. In this respect, it is interesting to note that the LG1 module expressed as a GST fusion protein exhibited a weak, but reproducible, binding activity to α3β1/α6β1 integrins. The putative synergy sites in the LG1 module remain to be explored.

Our data show that laminin-10 binds to integrins and α-dystroglycan through distinct LG modules within the G domain. Thus, laminin-10 may well be able to utilize both cell surface receptors simultaneously, although the linker segment between the LG3 and LG4 modules of laminin-10 and other laminin isoforms has been shown to be frequently cleaved in vivo and in vitro (Doi et al., 2002; Marinkovich et al., 1992; Talts et al., 2000), resulting in the loss of the α-dystroglycan-binding site. The physiological significance of this cleavage at the linker segment and the resulting inactivation of α-dystroglycan-binding activity remains to be elucidated, although it may be relevant to the assembly of laminin-10 into the basement membrane. There is some evidence that α-dystroglycan serves as a major cell surface receptor involved in the basement membrane assembly of laminins (Henry and Campbell, 1998). It is tempting, therefore, to speculate that when laminin-10 is secreted by the cell, it first binds to α-dystroglycan to facilitate its assembly to the basement membrane, but then subsequent cleavage between the LG3 and LG4 modules removes the α-dystroglycan-binding site, resulting in the transfer of laminin-10 to α3β1 and/or α6β1 integrins, the major laminin-10-binding integrins capable of eliciting a series of transmembrane signaling events (Gu et al., 2002; Gu et al., 2001). Although it is not clear how the cleavage between LG3 and LG4 affects the integrin-binding activity of LG3, Hirosaki et al. (Hirosaki et al., 2002) reported that the cleavage between LG3 and LG4 was associated with enhanced cell-adhesive activities of α3 chain-containing laminins. It remains to be defined whether

the processing at the linker segment between LG3 and LG4 regulates the integrin-binding activity of laminin-10.

Conclusion

To elucidate the integrin and α-dystroglycan binding sites of laminins, I produced many recombinant laminin-10 variants with C-terminal deletion or amino acid substitution within the G domain of laminin-10. I examined their binding activities to integrins, α-dystroglycan and heparin and identified LG4 module as the α-dystroglycan and heparin binding site, and analyzed the amino acid residues required for α-dystroglycan and heparin binding by site-directed mutagenesis. However I could not identify the integrin-binding site within laminin-10 or the recombinant laminin G domain fragments, which possess the integrin-binding activity.

In this chapter, I discuss the significance of α-dystroglycan and heparin binding to laminin-10 and the problems and prospects about the integrin-binding site(s) within laminins.

The structure of α-dystroglycan binding site within LG4 module

I have carried out a detailed mutational analysis of putative receptor binding sites in the laminin-10 LG4 module, which was found to be recognized by both α-dystroglycan and heparin. Through semiquantitative binding assays using a large number of mutant proteins, I have been able to identify the amino acid residues within the LG4 module that are involved in α-dystroglycan binding. When the most effective regions, i.e., L2, L3, L5, L6 and L9, are mapped on a 3D structural model of α2 LG4-5 modules (Fig. 12), they form a positively charged region on the surface of the α5 LG4 module, suggesting that they interact with adjacent sulfate groups along the sugar chain of α-dystroglycan. This feature is remarkably similar to α2 LG4-5 modules and α4 LG4 module (Hohenester et al., 1999; Wizemann et al., 2003; Yamashita et al., 2004). It is possible that these basic regions are generally conserved within laminin isoforms and act as α-dystroglycan binding site.

Previously Henry and Campbell (1998) have reported that basement membranes failed to form in dystroglycan-null embryoid bodies. They also demonstrated that anchorage of laminin-1 to the cell surface is mediated by dystroglycan, and dystroglycan is essential for basement membrane assembly. Therefore it has been accepted that dystroglycan could maintain high local concentrations of laminin-1 near the surfaces of cells and thus facilitate self-assembly of laminin-1. Since laminin-10 has the strong binding activity to α-dystroglycan, I hypothesize that dystroglycan concentrates laminin-10 on the cell surface, thereby facilitating its self-assembly.

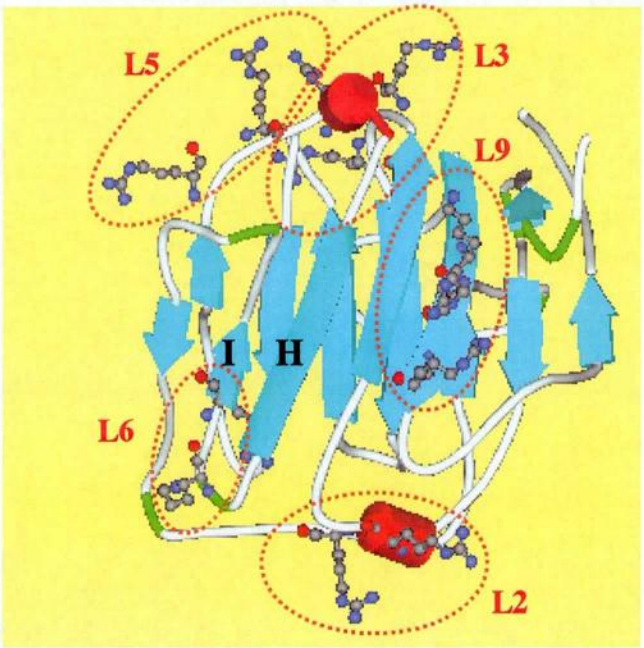


Fig. 12. The putative 3D model of α5LG4 module.

A three-dimensional model of the α5 LG4 module is generated with SwissModel (version 3.7 SP3) using the X-ray derived coordinates of the α2 LG4 module (ExPDB code1dyka) as a template. The localization of residues of the α5LG4 module involved in α-dystroglycan and heparin-binding is shown in ball-and-stick representation.

However, laminin-10 purified from the conditioned medium of various cultured cells is often proteolytically processed, losing LG4-5 modules which are required for dystroglycan-binding (Doi et al., 2002). This indicates that LG4-5 modules, the regions that bind to α-dystroglycan, were no longer associated with the parental laminin-10 molecule, suggesting that the processed laminin-10 is only recognized by integrin-type receptors, where binding is dependent in the presence of LG3 module. This assumption leads to an attractive possibility that proteases regulate laminin-10 activity through eliminating α-dystroglycan-binding activity after dystroglycan-mediated laminin-10 assembly.

The significance of heparin-binding activity within LG4 module

In the present study, I demonstrated that the LG4 module of laminin-10 (α5β1γ1) showed the strong affinity for heparin, and alanine mutagenesis of basic amino acid clusters in loops B/C (L2), C/D (L3), G/H (L5), **H/I** (L6), and L/N (L9) abolished heparin-binding, while other mutants showed no changes in heparin-binding activities. It has been reported that the LG4 module of laminin-1

(α1β1γ1) also showed the heparin-binding activity, and site-directed mutagenesis identified two basic clusters in loops F/G and **H/I** of α1LG4 as being responsible for the binding of heparin (Timpl et al., 2000). Interestingly, two heparin-binding sites of comparable strength have been demonstrated with LG1-3 and LG4-5 modules of laminin-2 (α2β1γ1). The heparin-binding site within LG4-5 modules mapped to the LG5 module, in which the lysine residues located in loops L/M and **H/I** were shown to participate in heparin-binding (Timpl et al., 2000). Taken together, it is likely that basic clusters in the H/I loop are conserved between α1, α2, and α5 chains, and make major contributions to heparin-binding. These observations emphasize the importance of the basic residues in H/I loop for heparin-binding, but do not exclude the participation of additional basic regions. Given the residual binding activity of L6 mutant, it seems that more than a single region is involved in heparin-binding.

My preliminary data show that GST-LG4 promotes cell attachment but not spreading (data not shown). Previously, Nielsen et al. (2000) also demonstrated that LG4 module of mouse laminin-10 was highly active to mediate cell attachment, and heparin inhibited the cell attachment to the LG4 module, whereas no inhibition was observed with EDTA or antibodies against the integrin β1 subunit. These results suggest that β1 integrins do not mediate cell attachment to LG4 module of laminin-10, and heparin-binding activity of the LG4 module is involved in the cell attachment activity. It is possible that cell surface heparin-like molecules are contributed to the cell attachment to LG4 module. It is interesting to note that the heparin-binding sites interact with cell surface receptors, such as syndecans. Syndecans are a family of transmembrane heparansulfate proteoglycans, and are involved in cell-extracellular matrix interactions, cell motility and focal adhesion assembly (Couchman and Woods, 1999; Rapraeger, 2000; Woods and Couchman, 2001; Yoneda and Couchman, 2003). It has been demonstrated that syndecan-1 was a receptor for the LG4 module of laminin-1 (Suzuki et al., 2003), and syndecans bound to the LG4 module of laminin-5 (α3β3γ2) and functioned as cell adhesion receptors (Okamoto et al., 2003; Utani et al., 2001). Therefore, it would be possible that the LG4 module of laminin-10 is involved in syndecans-mediated cell attachment. Moreover, I assume that the heparin-binding activity of LG4 module may assist in cell-laminin interactions, and is important for the biological activity of laminin-10.

Toward defining the integrin-binding site(s) within laminin-10

In the present study, I have demonstrated that rLN10ΔLG4-5 showed the integrin-binding activity, but rLN10ΔLG3-5 did not. These results implied that LG3 module is the integrin-binding site, though GST-LG3 and FN70K-LG3 did not exhibit the integrin-binding activity. However mutational analysis within LG3 module of rLN10 indicated that LG3 module is likely to be involved in the integrin-binding. Why did GST-LG3 and FN70K-LG3 show no integrin-binding activity?

The first possibility is that recombinantly expressed LG3 module such as GST-LG3 and FN70K-LG3 cannot fold properly when expressed alone, and therefore, is inactive in binding to integrins. Proper folding is necessary for protein activity. The second possibility is that single LG3 module is not sufficient for stable integrin-binding, and other LG modules including LG1 and LG2 would be necessary. In particular, LG1 module would be likely to play an important role in integrin-binding. Contribution of LG1 module to integrin-binding is illustrated by two lines of observations. First, GST-LG1 exhibited weak integrin-binding activity among the GST-LG modules. Second, mAb 4C7 which recognizes the α5 G domain and inhibits the integrin-binding to laminin-10 (Engvall et al., 1990; Tiger et al., 1997), seems to bind to LG1 module. I examined the binding activity of mAb 4C7 with rLN10 and its deletion mutants (Fig. 13A). A drastic decrease of binding activity of mAb 4C7 was observed in rLN10 lacking LG3-5 modules, but there remains a weak binding activity up to rLN10 lacking LG2-5 modules. These results raise the possibilities that mAb 4C7 recognizes the LG3 or LG1 module. Notably, mAb 4C7 is completely devoid of the binding activity to FN70K-LG3 (Fig. 13B). These results suggest that the epitope for mAb 4C7 may reside in the LG1 module, while LG3 module is necessary for mAb 4C7 to show full-binding activity. Since mAb 4C7 blocks the integrin-binding to laminin and GST-LG1 exhibited weak integrin-binding activity, LG1 module would be likely to contribute to integrin-binding. These results indicate that both LG1 and LG3 modules are required for complete integrin-binding. Based on these observations, it would be possible that the tandem array of LG1-3 modules functions as the integrin-binding site.

Previous report (Yu and Talts, 2003) demonstrated that LG1-3 modules of laminin-10 showed cell-adhesive activity, when expressed in 293 cells. However the cell-adhesive activity of α5 LG1-3 modules was 50-fold lower than intact laminin-10. With respect to the very low integrin-binding activity of LG1-3 modules, two possibilities are considered.

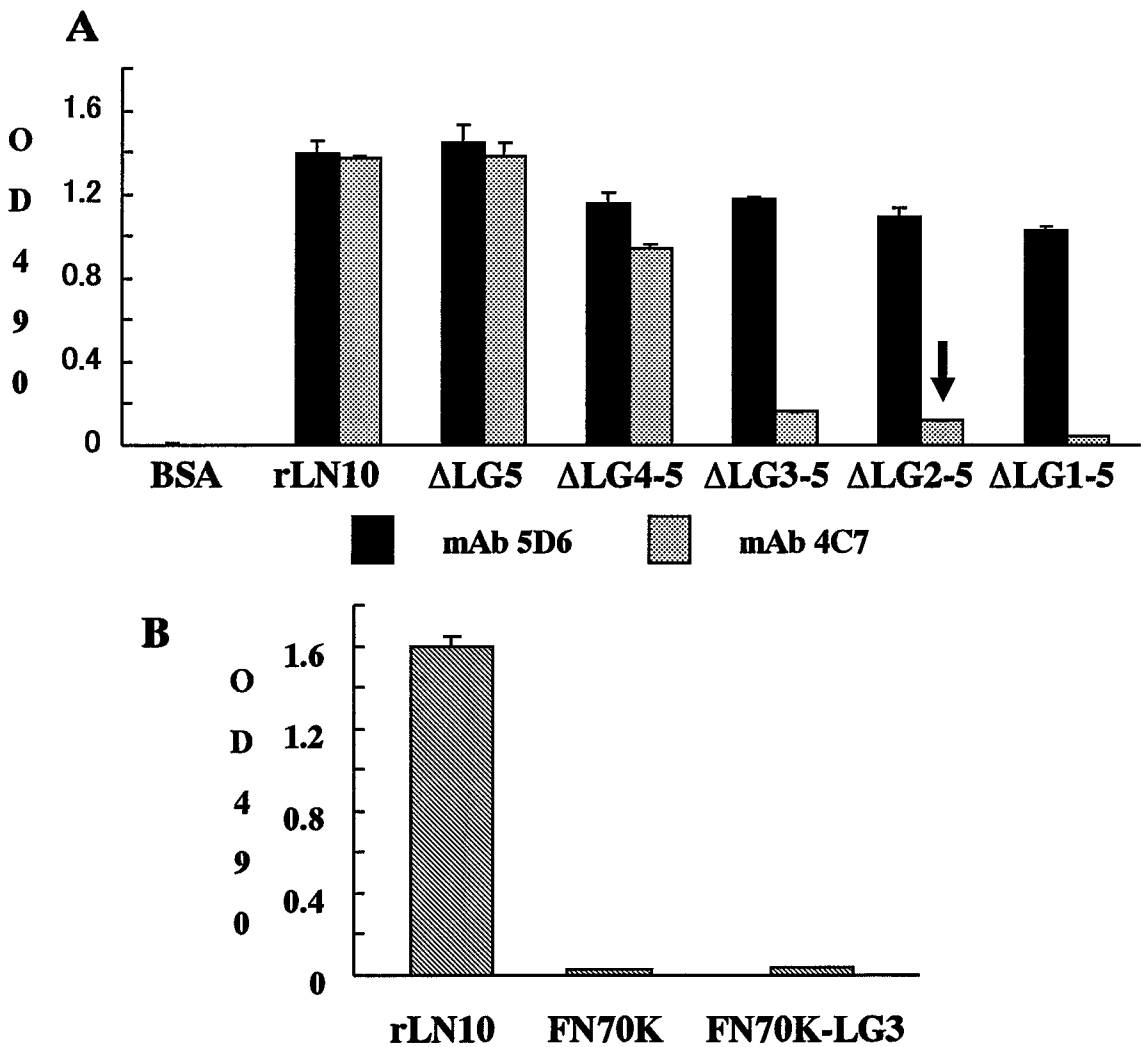


Fig. 13. Binding activity of mAb 4C7 to rLN10 deletion mutants and FN70K-LG3.

(*A*) 96-well microtiter plates were coated with 20 nM rLN10 deletion mutants, and coated proteins were detected with mAb 4C7 or 5D6. mAb 5D6 had binding activity to laminin-10 but did not inhibit the integrin-binding. Each *column* and *bar* represents the mean of triplicate assays and the standard deviation, respectively. (*B*) 96-well microtiter plates were coated with 80 nM rLN10, FN70K and FN70K-LG3, and coated proteins were detected with mAb 4C7.

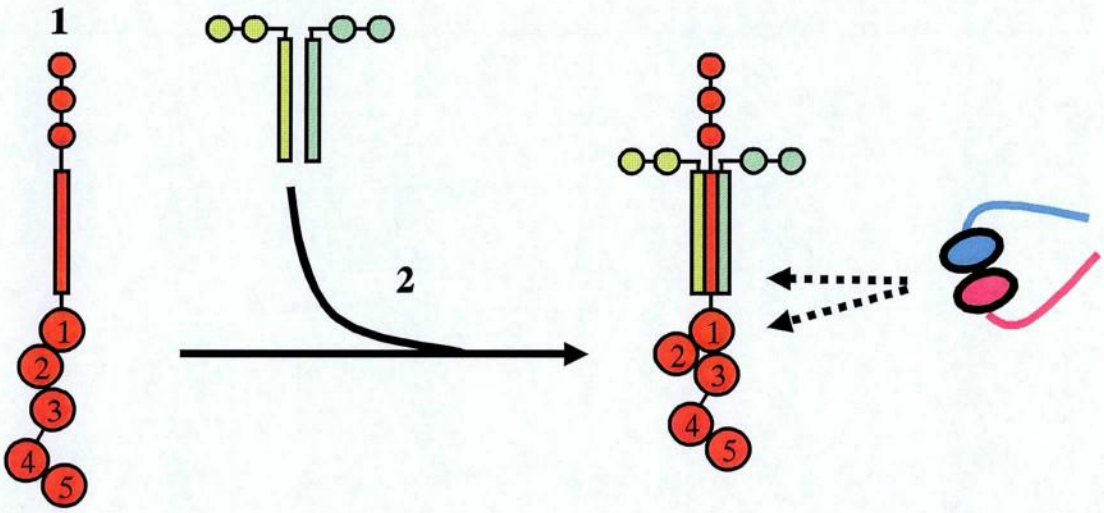
First, a tandem array of LG1-3 modules per se cannot assume the conformation active in integrin-binding. Second, the tandem array of LG1-3 modules cannot maintain proper global configuration; which is prerequisite for the high activity binding to integrins. It would be possible that global configuration of LG1 through LG3 modules is important for the integrin-binding activity. Regarding these two possibilities, it is interesting to note the two reports on the E8 fragment. E8

fragment (Fig. 2C) is produced by brief elastase digestion of laminin-1 (Edgar et al., 1984). The E8 fragment consists of C-terminal 220 residues of the β1 and γ1 chains, respectively, and of the coiled-coil domain and LG1-3 modules of the α1 chain. The E8 fragment possesses the ability to mediate cell-substratum adhesion which is dependent on β1 integrins on the cell surface, therefore, is considered to represent the major cell binding, i.e., integrin binding, domain of laminin-1 (Aumailley et al., 1987; Goodman et al., 1987; Nurcombe et al., 1989; Ziober et al., 1996). Deutzmann and co-workers (Deutzmann et al., 1990) demonstrated that separation of the E8 fragment into its α1 chain-derived fragment and the β1-γ1 dimer, resulted in a total loss of cell-adhesive activity. Interestingly, after in vitro recombination and refolding of the α1 fragment and the β1-γ1 dimer, the cell-adhesive activity was fully restored. Similarly, Kunneken and co-workers (Kunneken et al., 2004) showed very recently that a single truncated α3 chain, which corresponded to the α1 chain-derived fragment of E8 fragment and was expressed in 293 cells without the presence of β3-γ2 dimer, has no integrin-binding activity. When assembled with β3 and γ2 chains corresponding to β1 and γ1 portion of E8 fragment, however the α3 fragment regained the integrin-binding activity. These results indicated that recombinantly expressed LG1-3 modules folded properly, and heterotrimerization of the α chain with the β and γ chains within their coiled-coil domains is prerequisite for integrin-binding. These observations help explain the significant decrease of cell-adhesive and integrin-binding activity of the tandem array of LG1-3 modules, which did not associate with β and γ chains. Moreover these results indicate that formation of heterotrimerization through coiled-coil domains is indispensable for integrin-binding to laminin. I hypothesize that association of β and γ chains with α chain alters either the conformation of individual LG1-3 modules or the global configuration of the tandem array of LG1-3 modules, thereby activating the integrin-binding site(s). Alternatively, the requirement of heterotrimerization through the coiled-coil domain for integrin-binding may be explained by the direct interaction of the heterotrimeric coiled-coil region with integrins, suggesting that integrin recognizes bipartite binding sites with laminin-10, i.e., one in the G domain and the other in the coiled-coil region.

A model for the putative integrin-binding sites within laminin

Based on these considerations, I propose the following model for the β-γ dimer-dependant activation of the laminin G domain for integrin-binding. 1) LG1 module is not associated with LG3

module when the α chain is not associated with a β-γ dimer. 2) Association of the α chain with the β-γ induces global conformational change within the G domain, leading to juxtaposition of LG1 and LG3 modules which in turn allows either LG1 or LG3 module to assume an active conformation for integrin-binding. The binding sites for integrins may reside in either LG1 or LG3 module or in both modules. Alternatively, coiled-coil domain may well serve as one of integrin-binding sites.



For the past 20 years, researchers tried to map the integrin-binding site within laminins. Their studies are likely to reveal further complexities of laminin-integrin interactions. I think that determination of the crystal structure of the entire G domain is essential for better understanding of the comprehensive structure-function relationships of laminins.

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