Published in final edited form as:

Biochemistry. 2009 June 16; 48(23): 5375–5381. doi:10.1021/bi900542u.

Chain-Specific Heparin-Binding Sequences in the Laminin α Chain LG45 Modules

Kentaro Hozumi¹, Nobuharu Suzuki^{1,2}, Yoshihiko Uchiyama¹, Fumihiko Katagiri¹, Yamato Kikkawa¹, and Motoyoshi Nomizu^{1,*}

¹Laboratory of Clinical Biochemistry, Tokyo University of Pharmacy and Life Sciences, Hachioji, Tokyo 192-0392, Japan

²Laboratory of Cell and Developmental Biology, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, Maryland 20892-4370

Abstract

Laminin α chains contain five tandem globular modules (LG1-5) at the C-terminus. Here, we focused on the LG45 module, which play a critical biological role via binding to heparin/heparan sulfate, and examined their chain-specific heparin-binding affinity. The relative heparin-binding affinity of recombinant laminin α chain LG45 proteins was $\alpha 5 > \alpha 4 > \alpha 1 > \alpha 2$ and $\alpha 3$. The $\alpha 5$ chain LG45 module also promoted the strongest cell attachment. We screened heparin-binding sequences using the recombinant $\alpha 5$ LG45 protein and 43 synthetic peptides. Four peptides, A5G71 (GPLPSYLQFVGI) ($IC_{50} = 91.8 \mu M$), A5G77 (LVLFLNHGHFVA) ($IC_{50} = 7.0 \mu M$), A5G81 (AGQWHRVSVRWG) ($IC_{50} = 5.9 \mu M$), and A5G94 (KMPYVSLELEMR) ($IC_{50} = 0.84 \mu M$), inhibited the heparin-binding of rec- $\alpha 5$ LG45. Additionally, the same four peptides exhibited dose-dependent heparin-binding activity in a solid-phase assay. We found that the $\alpha 5$ chain LG45 module contains four heparin-binding sequences and this number is higher than that of the other LG45 modules ($\alpha 2$ and $\alpha 3$, 1 sequence; $\alpha 1$ and $\alpha 4$, 2 sequences). The data suggest that the active sequences identified from the synthetic peptide screening contribute to the heparin-binding activity of the LG45 module. Most of the heparin-binding sequences in the LG45 modules are located in the N-terminal regions of the LG4 module within the loop regions in the proteins. The data suggest that the N-terminal loop regions of the LG4 module are mainly involved in the heparin/heparan sulfate-mediated biological functions.

Keywords

laminin; basement membrane; heparin; peptide; chain specificity

Laminins are heterotrimeric basement membrane proteins that have multiple biological functions through interactions with other matrix molecules and cell surface receptors (1,2). Laminins consist of α , β , and γ chains that assemble into a triple-stranded coiled-coil structure. At least 16 isoforms of laminins, consisting of five α , three β , and three γ chains, have been identified (2). Laminins have diverse biological activities, including promotion of cell adhesion, cell migration, neurite outgrowth, and tumor metastasis. Five laminin α chains are tissue-specifically and/or developmental stage-specifically expressed. For example, the $\alpha 1$ chain is expressed in the blastocyst neuroectodermally derived tissues and in developing kidney in the early embryo (3,4). The $\alpha 2$ chain is expressed in both skeletal and cardiac muscle,

*To whom correspondence should be addressed: Motoyoshi Nomizu, Ph.D., Laboratory of Clinical Biochemistry, School of Pharmacy, Tokyo University of Pharmacy and Life Sciences, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, JAPAN. Phone/Fax: 81-426-76-5662, E-mail: nomizu@toyaku.ac.jp.

peripheral nerve, brain, and capillaries (5). The $\alpha 3$ chain is mainly localized in skin and in other epithelia (6,7). The $\alpha 4$ chain is detected in the microvasculature and in smooth muscle (8,9) and the $\alpha 5$ chain is expressed in multiple tissues during development, in adult microvasculature, and in various epithelia (10,11). The diversity of the α chain contributes critically to laminin isoform-specific functions (2).

The C-terminal globular domain (G domain) of the laminin α chains consists of five laminin G domain-like modules (LG1—5) and plays a critical role in the biological functions. The G domains are cleaved by endogenous proteolytic processing (12). The $\alpha 2$ chain G domain is processed in the LG3 module by the furin-type protease (13). The cleavage in the $\alpha 2$ chain LG3 module is required for clustering of acetylcholine receptors, contributing to neuromuscular junction formation in concert with agrin (13). The linker regions between the LG3 and LG4 modules of the $\alpha 3$ and $\alpha 4$ chains are cleaved, causing the C-terminal LG45 fragments to separate from the laminins. Plasmin promotes the cleavage of the $\alpha 3$ chain and releases the LG45 fragment (14). At wound edges in the skin, the leading keratinocytes migrate due to unprocessed laminin-332 containing the $\alpha 3$ chain LG1—5 module. In contrast, quiescent keratinocytes form hemidesmosomes by interaction of the processed laminin-332 releasing the LG45 module via integrins $\alpha 6$ (34) and $\alpha 3$ (31) (15,16). An anti-laminin $\alpha 4$ chain LG45 module antibody cannot detect the LG45 module in heart tissue and has been used to demonstrate that a proteolytic LG45 fragment is released from the heart tissue (17). The $\alpha 1$ chain LG45 module exists as a fragment in the ectoplacental cone of the early embryo (18). Taken together, proteolytic processing of the G domain regulates laminin functions in a cell- and tissue-type manner. Further, most of the α chains are cleaved between LG3 and LG4 in vivo, thus allowing the N-terminal portion, LG1—3 module, to mainly interact with integrins. The C-terminal portion, LG45 module, is mainly involved in heparin binding-related biological functions, including syndecan-mediated adhesion and migration.

We have systematically screened biologically active sequences in laminin molecules using recombinant proteins and a large set of synthetic peptides (19–23). We identified various heparin-binding sequences in the LG45 module, including the $\alpha 1$ chain, AG73 (RKRLQVQLSIRT, mouse laminin $\alpha 1$ chain residues 2719—2730) and AG75 (GLIYYVAHQNM, mouse laminin $\alpha 1$ chain residues 2735—2746) (24). AG73 promotes various biological activities and binds syndecan-1, a membrane-associated proteoglycan (19, 25–28). In the 2 chain, A2G78 was found to bind heparin and α -dystroglycan (N. Suzuki et al., manuscript submitted for publication). In the $\alpha 3$ chain, A3G75 (KNSFMALYLSKG, human laminin $\alpha 3$ chain residues 1411—1422) was identified as a heparin-binding sequence that promoted syndecan-2- and -4-mediated cell attachment and neurite outgrowth (29–31). In the $\alpha 4$ chain, A4G82 (TLFLAHGRLVFM, mouse laminin $\alpha 4$ chain residues 1514—1525) exhibited heparin- and syndecan-4-binding and cell attachment activity (32). In the $\alpha 5$ chain G domain, we identified several cell attachment sequences, including A5G77 (LVLFLNHGHFVA, mouse laminin $\alpha 5$ chain residues 3307—3318) and A5G81 (AGQWHRVSVRWG, mouse laminin $\alpha 5$ chain residues 3337—3348) (33,34). A5G77 inhibits epithelial branching morphogenesis of mouse submandibular gland ex vivo (35). A5G81 promotes syndecan-4-mediated cell attachment and induces tyrosine phosphorylation of focal adhesion kinase (33). To date, the heparin-binding sequences of the laminin $\alpha 5$ chain LG45 module have not been fully characterized.

Here, we evaluated heparin-binding of the LG45 modules of $\alpha 1$ — $\alpha 5$ chains using five recombinant proteins and found that a $\alpha 5$ chain LG45 recombinant protein had the strongest heparin affinity. We characterized heparin-binding sequences on the $\alpha 5$ chain LG45 module by a systematic peptide screening using rec- $\alpha 5$ LG45 and 43 synthetic peptides. We identified four heparin-binding sequences, indicating that the $\alpha 5$ chain LG45 module contains more

heparin-binding sequences than the other LG45 modules. The heparin-binding sequences may be important in chain-specific biological activities.

Experimental Procedures

Preparation of Recombinant Laminin α Chain LG45 Proteins

The recombinant proteins of the mouse laminin $\alpha 1$ (24), $\alpha 2$ (Suzuki et al., manuscript submitted for publication), laminin $\alpha 3$ (36), $\alpha 4$ (32), or $\alpha 5$ chain LG45 (33) modules with the human IgG Fc portion were prepared as previously described. The PCR primers with restriction enzyme sites (forward primers, *Avr* II; reverse primers, *Avr* II for $\alpha 1$ and $\alpha 4$ and *Bam* HI for $\alpha 2$, $\alpha 3$, and $\alpha 5$) were as follows: nucleotides 8158–9297 of the laminin $\alpha 1$ chain,

5'-GAGCCTAGGGCTACCACAGCCTGAACTG-3' (forward) and

5'-GAGCCTAGGCCCTCAGGCCCGGGCAGGAATG-3' (reverse); nucleotides 8158–9372 of the laminin $\alpha 2$ chain

5'-GAGCCTAGGGACCATGGTGCATGGCCCTTG-3' (forward) and

5'-GAGGGATCCCCGTTCCAGGGCCTTGGCAAATTAACC-3' (reverse); nucleotides 4099–5196 of the laminin $\alpha 3$ chain

5'-GAGCCTAGGGTGGCAAGATGGGAAGTCC-3' (forward) and

5'-GAGGGATCCCCGTGGTCAGGGCAGCCATTTCAG-3' (reverse); nucleotides 4538–5656 of the laminin $\alpha 4$ chain, 5'-

GAGCCTAGGGGCTCCAAGAGATTCCAC-3' (forward) and 5'-

GAGCCTAGGCCGGCTGTGGGACAGGAGTTGATG-3' (reverse); and nucleotides 9703–10905 of the laminin $\alpha 5$ chain

5'-GAGCCAGGGCAACCCAGCCAGGACCTTG-3' (forward) and

5'-GAGGGATCCCCATGCCAAAGTAGCGGGGAG-3' (reverse). The recombinant α chain LG45 modules (rec- $\alpha 1$ LG45 to rec- $\alpha 5$ LG45) were expressed in 293T cells by transfection using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). These proteins were purified from the conditioned medium using a Protein A-Sepharose column (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) and a heparin-Sepharose column (GE Healthcare Bio-Sciences Corp.). The eluted fraction was dialyzed with 20 mM Tris-HCl (pH 7.5) containing 150 mM NaCl. Protein concentrations were determined with the BCA assay kit (Thermo Fisher Scientific Inc., Rockford, IL).

Synthetic Peptides

All peptides were synthesized manually using the 9-fluorenylmethoxycarbonyl (Fmoc)-based solid-phase strategy and prepared in the C-terminal amide form as previously described (33). Amino acid derivatives and resins were purchased from Novabiochem (La Jolla, CA). The respective amino acids were condensed manually in a stepwise manner using 4-(2',4'-dimethoxyphenyl)-Fmoc-aminomethyl)-phenoxy resin. Dimethylformamide (DMF) was used during the synthesis as a solvent. For condensation, diisopropylcarbodiimide/*N*-hydroxybenzotriazole was employed, and for deprotection of *N*^a-Fmoc groups, 20% piperidine in DMF was employed. The resulting protected peptide resins were deprotected and cleaved from the resin using trifluoroacetic acid/thioanisole/*m*-cresol/ethanedithiol/H₂O (80:5:5:5:5, v/v) at 20 °C for 3 h. The crude peptides were precipitated and washed with diethyl ether and then purified by reverse-phase high-performance liquid chromatography (HPLC) using a C18 column with water/acetonitrile gradient containing 0.1% trifluoroacetic acid. The purity and identity of the peptides were confirmed by an analytical HPLC and by an electrospray

ionization mass spectrometer at the Central Analysis Center of the Tokyo University of Pharmacy and Life Sciences.

Effect of NaCl on the Heparin-Binding to Various α Chain LG45 Recombinant Proteins

The effect of NaCl on heparin-binding to the five LG45 recombinant proteins (rec-LG45) were examined. rec-LG45s (5 μ g) and heparin-Sepharose (1 mg) were mixed in 40 μ L of 10 mM Tris-HCl (pH7.4) and incubated for 1 h. The beads were pelleted by centrifugation and washed twice with binding buffer. The proteins bound to heparin-Sepharose were eluted with 40 μ L of binding buffer, including 100—800 mM NaCl by stepwise elution. Each eluate was analyzed by Western blotting (8% SDS—PAGE under reducing conditions) using biotinylated goat anti-human IgG Fc (1:1000 dilution) and a streptavidin-conjugated horseradish peroxidase (SA—HRP) (Sigma, St. Louis, MO) (1:2000 dilution) using an ECL kit (GE Healthcare Bio-Sciences Corp.).

Peptide inhibition of Heparin-Binding to rec- α 5LG45

The effect of peptides on the heparin-binding of rec- α 5LG45 was tested using heparin-Sepharose beads as previously described (24) with some modifications. The rec- α 5LG45 protein (3 μ g), heparin-Sepharose beads (1 mg, GE Healthcare Bio-Sciences Corp.), and peptide (20 μ g) were mixed in 70 μ L of 10 mM Tris-HCl (pH 7.4), containing 100 mM NaCl (binding buffer). After a 1 h incubation, the beads were pelleted by centrifugation. The supernate was removed, and the beads were washed twice with the binding buffer. The rec- α 5LG45 protein bound to the beads was extracted with SDS—PAGE sample buffer, analyzed by 8% SDS-PAGE under reducing conditions, and detected by western blotting using biotinylated goat anti-human IgG Fc (Jackson ImmunoResearch Laboratories, West Grove, PA) (1:1000 dilution) and a SA—HRP (1:2000 dilution) using an ECL kit.

Solid-Phase Heparin-Binding Assay Using Peptide-Coated Plates

A heparin-binding assay with peptide-coated plates was conducted using biotinylated heparin (Celsus Laboratories Inc., Cincinnati, OH) as previously described (N. Suzuki et al., manuscript submitted for publication) with some modifications. Various amounts of peptides in Milli-Q water (50 μ L) were coated onto 96-well ELISA plates (AGC Techno Glass, Chiba, Japan) and dried overnight at room temperature. The wells were washed with 0.05% Tween 20 in PBS (washing buffer) and then blocked with 3% bovine serum albumin (BSA) (Sigma) in PBS at room temperature for 2 h. After the sample had been washed, 10 ng of biotinylated heparin was added to the wells and incubated at 37 °C for 1 h. Then, 10 ng of streptavidin-conjugated horseradish peroxidase (Sigma) was added to the wells and incubated at 37 °C for 1 h. The bound protein was detected by TMB solution (Sigma) at 450 nm absorbance using a Model 550 Microplate Reader (Bio-Rad Laboratories, Hercules, CA).

Cell Attachment Assay

Ninety-six-well plates (Nalge Nunc, Rochester, NY) were coated with five rec-LG45s (2 μ g/well) and prepared as previously described(33) with some modifications. HT-1080 human fibrosarcoma cells (3×10^3 cells/well) were added to the wells and incubated for 120 min at 37 °C in a humidified atmosphere of 5% CO₂. After being washed with prewarmed PBS containing calcium and magnesium, cells were fixed and stained with a 0.2% crystal violet aqueous solution in 20% methanol for 10 min at room temperature. After the wells were washed with distilled water (two times), the attached cells were counted in three different fields using a BZ-8000 microscope (Keyence, Osaka, Japan).

Results

Heparin-Binding Affinity of Laminin α Chain LG45 Proteins

We prepared five recombinant laminin α chain LG45 proteins (rec- α 1LG45—rec- α 5LG45) using the 293T cells as described previously (24,31,32) (N. Suzuki et al., manuscript submitted for publication) (Figure 1A). The proteins correspond to the mouse laminin sequences except for the α 3 chain. Since mouse rec- α 3LG45 is rapidly cleaved during the expression and purification process, we used human rec- α 3LG45 instead of mouse protein in this study (84% homology vs mouse).

We examined the heparin-binding affinity of the recombinant proteins using heparin-Sepharose beads (Figure 1B). The proteins were mixed with heparin-Sepharose beads and washed with various concentrations of NaCl (from 100 to 800 mM). Then, the eluted protein was analyzed by SDS—PAGE (Figure 1B). The proteins were eluted with various NaCl concentrations: 600 mM for rec- α 5LG45, 400 mM for rec- α 4LG45, 300 mM for rec- α 1LG45, and 200 mM rec- α 2LG45. rec- α 3LG45 exhibited a broad elution pattern (from 100 to 800 mM NaCl). These results indicate that rec- α 5LG45 has the highest heparin-binding affinity.

Effect of Peptides on the Heparin-Binding of the rec- α 5LG45 Protein

Previously, we identified heparin-binding sequences in the LG45 module of the laminin α 1— α 4 chains using a systematic peptide screening method with a recombinant protein and synthetic peptides (31,32,37) (N. Suzuki et al., manuscript submitted for publication). Here, we focused on the α 5 chain LG45 module and screened heparin-binding sequences using the systematic peptide screening method with rec- α 5LG45 and synthetic peptides. Forty-six overlapping peptides covering the laminin α 5 chain LG45 module were prepared (Figure 2). The effect of the 43 soluble peptides on the interaction between rec- α 5LG45 and the heparin-Sepharose beads was evaluated (Figure 2). Four peptides, A5G71 (GPLPSYLQFVGI), A5G77 (LVLFNLNHHGFVA), A5G81 (AGQWHRVSVRWG), and A5G94 (KMPYVSLELEMR), significantly inhibited the heparin-binding of rec- α 5LG45, while rest of the soluble peptides did not (Figure 2). Scrambled peptides of A5G71, A5G77, A5G81, and A5G94, A5G71S (YLGQPLVPSIGF), A5G77S (FLHLVNFAFGHLV), A5G81S (WARGHQVRVGWS), and A5G94S (MSPYLEKLMERV), respectively, were also prepared and found to be inactive (Figure 2). These results indicate that the inhibitory effect of A5G71, A5G77, A5G81, and A5G94 on the heparin-binding of rec- α 5LG45 is sequence-specific.

Next, we examined their inhibitory effects on the heparin-binding of rec- α 5LG45 using various amounts of the four active peptides (Figure 3). These four active peptides exhibited a dose-dependent inhibitory effect with the following IC_{50} : 91.8 μ M (A5G71), 7.0 μ M (A5G77), 5.9 μ M (A5G81), and 0.84 μ M (A5G94) (Figure 3). These results suggest that the four sequences are important for heparin-binding in the α 5 chain LG45 module.

Heparin-Binding Activity of Peptides

We next examined the direct heparin-binding activity of the peptides using peptide-coated plates. In the solid-phase binding assay, biotinylated heparin was added to the peptide-coated plates. A5G77, A5G71, and A5G94 exhibited strong heparin-binding activity in a dose-dependent manner (Figure 4). A5G81 exhibited weak heparin-binding activity (Figure 4). In contrast, the scrambled peptides, A5G71S, A5G77S, A5G81S, and A5G94S, did not exhibit activity in this assay (Figure 4). These results further suggest that the heparin-binding activity of the peptides is sequence-specific.

We identified four heparin-binding sequences in the α 5 chain LG45 module by systematic peptide screening using a recombinant protein and a set of synthetic peptides. Previously, we

identified heparin-binding sequences in the LG45 modules of the $\alpha 1$ — $\alpha 4$ chains using similar methods. The $\alpha 5$ chain LG45 module contains a higher number of heparin-binding sequences compared with that of the other LG45 modules ($\alpha 2$ and $\alpha 3$ chains, one sequence; $\alpha 1$ and $\alpha 4$ chains, two sequences). To evaluate the relation between the number of heparin-binding sequences and biological function, we examined the cell attachment activity of the recombinant LG45 proteins.

Cell Attachment Activity of the Proteins

The cell attachment activity of the five recombinant proteins was examined using HT-1080 human fibrosarcoma cells. The cells were added to the protein-coated plates, and after a 1 h incubation, attached cells were counted (Figure 5). rec- $\alpha 1$ LG45 and rec- $\alpha 5$ LG45 promoted strong cell attachment, and rec- $\alpha 2$ LG45, rec- $\alpha 3$ LG45, and rec- $\alpha 4$ LG45 exhibited weak activity. These results suggest that heparin-binding activity contributes to cell binding and that the number of heparin-binding sequences correlates with biological function.

Discussion

Here, we focused on the functionally important laminin α chain LG45 modules and determined their chain-specific heparin-binding activity using recombinant proteins. The relative heparin-binding affinities of the proteins were as follows: rec- $\alpha 5$ LG45 > rec- $\alpha 4$ LG45 > rec- $\alpha 1$ LG45 > rec- $\alpha 2$ LG45 and rec- $\alpha 3$ LG45. Previously, we identified two heparin-binding peptides from the $\alpha 1$ LG45 and $\alpha 4$ LG45 modules and one peptide from the $\alpha 2$ LG45 and $\alpha 3$ LG45 modules (31,32,37) (N. Suzuki et al., manuscript submitted for publication). Here, four heparin-binding peptides were identified from the $\alpha 5$ chain LG45 module and are likely responsible for the strongest heparin-binding affinity among the laminin LG45 modules. The rec- $\alpha 1$ LG45, which contains only one heparin-binding sequence, promoted strong cell attachment similar to that of rec- $\alpha 5$ LG45. We previously identified two active peptides from the mouse laminin $\alpha 1$ LG4 module, AG73 for heparin/heparan sulfate binding and EF-1 (DYATLQLQEGR LHFMFDLG, mouse laminin $\alpha 1$ chain residues 2747–2765) for integrin $\alpha 2 \beta 1$ binding (24,37). The AG73 and EF-1 sequences cooperate and induce cell adhesion through the synergetic interaction of syndecan and integrin $\alpha 2 \beta 1$ (38). These results suggest that the strong cell attachment activity of rec- $\alpha 1$ LG45 was promoted by not only heparin-binding but also integrin $\alpha 2 \beta 1$ binding. Thus, the $\alpha 5$ chain LG45 module contains more heparin-binding sequence than the other α chain LG45 modules and is important in heparan sulfate proteoglycan-mediated biological functions.

Heparin-binding sequences in the α chain LG45 modules identified here and previously are listed (Figure 6). Most of the peptides contained basic amino acids, Arg, Lys, and His, except for A5G71. The basic amino acids are important for binding to acidic groups in the heparin. Additionally, most of the peptides contained aromatic amino acids, Tyr, Phe, Trp, and His, except for AG73, and β -branched amino acids, Ile and Val, except for A3G75. These hydrophobic amino acid groups, aromatic and β -branched amino acids, may contribute to the inter- or intramolecule interactions of the peptide and may provide the basic amino acid cluster for heparin binding. At this stage, no clear consensus sequences were observed. With these data taken together, we conclude that ionic, hydrophobic, and electrostatic interactions may be important for the heparin-binding activity of peptides.

We aligned amino acid sequences of the α chain LG45 modules on the basis of the previous report (12,39) and identified the locations of the 10 heparin-binding sequences (Figure 7). Three heparin-binding sequences are localized in the N-terminal region of laminin $\alpha 5$ chain LG4 module (Figure 7). The heparin-binding sequences in the $\alpha 1$ — $\alpha 4$ chain LG4 modules are also located in the N-terminal region (Figure 7). The N-terminal part of the LG4 module may play a critical role in the interaction with heparin/heparan sulfate proteoglycans. The different localization of these sites may provide a rationale for chain-specific biological function and/

or for the cell- and tissue-type specific activity. These heparin-binding peptides are useful for elucidating the mechanism of the laminin chain-specific biological activities.

The LG modules consist of a 14-stranded (β -sheet (A—N) sandwich structure (39) (Figure 7). The A5G77 as well as A3G75 and A4G82 are located in the connecting loop regions between β -strands and exposed on the protein surface in the LG4 module as described previously (12). A5G71 and A5G81 are also in the loop regions of the B and C strands and the G and H strands, respectively. The A5G94 sequence is a homologous region of AG73, the strongest heparin-binding peptide (24), and is in the loop regions of the B and C strands. All the heparin-binding peptides previously identified in the other LG4 modules are also located in the loop regions. The loop regions in the LG modules may be located at the molecular surface and interact with heparin/heparan sulfate proteoglycans. We previously described that AG73, A3G75, and A4G82 interact with syndecans, membrane-associated proteoglycans (26,31,32). A5G71, A5G77, A5G81, and A5G94 may interact with syndecans, but this has not been demonstrated. Previously, we cyclized biologically active peptides in loop regions to examine the structural requirements (37,40,41). The cyclic peptides enhanced the biological activity, suggesting that the loop structure of the peptides is important for the biological activities. Cyclic peptides of the $\alpha 5$ chain heparin-binding peptides have the potential to enhance the activity.

In this paper, we identified four heparin-binding sequences in the laminin $\alpha 5$ chain LG45 module. These heparin-binding peptides are useful in evaluating the biological functions of the laminin $\alpha 5$ chain. We also demonstrated that the laminin $\alpha 5$ chain LG45 module has the highest heparin-binding affinity compared with that of the other α chain LG45 modules. The laminin $\alpha 5$ chain may interact with heparin and/or heparan sulfate in vivo. These heparin-binding sequences as well as differences in the heparin-binding affinity of LG45 modules may contribute and regulate the chain-specific biological activities of laminins.

Acknowledgments

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Cultures, Sports, Science and Technology of Japan (17390024, 17014081, and 21750174).

REFERENCES

1. Colognato H, Yurchenco PD. Form and function: the laminin family of heterotrimers. *Dev Dyn* 2000;218:213–234. [PubMed: 10842354]
2. Miner JH, Yurchenco PD. Laminin functions in tissue morphogenesis. *Annu Rev Cell Dev Biol* 2004;20:255–284. [PubMed: 15473841]
3. Miner JH, Li C, Mudd JL, Go G, Sutherland AE. Compositional and structural requirements for laminin and basement membranes during mouse embryo implantation and gastrulation. *Development* 2004;131:2247–2256. [PubMed: 15102706]
4. Sasaki T, Giltay R, Talts U, Timpl R, Talts JF. Expression and distribution of laminin alpha1 and alpha2 chains in embryonic and adult mouse tissues: an immunochemical approach. *Exp Cell Res* 2002;275:185–199. [PubMed: 11969289]
5. Patton BL. Laminins of the neuromuscular system. *Microsc Res Tech* 2000;51:247–261. [PubMed: 11054875]
6. Fleischmajer R, Kuroda K, Utani A, Douglas MacDonald E, Perlish JS, Arikawa-Hirasawa E, Sekiguchi K, Sanzen N, Timpl R, Yamada Y. Differential expression of laminin alpha chains during proliferative and differentiation stages in a model for skin morphogenesis. *Matrix Biol* 2000;19:637–647. [PubMed: 11102753]
7. Hamill KJ, McLean WH. The alpha-3 polypeptide chain of laminin 5: insight into wound healing responses from the study of genodermatoses. *Clin Exp Dermatol* 2005;30:398–404. [PubMed: 15953081]

8. Hallmann R, Horn N, Selg M, Wendler O, Pausch F, Sorokin LM. Expression and function of laminins in the embryonic and mature vasculature. *Physiol Rev* 2005;85:979–1000. [PubMed: 15987800]
9. Petajaniemi N, Korhonen M, Korttesmaa J, Tryggvason K, Sekiguchi K, Fujiwara H, Sorokin L, Thornell LE, Wondimu Z, Assefa D, Patarroyo M, Virtanen I. Localization of laminin alpha4-chain in developing and adult human tissues. *J Histochem Cytochem* 2002;50:1113–1130. [PubMed: 12133914]
10. Kikkawa Y, Miner JH. Molecular dissection of laminin alpha 5 in vivo reveals separable domain-specific roles in embryonic development and kidney function. *Dev Biol* 2006;296:265–277. [PubMed: 16750824]
11. Miner JH, Cunningham J, Sanes JR. Roles for laminin in embryogenesis: exencephaly, syndactyly, and placentopathy in mice lacking the laminin alpha5 chain. *J Cell Biol* 1998;143:1713–1723. [PubMed: 9852162]
12. Timpl R, Tisi D, Talts JF, Andac Z, Sasaki T, Hohenester E. Structure and function of laminin LG modules. *Matrix Biol* 2000;19:309–317. [PubMed: 10963991]
13. Smirnov SP, McDearmon EL, Li S, Ervasti JM, Tryggvason K, Yurchenco PD. Contributions of the LG modules and furin processing to laminin-2 functions. *J Biol Chem* 2002;277:18928–18937. [PubMed: 11886875]
14. Goldfinger LE, Jiang L, Hopkinson SB, Stack MS, Jones JC. Spatial regulation and activity modulation of plasmin by high affinity binding to the G domain of the alpha 3 subunit of laminin-5. *J Biol Chem* 2000;275:34887–34893. [PubMed: 10956663]
15. Nguyen BP, Gil SG, Carter WG. Deposition of laminin 5 by keratinocytes regulates integrin adhesion and signaling. *J Biol Chem* 2000;275:31896–31907. [PubMed: 10926936]
16. Bachy S, Letourneur F, Rousselle P. Syndecan-1 interaction with the LG4/5 domain in laminin-332 is essential for keratinocyte migration. *J Cell Physiol* 2008;214:238–249. [PubMed: 17579341]
17. Talts JF, Sasaki T, Miosge N, Gohring W, Mann K, Mayne R, Timpl R. Structural and functional analysis of the recombinant G domain of the laminin alpha4 chain and its proteolytic processing in tissues. *J Biol Chem* 2000;275:35192–35199. [PubMed: 10934193]
18. Scheele S, Falk M, Franzen A, Ellin F, Ferletta M, Lonai P, Andersson B, Timpl R, Forsberg E, Ekblom P. Laminin alpha1 globular domains 4–5 induce fetal development but are not vital for embryonic basement membrane assembly. *Proc Natl Acad Sci U S A* 2005;102:1502–1506. [PubMed: 15668394]
19. Nomizu M, Kim WH, Yamamura K, Utani A, Song SY, Otaka A, Roller PP, Kleinman HK, Yamada Y. Identification of cell binding sites in the laminin alpha 1 chain carboxyl-terminal globular domain by systematic screening of synthetic peptides. *J Biol Chem* 1995;270:20583–20590. [PubMed: 7657636]
20. Nomizu M, Kuratomi Y, Malinda KM, Song SY, Miyoshi K, Otaka A, Powell SK, Hoffman MP, Kleinman HK, Yamada Y. Cell binding sequences in mouse laminin alpha1 chain. *J Biol Chem* 1998;273:32491–32499. [PubMed: 9829982]
21. Nomizu M, Kuratomi Y, Ponce ML, Song SY, Miyoshi K, Otaka A, Powell SK, Hoffman MP, Kleinman HK, Yamada Y. Cell adhesive sequences in mouse laminin beta1 chain. *Arch Biochem Biophys* 2000;378:311–320. [PubMed: 10860548]
22. Nomizu M, Kuratomi Y, Song SY, Ponce ML, Hoffman MP, Powell SK, Miyoshi K, Otaka A, Kleinman HK, Yamada Y. Identification of cell binding sequences in mouse laminin gamma1 chain by systematic peptide screening. *J Biol Chem* 1997;272:32198–32205. [PubMed: 9405421]
23. Nomizu M, Yokoyama F, Suzuki N, Okazaki I, Nishi N, Ponce ML, Kleinman HK, Yamamoto Y, Nakagawa S, Mayumi T. Identification of homologous biologically active sites on the N-terminal domain of laminin alpha chains. *Biochemistry* 2001;40:15310–15317. [PubMed: 11735413]
24. Suzuki N, Ichikawa N, Kasai S, Yamada M, Nishi N, Morioka H, Yamashita H, Kitagawa Y, Utani A, Hoffman MP, Nomizu M. Syndecan binding sites in the laminin alpha1 chain G domain. *Biochemistry* 2003;42:12625–12633. [PubMed: 14580209]
25. Hoffman MP, Engbring JA, Nielsen PK, Vargas J, Steinberg Z, Karmand AJ, Nomizu M, Yamada Y, Kleinman HK. Cell type-specific differences in glycosaminoglycans modulate the biological activity of a heparin-binding peptide (RKRLQVQLSIRT) from the G domain of the laminin alpha1 chain. *J Biol Chem* 2001;276:22077–22085. [PubMed: 11304538]

26. Hoffman MP, Nomizu M, Roque E, Lee S, Jung DW, Yamada Y, Kleinman HK. Laminin-1 and laminin-2 G-domain synthetic peptides bind syndecan-1 and are involved in acinar formation of a human submandibular gland cell line. *J Biol Chem* 1998;273:28633–28641. [PubMed: 9786856]
27. Kadoya Y, Nomizu M, Sorokin LM, Yamashina S, Yamada Y. Laminin alpha1 chain G domain peptide, RKRLQVQLSIRT, inhibits epithelial branching morphogenesis of cultured embryonic mouse submandibular gland. *Dev Dyn* 1998;212:394–402. [PubMed: 9671943]
28. Kim WH, Nomizu M, Song SY, Tanaka K, Kuratomi Y, Kleinman HK, Yamada Y. Laminin-alpha1-chain sequence Leu-Gln-Val-Gln-Leu-Ser-Ile-Arg (LQVQLSIR) enhances murine melanoma cell metastases. *Int J Cancer* 1998;77:632–639. [PubMed: 9679769]
29. Kato K, Utani A, Suzuki N, Mochizuki M, Yamada M, Nishi N, Matsuura H, Shinkai H, Nomizu M. Identification of neurite outgrowth promoting sites on the laminin alpha 3 chain G domain. *Biochemistry* 2002;41:10747–10753. [PubMed: 12196012]
30. Utani A, Momota Y, Endo H, Kasuya Y, Beck K, Suzuki N, Nomizu M, Shinkai H. Laminin alpha 3 LG4 module induces matrix metalloproteinase-1 through mitogen-activated protein kinase signaling. *J Biol Chem* 2003;278:34483–34490. [PubMed: 12826666]
31. Utani A, Nomizu M, Matsuura H, Kato K, Kobayashi T, Takeda U, Aota S, Nielsen PK, Shinkai H. A unique sequence of the laminin alpha 3 G domain binds to heparin and promotes cell adhesion through syndecan-2 and -4. *J Biol Chem* 2001;276:28779–28788. [PubMed: 11373281]
32. Okazaki I, Suzuki N, Nishi N, Utani A, Matsuura H, Shinkai H, Yamashita H, Kitagawa Y, Nomizu M. Identification of biologically active sequences in the laminin alpha 4 chain G domain. *J Biol Chem* 2002;277:37070–37078. [PubMed: 12130633]
33. Makino M, Okazaki I, Kasai S, Nishi N, Bougaeva M, Weeks BS, Otaka A, Nielsen PK, Yamada Y, Nomizu M. Identification of cell binding sites in the laminin alpha5-chain G domain. *Exp Cell Res* 2002;277:95–106. [PubMed: 12061820]
34. Nielsen PK, Gho YS, Hoffman MP, Watanabe H, Makino M, Nomizu M, Yamada Y. Identification of a major heparin and cell binding site in the LG4 module of the laminin alpha 5 chain. *J Biol Chem* 2000;275:14517–14523. [PubMed: 10799535]
35. Kadoya Y, Mochizuki M, Nomizu M, Sorokin L, Yamashina S. Role for laminin-alpha5 chain LG4 module in epithelial branching morphogenesis. *Dev Biol* 2003;263:153–164. [PubMed: 14568553]
36. Momota Y, Suzuki N, Kasuya Y, Kobayashi T, Mizoguchi M, Yokoyama F, Nomizu M, Shinkai H, Iwasaki T, Utani A. Laminin alpha3 LG4 module induces keratinocyte migration: involvement of matrix metalloproteinase-9. *J Recept Signal Transduct Res* 2005;25:1–17. [PubMed: 15960391]
37. Suzuki N, Nakatsuka H, Mochizuki M, Nishi N, Kadoya Y, Utani A, Oishi S, Fujii N, Kleinman HK, Nomizu M. Biological activities of homologous loop regions in the laminin alpha chain G domains. *J Biol Chem* 2003;278:45697–45705. [PubMed: 12933811]
38. Hozumi K, Suzuki N, Nielsen PK, Nomizu M, Yamada Y. Laminin alpha1 chain LG4 module promotes cell attachment through syndecans and cell spreading through integrin alpha2beta1. *J Biol Chem* 2006;281:32929–32940. [PubMed: 16945929]
39. Hohenester E, Tisi D, Talts JF, Timpl R. The crystal structure of a laminin G-like module reveals the molecular basis of alpha-dystroglycan binding to laminins, perlecan, and agrin. *Mol Cell* 1999;4:783–792. [PubMed: 10619025]
40. Kato-Takagaki K, Suzuki N, Yokoyama F, Takaki S, Umezawa K, Higo J, Mochizuki M, Kikkawa Y, Oishi S, Utani A, Nomizu M. Cyclic peptide analysis of the biologically active loop region in the laminin alpha3 chain LG4 module demonstrates the importance of peptide conformation on biological activity. *Biochemistry* 2007;46:1952–1960. [PubMed: 17261031]
41. Yokoyama F, Suzuki N, Haruki M, Nishi N, Oishi S, Fujii N, Utani A, Kleinman HK, Nomizu M. Cyclic peptides from the loop region of the laminin alpha 4 chain LG4 module show enhanced biological activity over linear peptides. *Biochemistry* 2004;43:13590–13597. [PubMed: 15491165]

Fig. 1.

Heparin-binding activity of recombinant LG45 proteins. (A) Five recombinant LG45 proteins were expressed in 293T cells as previously described (24,31,32) (N. Suzuki et al., manuscript submitted for publication). The purity of proteins was analyzed by a SDS—PAGE, followed by protein staining. (B) The rec-LG45s and heparin-Sepharose beads were mixed and incubated. The proteins bound to heparin-Sepharose were eluted with NaCl (100 – 800 mM). Duplicate experiments gave similar results.

peptide	sequence		peptide	sequence		peptide	sequence	
NONE			A5G85	HRVPRAERPQPY		A5G101	DGRWHRVAVIMG
A5G68	SRRSRQPSQDLA		A5G86	RPQPYTLSVGGLP		A5G102	VIMGRDTRLLEV
A5G69	TTPWLPGTIQDA		A5G87	GGLPASSYSSKL		A5G103	RLEVDTQSNHTTG
A5G70	IQDAYQFGGPLP		A5G88	SSKLPVSVGGLP		A5G104	HTTGRLPESLAG
A5G71	GPLPSYLQFVGI		A5G89	LKKLQLDKQPLRT		A5G105	SLAGSPALLHLG
A5G72	FVGISPSHRNRL		A5G90	LRTPTQMGVTP		A5G106	LHLGSLPKSSTA
A5G73	RNRLHLSMLVRP		A5G91	VSGPLEDGLFFPG		A5G107	SSTARPELPAYRG
A5G74	LVRPHAASQGLL		A5G92	FFPGSEGVVTLE		A5G108	LRKLLINGAPVN
A5G75	SQGLLLSTAPMSG		A5G93	VTLELPKAKMPY		A5G110	SVQIQGAVGMRG
A5G76	APMSGRSPSLVLF		A5G94	KMPYVSLELEMR		A5G111	SGTLALSKQGKA
A5G77	LVLFLNHGHFVA		A5G95	LEMRPLAAAGLI		A5G112	KQGKALTQRHAK
A5G78	GHFVAQTEGPGP		A5G96	AGLIFHLGQALA		A5G113	RHAKPSVSPLLWH
A5G79	GPGPRLQVQSRQ		A5G97	GQALATPYMQLKV		A5G71S*	YLGQPLVPSIGF
A5G80	VQSRQHSRAGQW		A5G98	MQLKVLTEQVLLQ		A5G77S*	FLHLVNAFGHLV
A5G81	AGQWHRVSVRWG		A5G99	VLLQANDGAGEF		A5G81S*	WARGHQVRVGWS	
A5G84	TWSQKALHHRVP		A5G100	AGEFSTWVTYPKL		A5G94S*	MSPYLEKLMERV	

Fig. 2.

Effect of peptides on rec- α 5LG45 protein—heparin binding. Sequences were derived from the mouse laminin α 5 chain G domain (position 3313—3718). Heparin-Sepharose beads (1 mg), peptide (20 μ g), and the rec- α 5LG45 protein (3 μ g) were incubated in 70 μ l of 10 mM Tris buffer (pH 7.4) at 4 °C for 1 h. After washing the beads, the rec- α 5LG45 bound to the heparin-Sepharose beads was analyzed by Western blotting. Active peptides are shown by bold. Triplicate experiments gave similar results. *Denotes scramble peptides of four active peptides.

Fig. 3.

Inhibitory effect of peptides on rec- α 5LG45 binding to the heparin. The heparin-binding of rec- α 5LG45 was inhibited by various amount of peptides and examined the IC₅₀ value of each peptide. The relative amount (%) of rec- α 5LG45 bound to the heparin-Sepharose bead was examined by SDS—PAGE and assessed by NIH image 1.63 software. IC₅₀ values of A5G71, A5G77, A5G81 and A5G94 were 91.8 μ M, 7.0 μ M, 5.9 μ M, and 0.84 μ M, respectively. Triplicate experiments gave similar results.

Fig. 4. Binding of biotinylated heparin to peptide-coated plates. 96-well plates were coated with various amounts of peptides. After blocking with BSA, biotinylated heparin (10 ng/75 μl) was added and incubated for 1 h. Then the biotinylated heparin bound to the peptides was detected by streptavidin-conjugated horseradish peroxidase. Triplicate experiments gave similar results.

Fig. 5.

Cell attachment activity of the recombinant α chain LG45 proteins. The proteins (2 μ g/well) were coated on the 96 well plates for 1h at room temperature. HT1080 cells (3000cells/well) were added and incubated for 1 h. Then attached cells were counted. Duplicate experiments gave similar results. Each value represents the mean of three separate determinations $\pm$ S.D. Statistical analyses were performed using an unpaired Student's t test. *Denotes a p value of <0.05 and **denotes a p value of <0.005 , N.S. : Not Significant.

chain	peptide	sequence											
$\alpha 1$	AG73	R	K	R	L	Q	V	Q	L	S	I	R	T
$\alpha 1$	AG75	G	L	I	Y	Y	V	A	H	Q	N	Q	M
$\alpha 2$	A2G78	G	L	L	F	Y	M	A	R	I	N	H	A
$\alpha 3$	A3G75	K	N	S	F	M	A	L	Y	L	S	K	G
$\alpha 4$	A4G82	T	L	F	L	A	H	G	R	L	V	F	M
$\alpha 4$	A4G83	L	V	F	M	F	N	V	G	H	K	K	L
$\alpha 5$	A5G71	G	P	L	P	S	Y	L	Q	F	V	G	I
$\alpha 5$	A5G77	L	V	L	F	L	N	H	G	H	F	V	A
$\alpha 5$	A5G81	A	G	Q	W	H	R	V	S	V	R	W	G
$\alpha 5$	A5G94	K	M	P	Y	V	S	L	E	L	E	M	R

Fig. 6.

List of heparin-binding peptides from the laminin α chain LG45 modules. Heparin-binding sequences identified here and previously reported are listed (24,31,32) (N. Suzuki et al., manuscript submitted for publication). Basic amino acids are boxed in black, aromatic amino acids are hexagons, and His residue is a hexagon in gray.

Fig. 7.

Active peptides and amino acid sequence alignment of the laminin α chain LG4 modules. The position of active peptides are mapped on the structure-based sequence alignment of LG modules of the α chain (12,39). β -strands are indicated by black arrows. The $\alpha 1$, $\alpha 2$, $\alpha 4$, and $\alpha 5$ chains are mouse laminin sequences (12,39). The $\alpha 3$ chain is the human sequence (31).

*Sequences of the $\alpha 5$ chain LG5 module examined in this paper are also indicated. Heparin-binding sequences are underlined.