Identification of a Major Heparin and Cell Binding Site in the LG4 Module of the Laminin α5 Chain*

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The G domain of the laminin α chains consists of five homologous G modules (LG1–5) and has been implicated in various biological functions. In this study, we identified an active site for cell and heparin binding within the laminin α5 G domain using recombinant proteins and synthetic peptides. Recombinant LG4, LG5, and LG4–5 modules were generated using a mammalian expression system. The LG4 and LG4–5 modules were highly active for cell binding, whereas the LG5 module alone showed only weak binding. Heparin inhibited cell binding to the LG4–5 module, whereas no inhibition was observed with EDTA or antibodies against the integrin β1 subunit. These results suggest that the LG4–5 module interacts with a cell surface receptor containing heparan sulfate but not with integrins. Solid-phase assays and surface plasmon resonance measurements demonstrated strong binding of the LG4 and LG4–5 modules to heparin with KD values in the nanomolar range, whereas a 16-fold lower value was determined for the LG5 module. Treatment with glycosidases demonstrated that N-linked carbohydrates on the LG5 module are complex-type oligosaccharides. The LG4–5 module, devoid of N-linked carbohydrates, exhibited similar binding kinetics toward heparin. Furthermore, cell binding was unaffected by removal of N-linked glycosylation. To localize active sites on the LG4 module, various synthetic peptides were used to compete with binding of the tandem module to heparin and cells. Peptide F4 (AGQWHRVSVRWG) inhibited binding, whereas a scrambled peptide of F4 failed to compete binding. Alanine replacements demonstrated that one arginine residue within F4 was important for cell and heparin binding. Our results suggest a critical role of the LG4 module for heparan sulfate-containing receptor binding within the laminin α5 chain.

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EXPERIMENTAL PROCEDURES

Construction of Expression Plasmids—Mouse cDNA was used as a template in polymerase chain reaction to amplify sequences encoding the laminin α5 LG4, LG5, and LG4–5 tandem modules. Polymerase chain reaction was performed with Ffu/Turbo DNA polymerase (Stratagene, La Jolla, CA) using the following primers: 1) GAG AGA CTC GAG AGC CAG GAC CTT GCC TGC ACG A; 2) GAG AGA GGA TCC GTT TCC TGA GGG GCA TCC GGC; 3) GAG AGA GGA TCC TTC CAG GGG GCC TGA
Expression and Purification of Recombinant Laminin α5 LG Modules—The expression plasmids were transfected into human embryonal kidney 293 cells (CRL-1573, ATCC) using FuGENE 6 (Roche Molecular Biochemicals), and stable transfected 293 cells were established by cloning and selection with 400 μg/ml of G418 (Geneticin Liquid, Life Technologies, Inc.). Secretion of recombinant laminin modules was analyzed by Western blotting of serum-free, conditioned medium (Dulbecco’s modified Eagle’s medium, Life Technologies, Inc.) using an anti-FLAG M2 monoclonal antibody (Sigma). For protein production, conditioned medium was collected with phenol-buffered saline and then harvested and replaced with serum-containing medium. Conditioned, serum-free medium was cleared of detached cells and debris through centrifugation, and proteinase inhibitors were added to final concentrations of 5 mM EDTA, 1 mM N-ethylmaleimide, and 1 mM phenylmethylsulfonyl fluoride. The conditioned medium was applied onto an anti-FLAG M2 column (Sigma), and the column was washed with 50 mM Tris-HCl, 10 mM NaCl, pH 7.5, followed by 50 mM Tris-HCl, 150 mM NaCl, pH 7.5. The FLAG-tagged proteins were eluted with 0.1 M glycine-HCl, pH 3.5, and neutralized with 100 mM Tris-HCl, pH 8.0, immediately after elution. Protein concentrations were determined using a BCA protein assay kit with bovine serum albumin as standard (Pierce). Synthetic Peptides—Peptides were synthesized as described previously (16) or obtained from the Facility for Biotechnology Resources (United States Food and Drug Administration). The peptides were prepared with a C-terminal amide. All peptides were purified by reverse-phase high performance liquid chromatography and characterized by mass spectrometry.

Characterization of Glycosylation—N-Glycanase F (1.4 unit) (Oxford Glycosystems, Inc., Bedford, MA), endoglycosidase H (10 milliunits) (Roche Molecular Biochemicals), and N-acetylgalactosaminidase (25 milliunits) (Glyco, Nova, CA) were used to investigate the presence of N-linked carbohydrates, high mannose sugars, and sialic acids, respectively. Samples were incubated for 18 h at 37 °C. The presence of O-glycosylation was examined by treatment with N-glycanase F (5 units) with the addition of 15 milliunits of N-acetylgalactosaminidase III after 15 h, and then 2 milliunits of O-glycosidase (Roche Molecular Biochemicals) were added after 3 h, and the incubation was continued for another 12 h at 37 °C. All samples were subsequently analyzed by SDS-PAGE. Enzymatic Deglycosylation for Binding Studies—Recombinant laminin α5 LG–5 tandem module (5 μg) in 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, was digested with 200 milliunits of N-glycanase F (Oxford Glycosystems, Inc.) or with 10 milliunits of N-acetylgalactosaminidase III for 18 h at 37 °C. Samples (1 μg) of digested and undigested tandem module incubated in parallel were analyzed by SDS-PAGE. The affinities for the deglycosylated and control tandem module for heparin were determined using surface plasmon resonance, as described below.

Heparin Binding Solid-phase Assay—Enzyme-linked immunosorbent assay plate wells were coated with 100 μl of heparin–BSA (10 mg/ml) (Sigma) overnight at 4 °C. Wells were blocked at room temperature (2 h) with 0.05 % Tris–HCl, pH 7.5, 0.15 % NaCl (Tris-buffered saline), 1 % BSA and then washed and incubated with recombinant proteins serially diluted in the same buffer overnight at 4 °C. After washing (Tris-buffered saline including 0.5 % Tween), bound FLAG-tagged proteins were detected with anti-FLAG monoclonal antibodies. After a further wash, bound antibody was detected with alkaline phosphatase-conjugated goat anti-mouse IgG (Pierce) and PNPP phosphatase substrate kit (Pierce). For competition experiments, biotinylated heparin (0.16 μM) (Celsius Laboratories, Inc., Cincinnati, OH) was mixed with synthetic peptides serially diluted in Tris-buffered saline, including 0.05 % Tween and 1 % BSA, and incubated in wells coated with recombinant laminin α5 tandem module (60 ng/50 μl, 19 μM) at room temperature, wells were washed, and bound biotinylated heparin was detected with alkaline phosphatase-conjugated streptavidin (Pierce).

Surface Plasmon Resonance—Biotinylated heparin (Celsius Laboratories, Inc.) at 40 μg/ml in 25 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 0.005 % surfactant P20 was immobilized on a streptavidin-coated sensor chip (Sensor Chip SA, BIAcore, Inc., Piscataway, NJ) at 10 μl/min for 4 min to an immobilization level of 300 resonance units. In the affinity measurements, recombinant proteins, at different concentrations, were injected on the heparin-coated surface at 30 μl/min (in 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.005 % surfactant P20, 25 °C), and the binding and dissociation were registered (2 min each) in a BIAcore 1000 instrument (BIAcore, Inc.). The streptavidin-heparin surface was regenerated by two successive injections of 30 μl of 20 mM NaOH containing 1 μM NaCl between each run. In control experiments with the same concentrations of recombinant proteins, but with a blank streptavidin sensor chip, no binding was seen. The obtained sensorgrams were analyzed by nonlinear least square curve fitting using BIAevaluation 2.1 software assuming single-site association and dissociation kinetics.

Cell Adhesion Assays—The human submandibular gland (HSG) cell line was cultured as described previously (13). Cells were detached with 0.02 % EDTA, washed with Dulbecco’s modified Eagle’s medium containing 1 % BSA, and resuspended to a concentration of 3.5 × 10^6/ml. Assays were performed in 96-well round-bottomed microtiter plates (Immulon-2HB, Dynex Technologies, Inc., Chantilly, VA). Wells were coated overnight at 4 °C with recombinant proteins or laminin 5 LG5, and LG4–5 Tandem Modules—Constructions were generated for expression of mouse laminin α5 LG modules in 293 cells with a FLAG-tag and a portion of the C-terminal of the laminin γ1 chain at the N- and C-terminal ends, respectively (Fig. 1A). The recombinant proteins were purified by anti-FLAG affinity chromatography. From 1 liter of serum-free culture medium, 0.4–2.5 mg of pure recombinant proteins were recovered. The preparations of the recombinant proteins were analyzed by SDS-PAGE (Fig. 1B). The apparent molecular masses for the tandem module and the LG5 module were slightly higher than the predicted sizes from the DNA coding sequence, suggesting the presence of post-translational modifications.

Characterization of Glycosylation—The sequence of the tandem module has two consensus motifs for N-linked carbohydrate, the N-acetylgalactosaminyl core tripeptide. After treatment with N-glycanase F and analyzed by SDS-PAGE (Fig. 1C). The sequenced heparin-like glycosaminoglycans, substrate-coated and preblocked wells were incubated with inhibitors for 30 min at 37 °C and washed before cells were added.

RESULTS

Expression and Purification of Recombinant Laminin α5 LG4, LG5, and LG4–5 Tandem Modules— Constructs were generated for expression of mouse laminin α5 LG modules in 293 cells with a FLAG-tag and a portion of the C-terminal of the laminin γ1 chain at the N- and C-terminal ends, respectively (Fig. 1A). The recombinant proteins were purified by anti-FLAG affinity chromatography. From 1 liter of serum-free culture medium, 0.4–2.5 mg of pure recombinant proteins were recovered. The preparations of the recombinant proteins were analyzed by SDS-PAGE (Fig. 1B). The apparent molecular masses for the tandem module and the LG5 module were slightly higher than the predicted sizes from the DNA coding sequence, suggesting the presence of post-translational modifications.

Characterization of Glycosylation—The sequence of the tandem module has two consensus motifs for N-linked carbohydrate (Asn-Xaa-(Ser/Thr)) located in the LG5 region. To address the possibility of N-linked carbohydrate, the tandem module was treated with N-glycanase F and analyzed by SDS-PAGE (Fig. 1C). The sequenced heparin-like glycosaminoglycans, substrate-coated and preblocked wells were incubated with inhibitors for 30 min at 37 °C and washed before cells were added.
removes sialic acid, the molecular mass of the tandem module was decreased 1–2 kDa (Fig. 1C, lane 2). The possible presence of O-linked carbohydrate was examined by O-glycosidase following treatments with N-glycanase F and N-acetylneuraminidase III. However, there was no difference in apparent molecular masses between tandem module treated by N-glycanase F alone and tandem module treated with N-glycanase F, N-acetylneuraminidase III, and O-glycosidase (data not shown). These results suggest that the tandem module is modified only by N-linked carbohydrate.

**Cell Binding Activity**—The proteolytic fragment E3 of the laminin α1 chain, which corresponds to the laminin α1 LG4–5 tandem module, has previously been shown to promote binding of HSG cells (13). These cells were used to analyze the adhesive properties of the recombinant laminin α5 LG modules and laminin containing the α5 chain (laminin-10/11). HSG cells showed similar binding to the LG4 module and the tandem module (Fig. 2). Weak cell binding was observed to the LG5 module. A recombinant control protein containing a FLAG-tagged portion of the C-terminal of the laminin α5 chain and the constructs used for expression of mouse recombinant proteins in 293 cells. B, nonreducing SDS-PAGE was performed on a Novex 10–20% SDS-polyacrylamide gel (Novex, San Diego, CA). Gels were stained with colloidal Coomassie G-250 (GelCode Blue, Pierce). Lane s, standard proteins (Mark 12 wide range protein standard, Novex), the molecular masses of which are indicated in kDa. Lane 1, LG4–5 tandem module; lane 2, LG4 module; lane 3, LG5 module. One to two μg of purified recombinant protein were loaded in each lane. C, nonreducing SDS-PAGE after treatment of LG4–5 tandem module with glycosidases. Lane 1, untreated LG4–5 tandem module; lane 2, LG4–5 tandem module treated with NAN III (the upper band is due to NAN III); lane 3, LG4–5 tandem module treated with N-glycosidase F (the lower band is due to N-glycosidase F).

**Effect of EDTA and an Inhibitory Monoclonal Antibody against β1 Integrin Subunit on Cell Binding**—Cell binding to the recombinant laminin α5 LG4–5 protein and to laminins containing either the α5 chain (laminin-10/11) or the α1 chain (laminin-1) was examined in the presence of EDTA and a blocking monoclonal antibody against the β1 integrin subunit. The antibody against the β1 integrin subunit and 10 mM EDTA showed only partial inhibition of HSG binding to laminin-10/11 (Fig. 3A) but complete inhibition of cell binding to laminin-1 (data not shown and Ref. 13). These results suggest that dif-
different cellular receptors mediated binding to laminin-1 and laminin-10/11. The antibody against the β1 integrin subunit showed no effect on binding to the tandem module (Fig. 3B). Furthermore, EDTA showed no inhibition, suggesting that HSG cell binding to the laminin α5 tandem module is not mediated by β1 integrins (Fig. 3B).

Effect of Heparin on Cell Binding—Because heparin binding activity has been localized to the tandem modules of the laminin α1 and α2 chains (11), cell binding to the recombinant laminin α5 LG4–5 tandem module was also examined in the presence of heparin and some other heparin-like glycosaminoglycans. Heparin and heparan sulfate showed no inhibition of HSG cell binding to laminin-10/11 (Fig. 4A) and laminin-1 (data not shown), consistent with previously reported results (13). Heparin and heparan sulfate were found to partly inhibit cell binding to the tandem module, whereas keratan sulfate was noninhibitory (Fig. 4B). These results suggest that HSG cell binding to the LG4–5 tandem module of the laminin α5 chain is mediated by heparan sulfate proteoglycans.

Binding to Heparin in Solid-phase Assay—The interactions with heparin were analyzed in a solid-phase assay using immobilized heparin-albumin and various concentrations of the recombinant laminin α5 LG modules. The highest binding activity was observed for the LG4 module (half-maximum at 10 nM) and the tandem module (half-maximum at 3 nM), whereas much lower binding activity was observed for the LG5 module (half-maximum not observed at concentrations up to 300 nM) (Fig. 5). No binding was observed for the fusion protein. Thus, the heparin binding activity correlated with the observed cell binding activity of the modules described above.

Binding to Heparin Using Surface Plasmon Resonance—The binding kinetics between immobilized biotinylated heparin and the laminin α5 tandem module and individual modules were measured directly by real-time biomolecular interaction analysis using surface plasmon resonance on a BIAcore™ system. Similar equilibrium dissociation constants ($K_D = 1.7–2$ nM) were obtained for the interactions of the LG4 module and the tandem module with heparin (Table I). Binding of the tandem module to the immobilized biotinylated heparin was inhibited with an excess of unlabeled heparin (not shown). The binding affinity of the LG5 module to heparin was 16-fold weaker ($K_D = 462$ nM). These results are consistent with the binding profiles determined by the heparin solid-phase assay and demonstrate that the major heparin binding site within the tandem module is located in the LG4 module.

Functional Impact of Glycosylation on Heparin Binding and Cell Binding Activity—To determine whether glycosylation is required for heparin and cell binding, recombinant laminin α5 LG4–5 tandem module was treated with neuraminidase and N-glycanase and tested for its heparin- and cell binding activity. The binding kinetics between immobilized biotinylated heparin and the deglycosylated tandem module were measured directly by surface plasmon resonance (Table I). The association and dissociation rate constants measured for the interaction between the tandem module and heparin corresponded to
Heparin and Cell Binding Site in the Laminin α5 G4 Module

Kinetic and affinity constants for interactions between recombinant laminin α5 LG modules and immobilized heparin

Data were obtained by surface plasmon resonance analysis. The kinetic constants were derived from several independent experiments on a streptavidin sensor chip with immobilized biotinylated heparin using 100–1000 nM of the various recombinant laminin α5 LG module preparations. The apparent dissociation constants ($K_D$) for the preparations were calculated from the ratio of the corresponding rate constants, $k_{on}/k_{off}$. NAN and GlyF represent treatment with neuraminidase-III and N-glycanase F, respectively. The extent of deglycosylation of the molecules was assessed by SDS-PAGE.

| Module | $k_{on} \times 10^3$ | $k_{off} \times 10^{-3}$ | $K_D$ |
|--------|----------------|-----------------|-------|
| LG4–5 (0.1–0.2) | 0.54 ± 0.2 | 192 ± 63 | 2.8 ± 1.4* |
| LG4–5NAN (0.1–0.2) | 0.56 ± 0.1 | 172 ± 24 | 3.4 ± 0.9 |
| LG4–5GlyF (0.1–0.2) | 0.17 ± 0.1 | 70 ± 14 | 4.1 ± 1.3 |
| LG4 (0.1–0.3) | 0.26 ± 0.1 | 149 ± 55 | 1.7 ± 0.3 |
| LG5 (0.4–1) | 0.32 ± 0.1 | 7 ± 0.1 | 46.2 ± 14.6 |

*Experimental variation is shown as standard deviation.

a dissociation constant of approximately 2.8 nM. Enzymatic removal of the sialic acids and the entire N-linked carbohydrate on the tandem module by neuraminidase and N-glycanase treatment, respectively, did not change the binding kinetics significantly ($K_D = 3.4–2.4$ nM). Furthermore, the removal of N-linked carbohydrate showed no effect on the cell binding properties (data not shown). These data suggest that N-linked glycosylation is not critical for receptor interactions involved in cell binding to the LG4–5 tandem module.

Localization of a Heparin Binding Site on the Laminin α5 LG4 Module—Protein interactions with heparin-like ligands are based on contacts between positively charged residues and negatively charged groups of heparin. In a number of protein domains (e.g. fibronectin repeats), the structure of the heparin-binding surfaces has been shown to consist of several Arg and Lys residues (17). Three possible heparin-binding sequences that contain clusters of Arg and Lys residues are located within the LG4 module sequence. We prepared a panel of LG4 module peptides derived from these sequences (Table I). Initially, we screened these peptides for direct heparin and cell binding activity using solid-phase assays. The peptide F4 (AGQWHRVSVRWG) was active both for HSG cell and heparin binding, whereas the peptide F1 showed weak cell binding activity but no heparin binding activity (data not shown). Next, we tested all the peptides to determine whether they affected the binding of biotinylated heparin to immobilized tandem module. Only peptide F4 was found to inhibit binding of heparin to the tandem module (Fig. 6), whereas none of the other peptides had any effect on the tandem module-heparin interaction, even when the peptides were almost 1000-fold molar excess over heparin (152 mM peptide, 0.16 mM biotin-heparin conjugate). Half-maximum inhibition was seen at 10–20 μM for the inhibitory peptide. The ability of the synthetic peptides to inhibit the binding of the tandem module to heparin was also studied using surface plasmon resonance. Only peptide F4 prevented the binding of the tandem module to immobilized heparin on the sensor chip, which supports the solid-phase data (data not shown).

Inhibition of cell binding to the laminin α5 LG4–5 tandem module and laminin-10/11 by the cell-binding peptides was also examined. Peptide F4 inhibited HSG cell binding to the tandem module, whereas peptide F1 exhibited no significant inhibition (Fig. 7). None of the peptides inhibited cell binding to laminin10/11, indicating that other cell binding sites are available on the intact molecule (data not shown). Together, these results suggest that the AGQWHRVSVRWG sequence is a major site for heparin and cell binding within the laminin α5 LG4–5 tandem module.

Identification of Important Residues of the F4 Sequence by Mutational Analysis—We next examined sequence specificity of F4 binding to heparin by mutational analysis. A scrambled peptide (P4S, Table III) failed to show competition with heparin, suggesting that heparin binding is sequence-specific. To analyze the contribution of the Arg residues to heparin and cell binding activity of the F4 peptide, individual Arg residues were replaced with Ala residues (Table III). The two peptides F4A (substitution of Arg6) and F4C (substitution of Arg6 and Arg10) did not compete at 20 μM with the LG4–5 tandem module.

| Peptide | Notation | Sequence | Residues |
|---------|----------|----------|----------|
| F1 | RNLHLSMLVRP | 3275–3286 |
| F2 | GPFRLQVQSRQ | 3222–3333 |
| F3 | VCSRHSRRAGW | 3239–3340 |
| F4 | AGQWHRVSVRWG | 3337–3348 |
| F5 | TWSQKALHHRVP | 3361–3372 |
| F6 | HRVPRAERPQFY | 3369–3380 |
| F7 | LKKQLDQLKQPLRT | 3406–3418 |

![Peptide concentration (μM)](image)

**Fig. 6.** Peptide inhibition of the laminin α5 LG4–5 tandem module-heparin interaction. Competition solid-phase assays were carried out using biotinylated heparin and immobilized laminin α5 LG4–5 tandem module. The amount of biotinylated heparin bound (relative to the amount bound in the absence of peptide) is shown versus concentrations of the seven peptides listed in Table II. Shown are results for peptides F1 (−), F2 (■), F3 (●), F4 (□), F5 (×), F6 (○), and F7 (+).

**Fig. 7.** Effects of peptides on cell binding to recombinant laminin α5 LG4–5 tandem module and laminin-10/11. HSG cells were preincubated for 10 min with peptide F4 (10 μg/ml (column 2) or F1 (10 μg/ml (column 3) and added to wells coated with laminin α5 LG4–5 tandem module (250 ng/50 μl, 79 nm). After 1 h, cell attachment was quantitated. Values are expressed as percentage of control without peptides (column 1) and are the mean of three different experiments. Duplicate experiments gave similar results.

**TABLE I**

Synthetic peptides from the sequence of the LG4 module of the mouse laminin α5 chain

Underlined sequence is active for heparin and cell binding.

| Notation | Sequence | Residues |
|----------|----------|----------|
| F1 | RNLHLSMLVRP | 3275–3286 |
| F2 | GPFRLQVQSRQ | 3222–3333 |
| F3 | VCSRHSRRAGW | 3239–3340 |
| F4 | AGQWHRVSVRWG | 3337–3348 |
| F5 | TWSQKALHHRVP | 3361–3372 |
| F6 | HRVPRAERPQFY | 3369–3380 |
| F7 | LKKQLDQLKQPLRT | 3406–3418 |
whereas peptide F4B (substitution of Arg<sup>6</sup>) competed with heparin binding to the tandem module (Fig. 8). In direct cell binding assays, the peptides F4A, F4C, and F4S showed no or weak cell attachment activity, whereas the activity for peptide F4B was only slightly reduced compared with F4 (Fig. 9). Taken together, these data indicate that Arg<sup>6</sup> is critical for both heparin and cell binding activity of F4.

**DISCUSSION**

Various ligands for the LG modules of the laminin α1 and α2 chains have been reported, including heparin, heparan sulfate of perlecan, syndecan, integrins, and dystroglycan, but binding to the LG modules of the newly discovered laminin α5 chain has not been studied. In this work, we have investigated the biological activities of recombinant LG4, LG5, and LG4–5 tandem modules from the G domain of the mouse laminin α5 chain. These recombinant modules were used to identify active sites and to compare with those previously identified in the laminin α1 and α2 chains. Here, we demonstrate heparin and cell binding activity in the LG4 module and identify the sequence responsible.

The LG modules of the laminin α5 chain were expressed in mammalian cells to ensure folding and posttranslational modifications. Folding of the recombinant LG module was indicated from the formation of intrachain disulfide bonds as shown by comparison of nonreducing and reducing SDS-PAGE (data not shown). The tandem module was modified by sialic acids attached to N-linked carbohydrate in accordance with two consensus sites for N-linked carbohydrate within the LG5 module, whereas O-linked carbohydrate was not detected. Previous characterization of the glycosylation of the recombinant laminin α2 LG4–5 tandem module has also shown that it is modified only by N-linked glycosylation (18). The N-glycosylation of the laminin α5 tandem module was insensitive to treatment with endoglycosidase H, indicating a complex type of N-linked carbohydrate.

The LG4 module and the LG4–5 tandem module promoted similar HSG cell binding, whereas less cell binding was observed to the LG5 module, suggesting that the LG4 module of the laminin α5 chain contributes the major cell binding site. A similar binding was observed with other cell types, including mouse neural crest cells and mouse B16-F10 melanoma cells (data not shown). Inhibitors of cell binding were used to identify the type of cell surface receptors mediating binding to the tandem module. EDTA and an inhibitory β<sub>1</sub> integrin subunit monoclonal antibody did not affect cell binding to the tandem module, although cell binding to laminin-10/11 was partially inhibited by both reagents. These results suggest that β<sub>1</sub> integrins do not mediate cell binding to the laminin α5 tandem module and that cell binding to laminin-10/11 is partly mediated by β<sub>1</sub> integrins, probably through a different domain. However, inhibition of cell binding with heparin-like glycosaminoglycans demonstrated the presence of heparin binding sites on the tandem module and suggests that membrane-bound heparan sulfate proteoglycans may mediate binding to this region of the laminin α5 chain.

The identity of the HSG cell surface molecules that bind to the laminin α5 LG4–5 tandem module remains to be determined, but likely candidates include syndecan-1 and α-dystroglycan. Syndecan-1 was identified as a receptor for laminin-1 (α1β1γ1) by Western blotting analysis of fractions from affinity chromatography of biotinylated HSG cell membranes (13). α-Dystroglycan was shown to bind to the laminin α5 G domain in ligand blotting inhibition experiments using laminin α5 G domain expressed in bacteria and soluble α-dystroglycan from aortic endothelial cells (8).

It is interesting that EDTA did not inhibit the binding of the laminin α5 LG4–5 module to HSG cells, because α-dystroglycan has been shown to bind extracellular matrix molecules, including laminin-1 and -2, perlecan, and agrin, in a calcium-dependent fashion. Heparin/heparan sulfate inhibits HSG cell binding to the laminin α5 LG4–5 module. It has been shown that heparin and α-dystroglycan binding sites may overlap but that the α-dystroglycan binding site is generally larger in agrin and the laminin α1 chain (12, 19). Furthermore, α-dystroglycan binding to laminin-2 (20, 21) and laminin α2 LG1–3 and LG4–5 is not inhibited by heparin (11). Thus, if the laminin α5 LG4–5 modules bind α-dystroglycan, the mechanisms of the binding may be different from that of the laminin α2 chain.

Heparin/heparan sulfate showed no effect on HSG cell bind-
ing to laminin-10/11 containing the α5 chain. This implies that multiple receptors exist and that other domains within laminin-10/11 have cell binding capability that can compensate for inhibition of the LG4–5 tandem module. These other domains may interact with integrins, as demonstrated by partial inhibition of cell binding to laminin-10/11 with EDTA or an inhibitory β1 subunit monoclonal antibody. Cell binding to laminin-10/11 may be mediated in part by the α5β1 integrin, which has recently been shown to mediate binding of a lung adenocarcinoma cell line to laminin-10/11 (7).

The carbohydrate moieties of the intact laminin-1 molecule have previously been found to affect cell spreading and neurite outgrowth, suggesting some interactions between cell surface molecules and carbohydrates. N-Linked carbohydrates from the tandem module did not affect the binding kinetics with heparin or cell binding. Therefore, the carbohydrate moieties within the tandem module are not critical for heparin binding and receptor interactions involved in cell binding.

The laminin α5 LG module and the tandem module showed similar affinities for heparin in solid-phase and surface plasmon resonance assays, whereas the LG5 module showed at least 16-fold weaker binding affinity. Recent solid-phase heparin binding studies of the corresponding recombinant modules of the laminin α1 chain show similar results (11, 12), suggesting that the heparin binding properties of the LG4 module are conserved between the laminin α1 and α5 chains. Interestingly, the heparin binding affinities of these modules within the laminin α2 chain are different, and the LG5 module is the major heparin binding site in this chain (11).

We identified a sequence, AGQWHIRSVRWG (peptide F4), within the LG4 module that inhibited both heparin and cell binding. One Arg residue (Arg6) was found by an alanine substitution to be involved in cell binding activity, and it is conserved between the mouse and human α5 chains but not in other laminin α-chain isoforms. The Arg6 alanine substitution had a significant effect on reducing cell binding and was critical for heparin binding, indicating that the binding sites for heparin and cell surface receptors may be similar. The contribution of Arg and Lys residues to ligand binding has recently been illustrated by the crystal structure of the laminin α1 carbohydrate moiety of the β1, β3, and α2 integrins (23). A jelly-roll fold, the spatially close, suggesting a critical role of the loops of the jelly-roll fold. The other Arg residue important for both heparin and cell binding activity of the F4 sequence (Arg6) corresponds to a Lys residue (Lys3027) in the α2 LG5 module important for binding to α-dystroglycan but not for heparin binding to the α2 LG5 module. One of the two heparin binding sites in the α1 LG4 module also aligns to the same loop (EYIKRRK), whereas the other site (GKGRTK) maps to a loop spatially close, suggesting a critical role of the loops of the jelly-roll fold. The other Arg residue important for both heparin and cell binding activity of the F4 sequence (Arg6) corresponds to a Lys residue (Lys3027) in the α2 chain located in the middle of β strand H. The importance of this Lys residue in the α2 chain remains to be determined. However, our results indicate a critical role of this position for receptor interactions in the α5 chain and suggest important binding functions within the β strands of the jelly-roll fold in addition to those in loop regions.

In conclusion, the present data represent the first mapping of heparin- and cell binding activity to the LG4 module of the mouse laminin α5 chain. Peptide competition experiments indicate that one Arg residue in the sequence AGQWHIRSVRWG of the LG4 module is important for cell surface receptor interactions. The identified residues are located close to sites previously mapped in the laminin α1 LG4 module, suggesting that different sites in the LG4 module of the laminin α1 and α5 chains are responsible for receptor interactions.

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