Laminin α1 Chain LG4 Module Promotes Cell Attachment through Syndecans and Cell Spreading through Integrin α2β1*

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The laminin α1 chain is a subunit of laminin-1, a heterotrimeric basement membrane protein. The LG4-5 module at the C terminus of laminin α1 contains major binding sites for heparin, sulfatide, and α-dystroglycan and plays a critical role in early embryonic development. We previously identified active synthetic peptides AG73 and EF-1 from the sequence of laminin α1 LG4 for binding to syndecan and integrin α2β1, respectively. However, their activity and functional relationship within the laminin-1 and LG4 as well as the functional relation between these sites and α-dystroglycan binding sites in LG4 are not clear. To address these questions, we created mutant recombinant LG4 proteins containing alanine substitutions within the AG73 (M1), EF-1 (M2, M3), and α-dystroglycan binding sites (M4, M5) and analyzed their activities. We found that recombinant proteins rec-M1 and rec-M5, containing mutations within M1 and M5, respectively, did not bind heparin or lymphoid cell lines expressing syndecans. These results suggest that LG4 binds to heparin and syndecans through M1 and M5. Rec-M1 and rec-M5 reduced fibroblast attachment, whereas mutant rec-M2 and rec-M3 retained cell attachment activity but did not promote cell spreading. Fibroblast attachment to rec-LG4 was inhibited by heparin but not by integrin antibodies. Spreading of fibroblasts on rec-LG4 was inhibited by anti-integrin α2 and β1 but not by anti-integrin α1 and α6. These results suggest that the M1 and M5 sites are necessary for cell attachment on LG4 through syndecans and that the EF-1 site is for cell spreading activity through integrin α2β1. In contrast, laminin-1-mediated fibroblast attachment and spreading were not inhibited by heparin or anti-integrin α2. Our findings indicate that LG4 has a unique function distinct from laminin-1 and suggest that laminin α1 LG4-5 may also be produced by a proteolytic cleavage in certain tissues where it exerts its activity.

Laminins are heterotrimeric basement membrane proteins that exert multiple biological functions through interactions with extracellular matrix molecules and with cell surface receptors. The regulation of these interactions are critical to many biological processes, including cell adhesion and migration, angiogenesis, tumor progression, and neurite outgrowth (1, 2). The laminin family contains at least 11 chains (five α, three β, and three γ chains) (2). The five laminin α chains share a large module at their C-terminal region (G domain), which contains five laminin G domain-like modules (LG1-5), and each module consists of about 200 amino acids (3, 4).

Laminin-1 (α1β1γ1; also called laminin-111 according to a recently proposed nomenclature) (5) was originally isolated from the Engelbreth-Holm-Swarm (EHS) tumor and is the major isoform present in early embryonic stages (6). The laminin α1 chain interacts with several integrins through the N terminus and C-terminal triple-helical region (7). Laminin α1 LG4-5 interacts with heparin/heparan sulfate, sulfatides, perlecans, fibulin-1, and α-dystroglycan (8–11). We also found that peptide AG73 (KRQLQVLSIRT, residues 2719–2730) from LG4 is active for cell attachment, promotes neurite outgrowth, and binds to syndecans, a membrane-associated heparan sulfate proteoglycan (12–16), and peptide EF-1 (DYATLQLQEGRHFMFDLG, residues 2747–2765), which consists of β-sheet strands with their connecting loop region of LG4, binds to integrin α2β1 (17). More recently, using synthetic peptide screening, active peptides from the LG4 of other laminin α chains have been identified for cell attachment and heparin and syndecan binding (15, 18–21). These studies revealed that the AG73 activities and sequence are unique in the α1 chain and that EF-1 is the only peptide active for integrin binding among the homologous peptides from other α chains. However, it is uncertain whether these sites are active in the LG4 and laminin-1 proteins. Mutagenesis analysis with recombinant laminin α1 LG4 revealed that two sequences, EYIKKK (K1 site, residues 2789–2793) and GKGRTK (K3 site, residues 2766–2770), which are located downstream of the AG73 and EF-1 sites, are active for heparin and α-dystroglycan binding of LG4 (22). These findings indicate that laminin α1 LG4 has several active sites and multiple biological functions through interacting with extracellular matrix and cell surface receptors. However, func-

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tional relationships between these active sites have not been elucidated.

Laminin α1 knock-out mice die around embryonic day E7.5 due to the lack of Reichert’s membrane and defective epiblast polarization (23). Recently, mutant mice that express a truncated laminin-1 molecule that is specifically missing the LG4-5 portion of the laminin α1 chain were generated (24). The mutant mice die from defective Reichert’s membrane, just like the laminin α1 knock-out mice. In addition, the ectoplacental cone of wild-type mice stain with antibodies specific to laminin α1 LG4 but not to the N terminus or to LG1-3, suggesting that laminin α1 may be cleaved to generate the LG4-5 molecule in vivo, as observed in laminin α2 (25, 26), α3 (27, 28), α4 (29), and α5 (30). Taken together, these results suggest that the laminin α1 LG4-5 tandem module is critical for mouse embryogenesis and may also serve as a distinct functional unit apart from the whole laminin-1 molecule in certain tissues.

In this report we prepared recombinant mouse laminin α1 LG4 proteins, which contained site-specific mutations within the AG73, EF-1, K1, and K3 sites and analyzed their activities for binding to heparin, syndecans, and integrins in relation to their activities for cell attachment and spreading. We found that the critical residues of the AG73 and α-dystroglycan binding sites are very closely located in the predicted three-dimensional structure and also bind to syndecan. On the other hand, the EF-1 site is not essential for cell attachment but is required for cell spreading through integrin α2β1 binding. This site is positioned on the opposite side from the syndecan binding sites in the LG4 module. Inhibition assays revealed that the laminin α1 LG4 module has a unique function distinct from the whole laminin-1 molecule. Our results suggest that the AG73 and α-dystroglycan sites within LG4 have an overlapping function, and the EF-1 site exerts its activity for cell spreading in concert with the AG73 and α-dystroglycan sites.

Experimental Procedures

Cells and Culture—293 EBNA cells (Invitrogen) and human foreskin fibroblasts were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS)5 (HyClone, Logan, UT), 100 units/ml penicillin, and 100 mg/ml streptomycin (Invitrogen). Human lymphoid cell line ARH-77 and its derivative cell lines, ARH-77-Con ARH-77-Synd-1, ARH-77-Synd-2, ARH-77-Synd-4, and ARH-77-Gpc-1, which express an empty vector, recombinant syndecan-1, -2, and -4 and glypican-1, respectively (31, 32), were grown in suspension culture in RPMI medium 1640 (Invitrogen) supplemented with 5% FBS, 100 units/ml penicillin, and 100 mg/ml streptomycin. All cells were maintained at 37 °C in a humidified, 5% CO2, 95% air atmosphere.

Construction of Expression Vectors and Site-directed Mutagenesis for LG4 and LG4-5—Mouse laminin α1 cDNA (3) was used as a template for PCR to amplify sequences encoding the laminin α1 LG4 module (Leu2683–Pro2874) and LG4-5 tandem module (Leu2683–Pro3060). PCR was performed with KOD Hot Start DNA polymerase (Novagen, Madison, WI) using primers 5′-CCC AAG CTT CTG CAC AGA GAA CAC GGG-3′ (forward), 5′-TAA ACT ATG CGG CCA TAGCAC CTG GCC ACA GC-3′ (LG4, reverse), or 5′-GAG AGA CTC GAG AGG GCT CAG GCC GGC AG-3′ (LG5, reverse). These primers contained restriction sites for HindIII and NotI for LG4 or HindIII and XhoI for LG4-5. The PCR product was cloned into pCR4 Blunt-TOPO® for sequencing (Invitrogen), and site-directed mutagenesis was performed using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The following amino acid residues of the laminin α1 LG4 module were substituted with alanine: Arg-2719, Lys-2720, and Arg-2721 within the AG73 site, Arg-2757 and Asp-2763 within the EF-1 site, and Lys-2766, Arg-2768, and Lys-2770 and Lys-2791, Arg-2792, and Lys-2793 within α-dystroglycan binding site. And mutate sites are named from M1 to M5 through the N to C terminus, and recombinant proteins were rec-M1 to -M5. All mutations were verified by DNA sequencing.

The expression vector for the LG4 module, pCEP4-MullPURD, is a modified vector of pCEP4-WT-His (33), a derivative of pCEP4 (Invitrogen), and contains a BM-40 signal peptide, a hexahistidine tag, and a multicloning site along with the cytomegalovirus promoter and enhancer. In this vector the original hygromycin resistance gene was replaced by the puromycin resistance sequence from the pPUR vector (BD Bioscience). HindIII site in puromycin was deleted by the fill-in method using the DNA Blunting Kit (Takara, Ohtsu, Japan), and the hygromycin resistance gene was replaced in pCEP4 by PURD at SpeI and NruI. Various mutated laminin α1 LG4 module sequences were cloned into the HindIII and NotI sites of pCEP4-Mul-PURD.

Site-directed mutagenesis of LG4-5 was also performed similar to LG4. Three amino acids, Arg-2719, Lys-2720, and Arg-2721, within AG73 were substituted with alanine individually or all together. The mutant LG4-5 sequences were cloned into the HindIII and XhoI sites of pSecTag2/Hygro B (Invitrogen). The recombinant proteins were prepared as previously reported (34).

Expression and Purification of Recombinant Proteins—The recombinant LG4 expression vectors were transfected into 293 EBNA cells using FuGENE 6 (Roche Applied Science). The transfected cells were maintained for 2 days, then split at a 1:6 ratio in 10% FBS in Dulbecco’s modified Eagle’s medium and incubated with 5 μg/ml puromycin (Sigma) for 3 days for selection. The selected cells were maintained with 0.5 μg/ml puromycin. To prepare recombinant proteins, nearly confluent cells were cultured without serum for 3 days, and the conditioned medium was collected. The medium was cleared of debris by centrifugation, and Complete EDTA-free (Roche Applied Science) was added to decrease proteolysis. Nickel-charged agarose resins (ProBond, Invitrogen) were equilibrated with Dulbecco’s PBS (D-PBS; Invitrogen), pH 8.0, containing 10 mm

5 The abbreviations used are: FBS, fetal bovine serum; rec-LG4, recombinant wild-type LG4; rec-M1, rec-M2, rec-M3, rec-M4, and rec-M5, recombinant LG4 mutated at position M1, M2, M3, M4, and M5, respectively. LG4-5, recombinant wild-type LG4-5; rec-A1, rec-A2, rec-A3, and rec-A4, recombinant LG4-5 mutated at M1; D-PBS, Dulbecco’s phosphate-buffered saline; RT, room temperature.
imidazole and added to the conditioned medium. After incubation at 4 °C for 60 min, the resins were transferred to a column and washed with D-PBS, pH 8.0, containing 20 mM imidazole. His-tagged laminin α1 LG4 module recombinant proteins were eluted with D-PBS, pH 8.0, containing 250 mM imidazole. Purified proteins were dialyzed against D-PBS and quantified using the BCA protein assay kit with bovine serum albumin as a standard (Pierce). Purity was determined by reducing SDS-PAGE followed by Coomassie G-250 Blue (GelCode Blue, Pierce) staining and judged to >95%.

**Synthetic Peptides**—The peptides AG73 and EF-1 were manually synthesized with an amide at the C terminus by the Fmoc (9-fluorenylmethoxycarbonyl) strategy as previously described (17, 35). Purity and identity of the peptides were confirmed by an analytical performance liquid chromatography and an electrospray ionization mass spectrometer at the Central Analysis Center, Tokyo University of Pharmacy and Life Science.

**Biotinylated Heparin Binding Assay**—Heparin binding assays were performed in 96-well round-bottomed microtiter plates (Immuno-2HB, Dynax Technologies, Inc., Chantilly, VA) as previously described (15). Wells were coated with recombinant laminin α1 LG4 proteins in 50 μl of 10 mM Tris-HCl, pH 7.4, 100 mM NaCl, and 1 mM CaCl₂ overnight at 4 °C, and then biotinylated heparin, with an average mass of 12.5 kDa (Heparin-BH, Celsus Laboratories, Inc., Cincinnati, OH), was detected with streptavidin-horseradish peroxidase (Invitrogen). After incubation with the enzyme substrate (TMB-ELIZA, Pierce), the reaction was stopped by 1 M H₂SO₄, and the absorbance at 450 nm was measured.

**Antibodies**—Mouse monoclonal antibodies against human integrins α1 (FB12), α2 (P1E6), and β1 (6S6), rat monoclonal antibody against human integrin α6 (NK1-GoH3), and rabbit polyclonal antibody against human integrin α2 were purchased from Chemicon International, Inc. (Temecula, CA). Mouse monoclonal antibody against human vinculin (hVIN-1) was purchased from Sigma, and Cy3-conjugated AffiniPure F(ab')2 fragment donkey anti-mouse and anti-rabbit IgG (H+L) were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Glove, PA).

**Cell Attachment Assay**—96-Well round-bottomed microtiter plates (Immuno-2HB) were coated with recombinant proteins or laminin-1 from Engelbreth-Holm-Swarm (EHS) tumor plates (Immulon-2HB) were coated with recombinant protein (West Glove, PA). Cells were detached with 0.02% trypsin, EDTA (Invitrogen). Cell suspension was eluted with D-PBS, pH 8.0, containing 20 mM imidazole. Puriﬁed proteins were dialyzed against D-PBS and quantified using the BCA protein assay kit with bovine serum albumin as a standard (Pierce). Purity was determined by reducing SDS-PAGE followed by Coomassie G-250 Blue (GelCode Blue, Pierce) staining and judged to >95%.

**Cell Spreading Assay**—Glass Chamber Slides (Nalge Nunc International, Naperville, IL) were coated with proteins (3 μg/well) and prepared as described for the cell attachment assay. Human foreskin fibroblasts (2 × 10⁵ cells/well) were added to the wells and incubated for 150 min at 37 °C in a humidified atmosphere of 5% CO₂. After washing with prewarmed D-PBS containing calcium and magnesium (Invitrogen), cells were ﬁx with 4% paraformaldehyde D-PBS for 10 min at 37 °C. Fixed cells were mounted with GEL/MOUNT (Biomeda, Foster City, CA) and examined under an Axiovert 135 microscope (Carl Zeiss, Thornwood, NY) equipped with an AxioCam HRc CCD camera (Carl Zeiss). Digital images were obtained using MetaMorph 6.16 software.

**Immunofluorescence Assay of Cultured Cells**—Fibroblasts were prepared as described for the spreading assay. Cells were ﬁxed and permeabilized with prewarmed D-PBS containing calcium and magnesium (Invitrogen), 4% paraformaldehyde, 0.5% Triton X-100, and 5% sucrose for 3 min at 37 °C. Then, for further ﬁxation, slides were treated with 2% paraformaldehyde containing 5% sucrose D-PBS with calcium and magnesium for 10 min at 37 °C and blocked in D-PBS, 20% donkey serum (Jackson ImmunoResearch Laboratories, Inc.), and Mouse on Mouse (MOM) blocking reagent (Vector Laboratories, Inc., Burlingame, CA) as recommended by the manufacturer. Cells were incubated with anti-vinculin or integrin antibody in D-PBS, 0.05% Tween 20, and 7% MOM protein concentrate (Vector Laboratories, Inc.) to 10 μg/ml for 60 min at RT or 4 °C overnight. Bound antibodies and F-actin were visualized by secondary antibody diluted to 1:100 and Alexa Fluor 488 phalloidin (Invitrogen) diluted to 1:100 in D-PBS, 5% donkey serum, and 0.05% Tween 20 for 30 min at RT. After washing, cells were mounted with VECTASHIELD (Vector Laboratories) and examined under a LSM510 ﬂuorescent confocal microscope (Carl Zeiss).

**RESULTS**

**Mutant Recombinant Laminin α1 LG4 and LG4-5 Proteins**—Laminin α1 LG4-5 contains two tandem repeat modules, LG4 and LG5. We ﬁrst prepared recombinant LG4-5, LG4, and LG5 modules of laminin α1 and tested their activity for cell attach-
ment. LG4-5 and LG4 promoted cell attachment, but LG5 did not (see Fig. 3B and data not shown). These results suggest that LG4 plays a critical role for cell binding activity of laminin/α1. We, therefore, primarily focused on the LG4 module in this study.

We prepared five mutant LG4 proteins (rec-M1 to M5) with alanine substitutions; rec-M1 has a mutation within the AG73 sequence, rec-M2/-M3 has a mutation within the EF-1 sequence, and rec-M4/-M5 has a mutation within α-dystroglycan binding regions (Fig. 1, Table 1). Mutant rec-M1 protein has alanine substitutions at three consecutive positively charged residues, Arg-2719, Lys-2720, and Arg-2721 (M1 site), within AG73 that were shown to be critical for peptide AG73 cell attachment and heparin binding activity (14–16). Mutant rec-M2 and rec-M3 proteins contained a single alanine substitution at Arg-2757 (M2 site) and Asp-2763 (M3 site), respectively, which were important for peptide EF-1 integrin α2β1 binding activity (17). In addition, two mutant proteins, rec-M4 and rec-M5, were created within α-dystroglycan binding sites by alanine substitutions at the three positively charged residues in each site, Lys-2766, Arg-2768, and Lys-2770 (M4 site) and Lys-2791, Arg-2792, and Lys-2793 (M5 site), respectively (22). We also created four mutations within the M1 site of AG73 in recombinant LG4-5; alanine was substituted at residues Arg-2719, Lys-2720, and Arg-2721 either singly or all together. These mutant proteins were purified from the conditioned media of 293 EBNA cells or Cos7 cells that had been transfected with the expression vectors as described under “Experimental Procedures.” The purified proteins showed a single band with 90% purity in SDS-PAGE analysis under a reduced condition and also under a nonreduced condition (Fig. 1B).

Heparin Binding of rec-LG4 and Mutant LG4 Proteins—First we tested heparin binding of the recombinant wild-type LG4 protein (rec-LG4) and the mutant proteins (rec-M1 to rec-M5) using biotinylated heparin in a solid phase binding assay (Fig. 2). The purified proteins showed similar results...
using a heparin beads affinity assay (data not shown). These results indicate that the M1 sequence of AG73 and M5 sequence of the α-dystroglycan site is critical for heparin binding of LG4 and suggest that the EF-1 site is not involved in heparin binding of LG4. All of the basic charged residues (Arg-2719, Lys-2720, and Arg-2721) of the M1 site are critical for heparin binding since mutant LG4-5 with single substitutions at each of these residues lost heparin binding activity (data not shown).

**Binding of rec-LG4 and Mutant LG4 and LG4-5 Proteins to Lymphoid Cell Lines Expressing Syndecans-1, -2, and -4, and Glypican-1**—To assess the interaction of wild-type LG4 to cell surface heparan sulfate proteoglycans, we analyzed cell attachment activity of the LG4 proteins using ARH-77 B-lymphoid cell lines, which specifically express syndecan-1, -2, and -4 and glypican-1 on the cell surface (Fig. 3A) (31, 32). Wild-type rec-LG4 did not attach to parental ARH-77 cells (data not shown) or mock-transfected ARH-77 (ARH-Con) cells, which do not express cell surface heparan sulfate proteoglycans or integrin α2β1. However, rec-LG4 attached to cells that express syndecan-1 (ARH-Synd-1), syndecan-2 (ARH-Synd-2), and syndecan-4 (ARH-Synd-4) but not to glypican-1-expressing cells (ARH-Gpc-1).

To identify syndecan binding sites of LG4, we examined cell attachment activity of mutant LG4. We found that mutant rec-M1, rec-M4, and rec-M5 proteins do not attach to any of the syndecan-expressing cell lines, ARH-Synd-1, ARH-Synd-2, and ARH-Synd-4 (Fig. 3A). In contrast, rec-M2 and rec-M3 attached to these syndecan-expressing cells. None of the mutant LG4s attach to ARH-Gpc-1 cells similar to rec-LG4. These results indicate that LG4 attaches to cells expressing syndecan-1, -2, or -4 but not to cells that express glypican-1. The M1, M4, and M5 sites are essential for these interactions, and a mutation at any of these sites destroys the activity of LG4 attachment to the syndecan-expressing lymphoid cells. Our data also suggest that the EF-1 site is not necessary for LG4 attachment to syndecan-expressing cells.

To confirm the significance of the M1 site within LG4-5 for syndecan binding, we analyzed cell attachment of mutant LG4-5 proteins using ARH-Synd-1 cells (Fig. 3B). We found that mutant LG4-5 containing a single substitution at each of three RKR residues of the M1 site failed to attach to the cells. These results indicate that each of these basic residues is critical for syndecan binding of LG4-5 and suggest that the LG5 module is not involved in this activity.

**Fibroblast Attachment and Spreading on rec-LG4, Mutant LG4 Proteins, and Laminin-1**—To identify the active sites of LG4 that are important for cell attachment and spreading and their functional relationship, we analyzed the activities of mutant LG4 proteins for cell attachment and spreading using human foreskin fibroblasts, which express both syndecans (18) and integrin α2β1 (36). Fibroblasts attached to laminin-1, rec-LG4, rec-M2, and rec-M3 in a dose-dependent manner (Fig. 4A). However, fibroblasts did not attach to rec-M1, rec-M4, or rec-M5 substrates. These results indicate that heparin binding sites M1, M4, and M5 are all required for fibroblast attachment of rec-LG4, whereas the M2 and M3 sites are not necessary for fibroblast attachment.

We next analyzed heparin, EDTA, and peptide AG73 and EF-1 inhibition of fibroblast attachment on rec-LG4 and mutant LG4 proteins. Fibroblast attachment to rec-LG4 was significantly inhibited by heparin and the AG73 peptide but not by EDTA or the EF-1 peptide (Fig. 4B). In contrast, fibroblast attachment to laminin-1 was significantly inhibited by EDTA as previously reported (37) but not by heparin, AG73, or EF-1 (Fig. 4C). These results suggest that rec-LG4 attaches to fibroblasts through syndecans, and laminin-1-mediated fibroblast attach-

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**TABLE 1**

Mutant recombinant laminin α1 LG4 proteins

| Mutant | Alanine mutated sequence positions* | Name of mutation site in this study | Name of peptide and site | Binding molecule | Reference |
|--------|-------------------------------------|------------------------------------|--------------------------|-----------------|-----------|
| rec-M1 | Arg-2719, Lys-2720, Arg-2721        | M1                                 | AG73*                   | Heparin, syndecans | 12, 16    |
| rec-M2 | Arg-2757                            | M2                                 | EF-1*                   | Integrin α2β1    | 17        |
| rec-M3 | Asp-2763                            | M3                                 | EF-1*                   | Integrin α2β1    | 17        |
| rec-M4 | Lys-2766, Arg-2768, Lys-2770        | M4                                 | K3*                     | Heparin, α-Dystroglycan | 22     |
| rec-M5 | Lys-2791, Arg-2792, Lys-2793        | M5                                 | K1*                     | Heparin, α-Dystroglycan | 22     |

* The sequence positions and sites are from Andac et al. (22).  
* These active peptides were derived from the laminin α1 LG4 module. The LG4 sequence is shown in Fig. 1A.
FIGURE 3. Lymphoid cell attachment on rec-LG4, mutant LG4, and LG4-5 recombinant proteins. A, lymphoid cell lines ARH-Synd-1, ARH-Synd-2, ARH-Synd-4, ARH-Gpc-1, and ARH-Con, which express recombinant syndecan-1, syndecan-2, syndecan-4, glypican-1, and an empty vector, respectively, were plated on 96-well dishes coated with various recombinant LG4 proteins. B, recombinant LG4-5 proteins, which have various substitution mutations at M1 site (RKR) of AG73, were analyzed for ARH-Synd-1 cell attachment. Alanine was substituted at each or all of the RKR residues. Attached cells were stained with 0.2% crystal violet and dissolved in 1% SDS solution. The absorbance at 570 nm was measured. Experiments were in triplicate.*, p < 0.001.
ment is primarily through interactions with integrins such as α6β1, whose binding sites are located within the C-terminal triple-helical region of laminin α1 (38).

We also assessed the cell spreading activity of recombinant LG4 proteins and laminin-1 (Fig. 5). Laminin-1 and rec-LG4 induced fibroblast cell spreading, although rec-LG4 was slightly less active than laminin-1 (Fig. 5, A and B). Both rec-M2 and rec-M3 promoted cell attachment as described above, but rec-M3 did not spread well, whereas rec-M2 showed weak cell spreading activity (Fig. 5, C and D). Rec-M1, rec-M4, and rec-M5 did not show cell attachment and spreading (Fig. 5, C, F, and G). These results suggest that the M3 site is involved in the cell spreading activity of LG4.

Organization of Actin Stress Fibers and Vinculin Localization of Fibroblasts on rec-LG4, Mutant LG4 Proteins, and Laminin-1—We next looked at the organization of actin stress fibers and vinculin localization to focal contacts of fibroblasts on laminin-1, rec-LG4, rec-M2, and rec-M3 by immunostaining (Fig. 6). Fibroblasts on laminin-1 formed well organized actin stress fibers and focal contacts containing vinculin (Fig. 6A). The cells on rec-LG4 also formed actin stress fibers and focal contacts but to a lesser degree than those on laminin-1 (Fig. 6B). Fibroblasts weakly spread on rec-M2, but there were a few organized stress fibers and focal contacts containing vinculin (Fig. 6C). Fibroblasts did not spread well on rec-M3 (Fig. 6D), and there was actin accumulation at the edge of cells but no accumulation of vinculin. These results indicate that the M2 and M3 sites are important for cell spreading and focal contacts.

Effect of Heparin, EDTA, and Peptides AG73 and EF-1 on Fibroblast Spreading on rec-LG4 and Laminin-1—We examined the effect of heparin and EDTA on fibroblast spreading on the rec-LG4 substrate and compared their effects on laminin-1. Heparin inhibited fibroblast attachment on rec-LG4 (Fig. 7C) but not on laminin-1 (Fig. 7D). EDTA inhibited cell spreading on rec-LG4 but not cell attachment (Fig. 7E). In contrast, fibroblast attachment on laminin-1 was completely inhibited by EDTA (Fig. 7F). This indicates that LG4-mediated fibroblast attachment is dependent on heparin and syndecan interactions as shown in Fig. 4, but cell spreading on rec-LG4 is required for cation-dependent binding. Laminin-1-mediated cell attachment and spreading are required for cation-dependent binding.

Next we examined AG73 and EF-1 inhibition of cell spreading when fibroblasts were plated on rec-LG4 and laminin-1 (Fig. 7, G–J). Peptide AG73 reduced focal contacts and actin

FIGURE 4. Fibroblast attachment and inhibition of attachment on rec-LG4, mutant LG4 proteins, and laminin-1. A, fibroblasts were plated on 96-well dishes coated with various recombinant LG4 proteins and laminin-1. Attached cells were stained with 0.2% crystal violet, and the absorbance at 570 nm was measured. The effects of heparin, EDTA, and peptides AG73 or EF-1 on fibroblast attachment to rec-LG4 (B) and laminin-1 (C) were analyzed. In these assays 10 μg/ml heparin, 5 mM EDTA, and 100 μg/ml peptides were added to the cell suspension and incubated for 10 min at 37 °C before cell attachment was assayed as in A. *, p < 0.001.
stress fibers on rec-LG4 but not on laminin-1 (Fig. 7, G and H). In contrast, peptide EF-1 strongly inhibited cell spreading on rec-LG4 and weakly inhibited it on laminin-1 (Fig. 7, I and J).

These results indicate that the mechanisms of cell attachment and spreading are different between LG4 and laminin-1.

**Fibroblast Spreading on rec-LG4 and Laminin-1 through Integrin—Cell-matrix interactions via integrins are critical for organizing actin stress fibers and focal contacts.** We examined the effect of anti-integrin antibodies on fibroblast spreading on rec-LG4 and laminin-1 using double immunostaining for actin stress fibers and vinculin (Fig. 8A). Fibroblast spreading on rec-LG4 was inhibited by anti-integrin α2 and β1 antibodies but not by anti-integrin α1 or α6 antibodies. However, anti-integrin β1 and α6 antibodies inhibited cell attachment spreading on laminin-1, but neither anti-integrin α2 nor α1 antibodies inhibited cell spreading. Immunostaining of fibroblasts with anti-integrin antibodies showed that integrin α2 was localized at focal contacts on the LG4 substrate but not the laminin-1 substrate, whereas integrin β1 was present at
focal contacts on both substrates (Fig. 8B). Taken together these results indicate that integrin α2β1 plays a critical role in organizing actin stress fibers and focal contacts on LG4 and that integrin α6β1 is important for fibroblast attachment and spreading on laminin-1 as previously reported (38, 39).

DISCUSSION

The laminin α1 chain G domain interacts with extracellular matrix molecules and receptors and is implicated in cellular processes and assembly of the basement membrane (2). Several active sites of laminin α1 LG4 have been identified. It has been shown that the synthetic peptide AG73 promotes cell attachment and heparin and syndecan binding (12–16). More recently EF-1 was identified as an active peptide for integrin α2β1 binding (17). However, it is not clear whether these sites are active in LG4 and laminin-1 proteins or the nature of their functional relationship. In addition, two active sites, M4 and M5, located at the C-terminal region of LG4 have been identified as essential for heparin and α-dystroglycan binding (22). However, their cell attachment activity, syndecan binding, and functional relations with peptides AG73 and EF-1 are unknown. Here, we report that the M1 site in the AG73 sequence and the M4 and M5 sites for α-dystroglycan binding are utilized for primary cell attachment through syndecan and that the EF-1 site is critical for cell spreading through integrin α2β1. We also found that the mechanism of LG4-mediated fibroblast attachment is distinct from that of the whole laminin-1 in cell culture.

The mutation analyses revealed that the M1, M4, and M5 sites are all required for heparin binding of LG4 (though to a lesser extent with the M4 site) and that these sites are critical for the attachment of LG4 to lymphoid cells expressing syndecan-1, -2, and -4 but not to cells that express glypican-1. These results suggest that LG4 interacts with syndecans and not with glypican-1. These cell lines have been shown to produce heparan sulfate chains attached to the protein core of syndecans and glypican-1. The specific interaction of LG4 with syndecans may require not only heparan sulfate chains but also a core protein sequence of syndecans. It may also require different modifications of heparan sulfate chains between syndecans and glypican-1. The inhibition and mutation analyses also showed that the M2 and M3 sites in the EF-1 sequence are not essential for cell attachment but are required for fibroblast spreading on LG4. When LG4 was used as a substrate, anti-integrin α2 and β1 antibodies inhibited cell spreading but not cell attachment, whereas heparin inhibited cell attachment. These results suggest that the syndecan-binding sites M1, M4, and M5 are primarily utilized for cell attachment on LG4. A triple-amino acid substitution mutation may cause substantial conformational change that inactivates the biological function of mutant proteins. However, we found that even a single substitution mutation within each of the M1 sequences abolished heparin and cell binding activity of LG4-5 (Fig. 3B). In addition, the circular dichroism spectrum showed that there was no significant difference in conformation between the mutant LG4 proteins and wild-type LG4 protein (data not shown). These results suggest that the overall structure of the mutant LG4 proteins is likely maintained.

The crystal structure of the laminin α2 chain LG4-5 modules has been determined (40, 41). Because α2 LG4-5 shows 41% sequence homology to α1 LG4-5, we can predict the three-dimensional structure of the laminin α1 LG4 module (Fig. 1C). The M1 and M5 sites are located at the same edge in the three-
dimensional structure of LG4 and share an exclusive surface. This information together with our results that mutations in either one of the sites reduced syndecan and heparin interactions of LG4 (Figs. 2 and 3) suggest that the M1 site in AG73 and M5 in the α-dystroglycan binding region work in concert for binding of the LG4 module to syndecans. On the other hand, the M3 site, which is crucial for cell spreading, is located at the opposite side from M1 and M5, suggesting these sites may function independently for spreading.

Cell attachment and spreading are essential cellular processes for cell growth, migration, and invasion (42, 43). Cell spreading involves rapid rearrangement of actin stress fibers through integrins to form actin stress fibers. These signals are induced at integrin clustering points, called focal contacts, consisting of several molecules such as integrins, vinculin, paxillin, and actin, and integrins are thought to stabilize focal contacts by integrin-mediated “outside-in” signals (44, 45). Fibroblasts on laminin-1 form organized actin stress fibers and focal contacts as previously reported (46). Fibroblasts on rec-LG4 also form actin stress fibers and focal contacts (Fig. 6B). Fibroblasts on rec-M2 decreased the formation of actin stress fibers and focal contacts, and fibroblasts on rec-M3 completely lost these activities. Furthermore, focal contacts in fibroblasts on rec-LG4 were eliminated by anti-integrin α2 and β1 antibodies, whereas anti-integrin α1 and α6 antibodies had no effect (Fig. 8A). When fibroblasts on rec-LG4 were stained with anti-integrin α2 or β1 antibodies, accumulation of integrin α2 or β1 at focal contacts was observed (Fig. 8B). These results suggest that integrin α2β1 mediates fibroblast cell spreading on LG4. On the other hand, fibroblast attachment and spreading on laminin-1 were not inhibited by heparin and anti-integrin α2 antibody but were inhibited by anti-integrin α6 and β1 antibodies, suggesting that the integrin α6β1 binding site located at the C-terminal region of laminin-1 is the primarily site for cell attachment and spreading when laminin-1 is used as a substrate. Thus, the mechanism of cell attachment and spreading is different in laminin-1 and LG4. Recently, laminin α1 LG4-5-deficient mice were created (24). In these mutant mice, Reichert’s basement membrane was not formed, similar to whole laminin α1-deficient mice (23), despite the presence of the truncated N-terminal part of the laminin α1 chain, suggesting a critical role for laminin α1 LG4-5 in basement membrane assembly. The embryonic basement membrane between the visceral endoderm and epiblast was also not formed well, resulting in defects of epiblast development, suggesting an important function of laminin α1 LG4-5 for epiblast differentiation. LG4, as a part of laminin-1, may exert its activity in different cell types and developmental stages and may function as an anchoring site between the cell and matrix to form basement membrane in vivo.

**FIGURE 8.** Effects of integrin on actin stress fibers and focal contacts. A, fibroblasts were plated on 8-well glass chamber with 3 µg/well of rec-LG4 (a, c, e, and g) or laminin-1 (b, d, f, and h) in the presence of 10 µg/ml anti-integrin antibodies α1 (a and b), α2 (c and d), α6 (e and f), or β1 (g and h). Selected actin stress fibers and vinculin accumulation were visualized as in Fig. 6. B, immunostaining of fibroblasts plated on rec-LG4 (a and c) or laminin-1 (b and d) with anti-integrin α2 (a and b) and β1 antibodies (c and d). Integrin α2 was present in focal contacts on rec-LG4 but not on laminin-1. Integrin β1 was present in focal contacts on both rec-LG4 but not on laminin-1. Bar, 20 µm.
Spreading of fibroblasts on rec-LG4 was inhibited by EDTA and anti-integrin α2 or β1 antibodies, whereas fibroblasts cultured on rec-M3 did not spread. In contrast, cell attachment of fibroblasts on rec-LG4 was inhibited by heparin, and rec-M1 and rec-M5 did not show cell attachment activity. These results may suggest that fibroblast attachment and spreading on LG4 occurs in two steps; fibroblasts initially bind to syndecans, which induces focal enrichment of integrin α2β1 and then results in spreading. A similar stepwise mechanism for cell adhesion involving a cysteine-rich domain of ADAM12, a member of the transmembrane cell adhesion receptor ADAMs family, was proposed. A cysteine-rich domain of ADAM12 binds syndecan that promotes mesenchymal cell spreading through integrin β1 (47). In addition, several groups reported that the organization of actin stress fibers and focal contacts through integrin signals requires syndecans (48–52). For example, melanoma cell attachment to fibronectin through integrin α5β1 requires syndecan-4 clustering to organize focal contacts and actin stress fibers and up-regulation of protein kinase Cα signaling (48). It has also been reported that integrin αvβ3 signaling requires the accumulation of syndecan-1 for carcinoma cell attachment to vitronectin (49), and integrin α5β1 signaling requires the accumulation of syndecan-2 for carcinoma cell attachment to fibronectin (50). A similar cooperation between syndecans and integrin may regulate LG4-mediated cellular processes.

Cleavage of the laminin α chain G domain by endogenous proteolytic processing has been reported (25–30). Unprocessed laminins and cleaved G domain fragments may have distinct functions in vitro and in vivo. For example, laminin-5 containing the α3 chain without LG4-5 promotes keratinocyte adhesion, but the α3 chain with LG4-5 induces keratinocyte migration to the leading edge in the wound (27). The cleavage in the α2 LG3 module is required for clustering of acetylcholine receptors and for neuromuscular junction formation in concert with agrin (26). Laminin α1 may also be cleaved in the G domain since anti-LG4-5-specific antibody stained the epicalontal cone of laminin α1 LG4-5-deficient mice, but antibodies specific to the N-terminal laminin α1 chain failed to stain, suggesting that the LG4-5 fragment is generated for epicalontal cone development (24). Thus, the cleavage product, laminin α1 LG4, may have a unique functional role in development, cellular processes, and basement membrane formation. Laminin-1 is a large multifunctional domain protein. Its proteolytic cleavage would provide a mechanism by which new in vivo function was generated at specific times in development.

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