Cyclic Peptides from the Loop Region of the Laminin α4 Chain LG4 Module Show Enhanced Biological Activity over Linear Peptides†

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ABSTRACT: Laminins, heterotrimeric glycoproteins in the basement membrane, are involved in diverse biological activities. So far, five α, three *β*, and three *γ* chains have been identified, and at least 15 laminin isoforms exist composed of various combinations of the different three chains. The major cell-surface receptors for laminins are integrins and proteoglycans, such as dystroglycans and syndecans. Previously, we reported that synthetic peptide A4G82 (TLFLAHGRLVFM, mouse laminin α4 chain residues 1514−1525) showed strong cell attachment and syndecan binding activities. On the basis of the crystal structure of the LG module and sequence alignment, A4G82 is located in the connecting loop region between *β*-strands E and F in the laminin α4 chain LG4 module. Here, we have focused on the structural importance of this E−F loop region for the biological activity of the α4 chain LG4 module. To determine the importance of the loop structure, we synthesized peptide A4G82X (*cyclo*-A4G82X, Cys-TLFLAHGRLVFX-Cys, X= norleucine), which was cyclized via disulfide bridges at both the N- and C-termini. The cyclic peptides derived from A4G82X inhibited the heparin binding activity of the α4 chain G domain and promoted HT-1080 cell attachment better than the corresponding linear peptides. We determined FLAHGRLVFX as a minimal sequence of *cyclo*-A4G82X important for cell adhesion and heparin binding using a series of truncated peptides. Moreover, HT-1080 cell attachment to the cyclic peptides was more efficiently blocked by heparin than cell attachment to the linear peptides. Furthermore, the cyclic peptides showed significantly enhanced syndecan-2-mediated cell attachment activity. These results indicate that the activity of A4G82 is highly conformation-dependent, suggesting that the E−F loop structure is crucial for its biological activity.

Laminins, multifunctional heterotrimeric glycoproteins that are found mainly in basement membranes, consist of three distinct chains, α, *β*, and *γ*. Five α, three *β*, and three *γ* chains have been identified, and at least 15 laminin isoforms have been reported that contain various combinations of α, *β*, and *γ* chains (*1*−*4*). Laminins have diverse biological activities, including promotion of cell adhesion, migration, neurite outgrowth, angiogenesis, tumor metastasis, and wound healing (*5*). So far, more than 20 cell surface receptors have been determined for laminins, such as integrins, dystroglycans, and syndecans (*6*). Several biologically active sites in laminin-1, the best characterized laminin isoform consisting of α1, *β*1, and *γ*1 chains, have been found by means of proteolytic fragments, recombinant proteins, and synthetic peptides (*7*, *8*). Previously, cell adhesive sequences in laminin-1 were systematically screened by using 673 overlapping synthetic peptides covering the entire protein (*9*−*12*). Most of the active peptides were located in the globular domains and found to play an important role in binding to cell surface receptors in a cell-type-specific manner (*13*, *14*). Several peptides were found to interact with integrins and syndecans (*15*−*18*).

The laminin α chains are generally the larger of the three subunits and contain a unique C-terminal globular domain, called the G domain. The G domain contains five tandem laminin G-like modules (LG1−LG5). In vertebrates, the laminin α chains are expressed in a tissue- or developmental-stage-specific manner or both (*5*). The α4 chain is mainly expressed in endothelial cells and in adipocytes, which originate from mesenchymal components (*19*−*21*). The α4 chain is also found in adult tissues, such as the brain, spleen, liver, kidney, and testis (*22*). Recently, we identified several biologically active sequences in the mouse and human laminin α4 chain G domains using recombinant proteins and a large number of synthetic peptides, which cover the entire

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α4 chain G domain. Some of the active peptides were found to interact with either syndecan-2 or syndecan-4, transmembrane heparan-sulfate proteoglycans (*23, 24*). Site-direct mutagenesis analysis of recombinant laminin α4 chain LG4 protein demonstrated that the arginine residue in position 1520 was critical for the heparin binding activity (*25, 26*). This arginine residue is included in synthetic peptide A4G82 (TLFLAHGRLVFM, mouse laminin α4 chain residues 1514−1525), the most potent heparin binding and cell adhesive peptide in the α4 chain LG4 module.

The crystal structure of the laminin α2 chain LG5 module revealed that it forms a 14-stranded β-sheet (A−N) sandwich structure (*27*). On the basis of structure-based sequence alignment, the A4G82 peptide is located between β-strands E and F in the α4 chain LG4 module and forms a connecting loop structure in the native protein (*23, 28*). The connecting loop regions between β-strands E and F in the α1−α5 chain LG4 modules showed various biological activities (*29*). The E−F connecting loop regions in the α chain LG4 modules play an important biological role.

In this paper, we focused on the structural significance of the connecting loop region between β-strands E and F in the α4 chain LG4 module. To examine the biological activities of this E−F loop region, we prepared a cyclic peptide derived from the A4G82X peptide, in which a methionine residue was replaced with norleucine. We also prepared a series of N-terminally truncated cyclic A4G82X peptides. These cyclic peptides inhibited the heparin binding activity of the laminin α4 chain G domain recombinant protein and promoted HT-1080 human fibrosarcoma cell attachment in a dose-dependent manner better than the corresponding linear peptides. Further, HT-1080 cell attachment activity to these peptides was heparin-dependent and involved binding to cell-surface syndecan-2.

MATERIALS AND METHODS

Synthetic Peptides. All linear peptides were synthesized manually using an Fmoc-based solid-phase strategy and prepared in the C-terminal amide form as previously described (*10*). 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 or NovaSyn TGR resin (Novabiochem). Dimethylformamide was used during the synthesis as a solvent. For condensation, diisopropylcarbodiimide/*N*-hydroxybenzotriazole was employed, and for deprotecton of N^{α}-Fmoc groups, 20% piperidine in dimethylformamide was employed. The following side chain protecting groups were used: for asparagine, glutamine, and histidine, trityl; for aspartic acid, glutamic acid, serine, threonine, and tyrosine, *tert*-butyl; for arginine, 2,2,5,7,8-pentamethylchroman-6-sulfonyl or 2,2,5,7,8-pentamethyldihydrobenzofuran-5-sulfonyl; for lysine, *tert*-butoxycarbonyl. The resulting protected peptide resins were deprotected and cleaved from the resin using trifluoroacetic acid/thioanisole/*m*-cresol/ethanedithiol/H_2O (80:5:5:5:5, v/v/v/v/v) at 20 °C for 3 h. The crude peptides were precipitated and washed with ethyl ether and then purified by reverse-phase high performance liquid chromatography (HPLC) using a Mightysil RP-18 column (Kanto Chemical Co., Inc., Tokyo, Japan) and a gradient of water/acetonitrile containing 0.1% trifluoroacetic acid.

Cyclic peptides were synthesized using the protocol described above with some modifications. Since the methionine residue is easily oxidized during the cyclization step, the methionine residue was replaced with norleucine. Protected peptide resins were prepared on a NovaSyn TGR resin by the Fmoc-based solid-phase peptide synthesis. Treatment of the protected peptide resin with a solution of trifluoroacetic acid/1,2-ethanedithiol/H_2O (95:2.5:2.5, v/v/v) at 20 °C for 3 h afforded a linear peptide, which was precipitated with ethyl ether. The linear peptides were cyclized by oxidation in a mixture of H_2O/acetic acid/dimethyl sulfoxide (45:10:45 v/v/v) at room temperature for 2 days and purified by reverse-phase HPLC using a Cosmosil 5C18-ARII column (Nakalai Tesque, Kyoto, Japan) with a gradient of water/acetonitrile containing 0.1% trifluoroacetic acid.

Purity and identity of the peptides were confirmed by analytical HPLC and by either a fast atom bombardment mass spectrometer (the GC-MS and NMR Laboratory, Graduate School of Agriculture, Hokkaido University) or an ion-spray mass spectrometer.

Recombinant Protein. A recombinant protein containing the mouse laminin α4 chain G domain (residues 833−1815) with the c-Myc sequence at the C-terminus (rec-α4G) was expressed using *dhfr*-deficient Chinese hamster ovary (CHO) cells. The protein was purified using a heparin affinity column (HiTrap, Amersham Biosciences, Uppsala, Sweden) and gel filtration chromatography (Superdex 200, Amersham Biosciences) as described previously (*23, 25*).

Conditioned medium of CHO cells expressing rec-α4G (1000 mL) was collected. Since laminin α chain G domains have high affinity for heparin, heparin affinity chromatography was used for the purification of rec-α4G in the first step. The heparin affinity column was equilibrated in 10 mM Tris-HCl (pH 7.4) containing 150 mM NaCl, 2 mM EDTA, and 0.5 mM *N*-ethylmaleimide (buffer A). After the conditioned medium was applied to the heparin affinity column, rec-α4G was eluted at salt concentrations of 400 mM, 450 mM, 500 mM, and 1 M, and each fraction was analyzed by SDS gel electrophoresis. Based on the analysis of the SDS−PAGE, the fractions eluted at the 450 and 500 mM salt concentration were further purified using gel filtration chromatography. The high purity of rec-α4G was confirmed by both SDS−PAGE and Western blotting analyses. The protein concentration was determined by BCA assay (Pierce, Rockford, IL) with bovine serum albumin (BSA) as standard. The yield of rec-α4G was approximately 1.4 mg.

Cells and Culture. HT-1080 human fibrosarcoma cells and 293T human renal epithelial cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin (Invitrogen). 293T cells stably overexpressing syndecan-2 or glypican-1 were cultured in the presence of 0.4 μg/mL puromycin (Sigma, St. Louis, MO) as described previously (*18*). The cells were maintained in a humidified 5% CO_2, 95% air atmosphere.

Effect of the Peptides on Heparin Binding Activity of rec-α4G. The effect of peptides on the heparin binding of rec-α4G was tested using heparin−Sepharose beads as previously described (*23*). The rec-α4G protein (1.2 μg) was incubated with heparin−Sepharose beads (1 mg, Amersham Biosciences) in 70 μL of 10 mM Tris-HCl (pH 7.4)

Table 1: Biological Activities of the Linear and Cyclic Peptides Derived from A4G82

Peptide	Sequence[a] (X= Nle)	rec-α4G inhibition		Heparin binding[d]	Cell attachment[e]
		Heparin binding[b]	Cell attachment[c]		
A4G82	TLFLAHGRLVFM	+ (96 μM)	++	N. D.	+
A4G82X	TLFLAHGRLVFX	+ (68 μM)	++	+	+
A4G82Xa	LFLAHGRLVFX	+ (127 μM)	++	+	+
A4G82Xb	FLAHGRLVFX	-	++	+ (weak)	++
A4G82Xc	LAHGRLVFX	-	-	-	-
cyc-A4G82X	CTLFLAHGRLVFXC	++ (16 μM)	++	++	++
cyc-A4G82Xa	CLFLAHGRLVFXC	++ (31 μM)	-	++	+
cyc-A4G82Xb	CFLAHGRLVFXC	+ (105 μM)	++	++	+++
cyc-A4G82Xc	CLAHGRLVFXC	-	-	-	-
AG73[f]	RKRLQVQLSIRT	N. D.	N. D.	++	+++

[a] Sequence of the synthetic peptides are given in the single-letter code. All peptides have C-terminal amides. The single letter X denotes the norleucine residue. [b] For rec-α4G heparin binding inhibiiton assays, heparin binding was evaluated as shown in Figure 1: ++, completely inhibited; +, weakly inhibited; −, no inhibition; ND, not determined. IC_{50} values are indicated in parentheses. Duplicate experiments gave similar results. [c] For rec-α4G cell attachment inhibition assays, wells were coated with rec-α4G (0.5 μg/well) on 96-well plates, and cells were preincubated with 0.1 mg/mL of each peptide as described under Materials and Methods. Inhibition of cell attachment was evaluated on the following subjective scale: ++, completely inhibited; +, moderately inhibited; −, no inhibition; ND, not determined. Triplicate experiments gave similar results. [d] For heparin binding assay, heparin−Sepharose beads (1 mg) and each peptide (200 μM) were incubated for 1 h. The amount of peptides bound to the beads was quantified by the amino acid analysis. Heparin binding activity was evaluated on the following subjective scale: ++, binding comparable to that of AG73; +, weak binding compared with that of AG73; −, no binding; ND, not determined. Duplicate results gave similar results. [e] For cell attachment assays, various amount of peptides were coated on 96-well plates as described under Materials and Methods. Cell attachment was evaluated on the following subjective scale: +++, adhesion comparable to adhesion to AG73; ++, moderate adhesion compared with adhesion to AG73; +, weak adhesion; −, no adhesion. Triplicate experiments gave similar results. [f] AG73 (RKRLQVQLSIRT, α1 chain, residues 2719-2730) (*9*) was used as a positive control.

containing 100 mM NaCl (buffer B) in the presence of various concentrations of peptides at 4 °C for 1 h, and then, the beads were collected by centrifugation. The supernatant was removed, and the beads were washed twice with buffer B. The rec-α4G protein bound to the beads was extracted with SDS−PAGE sample buffer (10 μL) and analyzed by SDS−PAGE in 8% acrylamide gels under reducing conditions, followed by staining with Coomassie brilliant blue. The intensity of the bands was quantified by NIH image 1.62 software. Assays were carried out in duplicate, and each experiment was repeated at least twice.

Heparin Binding Activity of the Peptides. Heparin binding activity of the peptides was tested using heparin−Sepharose beads. Heparin−Sepharose beads (1 mg) were incubated with various peptides (200 μM) in 70 μL of buffer B for 1 h at 4 °C. After centrifugation, the supernatant was removed, and the beads were washed twice with buffer B. Then, the amount of peptides bound to the heparin−Sepharose beads was quantified by amino acid analysis (Center for Instrumental Analysis, Hokkaido University). Assays were carried out in triplicate.

Cell Attachment Assay. Cell attachment assays were performed in 96-well plates (Nunc, Inc., Naperville, IL) coated with either various amounts of synthetic peptides or the rec-α4G protein. For peptide coating, various amounts of peptides in 50 μL of Milli-Q water were added to the wells and dried overnight at room temperature. For rec-α4G coating, various amounts of rec-α4G in 50 μL of buffer A were incubated overnight at 4 °C. The substrate-coated wells were blocked with 150 μL of 1% BSA in DMEM at room temperature for 1 h and then washed twice with DMEM containing 0.1% BSA. Cells were detached with 0.02% trypsin−EDTA (Invitrogen) and recovered with DMEM containing 10% FBS at 37 °C for 20 min. After being washed with DMEM, cells (20 000 cells of HT-1080, 30 000 cells of 293T in 100 μL) were added to the wells and incubated at 37 °C for 1 h. The attached cells were stained with 0.2% crystal violet aqueous solution in 20% methanol for 10 min. After the wells were washed with Milli-Q water, 1% SDS (150 μL) was used to dissolve the stained cells, and the optical density at 570 nm was measured using a model 550 microplate reader (Bio-Rad Laboratories, Hercules, CA).

For inhibition of cell attachment with heparin or peptides, HT-1080 cells were incubated for 30 min at 37 °C in the presence of various concentrations of heparin or peptides. Attached cells were measured as described above. All assays were carried out in triplicate, and each experiment was repeated at least three times.

RESULTS

Effect of the Linear and Cyclic Peptides Derived from A4G82 on the Heparin Binding Activity of rec-α4G. Previously, the A4G82 sequence, located on the LG4 module of laminin α4 chain, was shown to have a strong inhibitory effect on the heparin binding activity of rec-α4G (*23*). Here, we first examined the inhibitory effect of A4G82 and the linear and cyclic A4G82X peptides on the heparin binding of rec-α4G (Table 1). A4G82 inhibited rec-α4G heparin

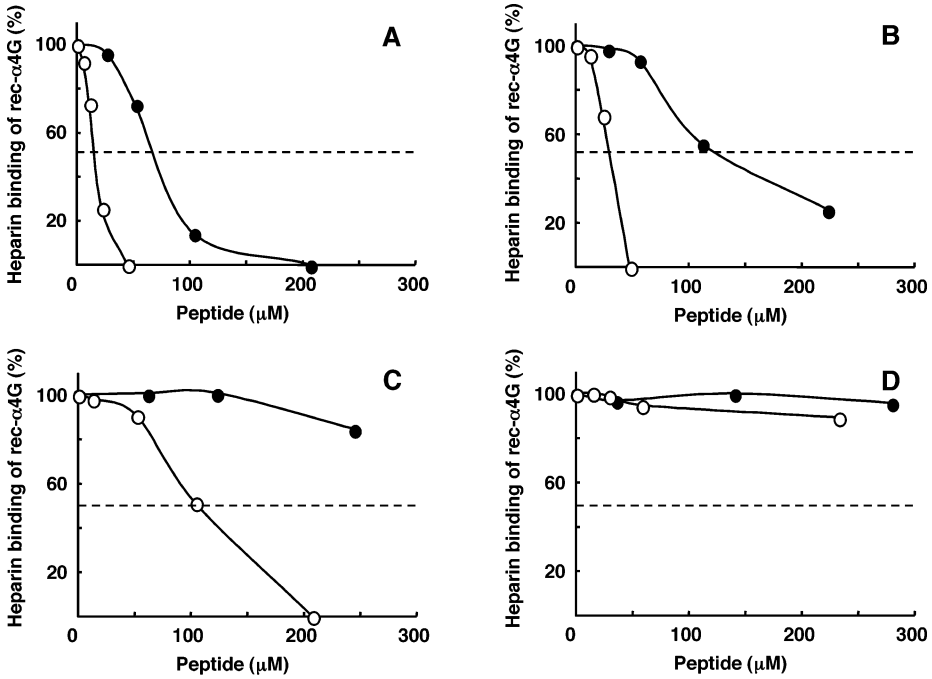

FIGURE 1: Effect of peptides on heparin binding to rec-α4G. Heparin−Sepharose beads (1 mg), together with various amount of peptide and rec-α4G (1.2 μg), were incubated in 70 μL of 10 mM Tris buffer (pH 7.4) at 4 °C for 1 h. After the beads were washed, the rec-α4G bound to the heparin−Sepharose beads was analyzed by SDS−PAGE. The intensity of the bands was quantified using NIH image 1.62 software. Filled and open circles in each panel indicate linear and cyclic peptides, respectively. The panels show the inhibitory effect of the peptides (A) A4G82X and *cyclo*-A4G82X, (B) A4G82Xa and *cyclo*-A4G82Xa, (C) A4G82Xb and *cyclo*-A4G82Xb, and (D) A4G82Xc and *cyclo*-A4G82Xc. Duplicate experiments showed similar results. IC_{50} values were as follows: A4G82X (68 μM), *cyclo*-A4G82X (16 μM), A4G82Xa (127 μM), *cyclo*-A4G82Xa (31 μM), and *cyclo*-A4G82Xb (105 μM). Dashed lines indicate IC_{50} values.

binding activity, and the IC_{50} value was 96 μM. A4G82X inhibited the heparin binding of rec-α4G in a dose-dependent manner, and the IC_{50} value was 68 μM. These results suggest that the inhibitory effect of A4G82X is comparable to that of A4G82 and that the methionine residue is replaceable with norleucine without loss of activity. The *cyclo*-A4G82X peptide showed a strong dose-dependent inhibitory effect on heparin binding to rec-α4G (Figure 1A). *cyclo*-A4G82X showed a much stronger inhibitory effect (IC_{50} = 16 μM) than A4G82X (Figure 1A). These results suggest that this loop region is important for the heparin binding activity of the laminin α4 chain LG4 module and that the loop structure may be important for the activity.

Critical Sequence in the Cyclic A4G82 Peptide for Heparin Binding Activity. To determine the minimal sequence in the *cyclo*-A4G82X peptide for the heparin binding activity, a series of N-terminally truncated peptides were prepared and evaluated for their inhibitory effect on the heparin binding to rec-α4G (Figure 1B−D). A4G82Xa (IC_{50} = 127 μM), in which the N-terminal threonine residue was deleted from A4G82X, had a reduced inhibitory effect. Neither A4G82Xb, in which the N-terminal leucine residue was deleted from A4G82Xa, nor A4G82Xc, in which the N-terminal phenylalanine residue was deleted from A4G82Xb, inhibited the heparin binding of rec-α4G. On the other hand, *cyclo*-A4G82X (IC_{50} = 16 μM) and *cyclo*-A4G82Xa (IC_{50} = 31 μM) showed a stronger inhibitory effect than the linear peptides. *cyclo*-A4G82Xb (IC_{50} = 105 μM) still had a strong inhibitory effect, while the linear peptide was inactive. The inhibitory effect of these active peptides was dose-dependent. Previously, it was reported that A4G82g, in which the C-terminal methionine residue was deleted from A4G82, completely lost heparin binding (data not shown) and cell attachment activity (*23*). Taken together, the results demonstrate that FLAHGRLVFX in A4G82X is a crucial sequence for the inhibitory effect on rec-α4G heparin binding. The results shown above also demonstrate that the cyclic peptides had an increased inhibitory effect over the linear peptides. We conclude that the activity of these peptides depends on a loop conformation.

Heparin Binding Activity of the Peptides. To test whether cyclizing the peptides increases heparin binding activity, we quantified the amount of the peptides bound to heparin−Sepharose beads by amino acid analysis (Figure 2). The AG73 peptide (RKRLQVQLSIRT, mouse laminin α1 chain residues 2719−2730), known to have a strong heparin binding activity, was used as a positive control (*14*, *30*). A4G82X and A4G82Xa showed a strong heparin binding activity, but A4G82Xb had weaker activity. The heparin binding activity of *cyclo*-A4G82X and *cyclo*-A4G82Xa was increased, and *cyclo*-A4G82Xb showed a significant increase in heparin binding activity compared to the linear peptide. The heparin binding activity of A4G82Xc and *cyclo*-A4G82Xc was lost as expected on the basis of the results of Figure 1. These results demonstrate that certain peptides had a strong heparin binding activity and cyclizing the linear peptides further increased the activity.

Effect of the Peptides on rec-α4G-Induced Cell Attachment Activity. Next, we evaluated the inhibitory activity of the peptides to rec-α4G-induced cell attachment (Figure 3). A4G82X, A4G82Xa, A4G82Xb, *cyclo*-A4G82X, and *cyclo*-A4G82Xb showed strong inhibitory effects on HT-1080 human fibrosarcoma cell attachment to rec-α4G. In contrast, *cyclo*-A4G82Xa had weaker inhibitory activity for rec-α4G cell attachment. Both A4G82Xc and *cyclo*-A4G82Xc did not have much effect on HT-1080 cell attachment to rec-α4G,

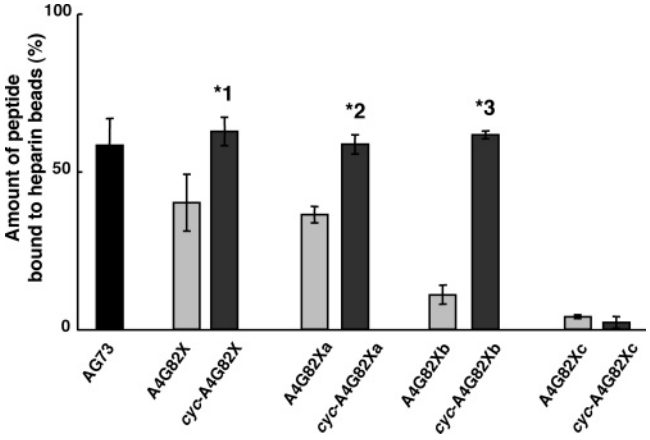

FIGURE 2: Heparin binding activity of peptides. Heparin−Sepharose (1 mg) and peptides (200 μM) were incubated in 70 μL of 10 mM Tris buffer (pH 7.4) at 4 °C for 1 h. After the beads were washed, the peptides bound to the heparin beads were quantified by amino acid analysis. The ratio of the bound peptides to the total amount of the peptides was calculated. Each value represents the mean of three separate determinations ± SD. Triplicate experiments gave similar results. *1 denotes $p < 0.05$; *2 denotes $p < 0.001$; *3 denotes $p < 0.0001$.

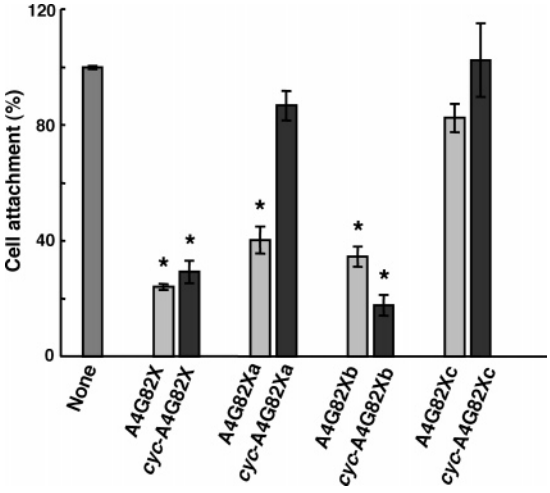

FIGURE 3: Effect of peptides on HT-1080 cell attachment to rec-α4G. Ninety-six-well plates were coated with rec-α4G (0.5 μg/well). The assay was carried out in the presence of 0.1 mg/mL added peptides. After a 30-min incubation at 37 °C, the attached cells were stained with crystal violet and lysed by 1% SDS. The OD (570 nm) was measured. Each value represents the mean of three separate determinations ± SD. Triplicate experiments gave similar results. Asterisk denotes $p < 0.0001$.

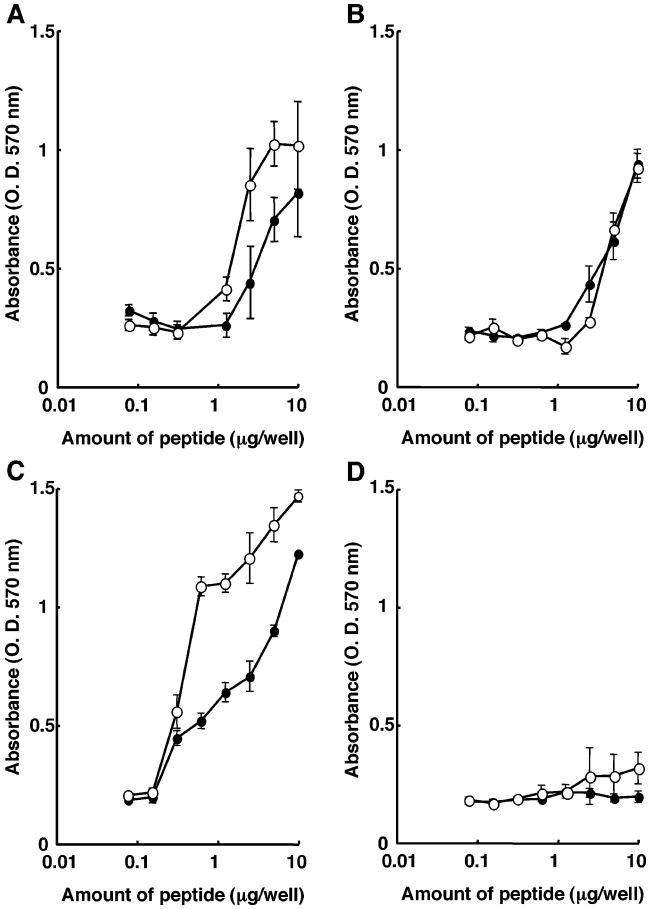

FIGURE 4: HT-1080 cell attachment activity to the peptides. Ninety-six-well plates were coated with various amount of peptides, and HT-1080 human fibrosarcoma cells were added to the wells. After a 1-h incubation, the attached cells were stained with crystal violet and lysed with 1% SDS. OD_{570} was measured. Filled and open circles in panels indicate linear and cyclic peptides, respectively. Cell attachment activities of (A) A4G82X and *cyclo*-A4G82X, (B) A4G82Xa and *cyclo*-A4G82Xa, (C) A4G82Xb and *cyclo*-A4G82Xb, and (D) A4G82Xc and *cyclo*-A4G82Xc are shown. Each value represents the mean of three separate determinations ± SD. Triplicate experiments gave similar results.

as expected. These results indicate that these cyclic peptides can function to block attachment to the recombinant protein as effectively as the linear peptides.

Cell Attachment Activity of the Cyclic Peptides Derived from A4G82X. To evaluate cell attachment activity and determine the active core sequence of *cyclo*-A4G82X, we tested the *cyclo*-A4G82X and its N-terminally truncated peptides, as well as the linear ones, for HT-1080 cell attachment activity on peptide-coated plates. A4G82X promoted cell attachment activity in a dose-dependent manner (Figure 4A). A4G82Xa promoted cell attachment activity similar to that of A4G82X (Figure 4B). A4G82Xb showed strong cell attachment activity comparable to that of A4G82X (Figure 4C), but A4G82Xc had no activity (Figure 4D). *cyclo*-A4G82X and *cyclo*-A4G82Xb showed an enhanced cell adhesive activity over the linear peptides (Figure 4A,C). The cell attachment activity of *cyclo*-A4G82Xa was strong but similar to that of the linear peptide, A4G82Xa (Figure 4B). *cyclo*-A4G82Xc did not promote HT-1080 cell attachment activity (Figure 4D). These results demonstrate that the cell attachment active core sequence of the *cyclo*-A4G82X peptide is FLAHGRLVFX, the same as that of the linear A4G82X peptide.

Effect of Heparin on HT-1080 Human Fibrosarcoma Cell Attachment to the Peptides. Next, we evaluated the effect of heparin on HT-1080 cell attachment to the peptides (Figure 5). Heparin strongly inhibited HT-1080 cell attachment to A4G82X, A4G82Xa, A4G82Xb,*cyclo*-A4G82X, *cyclo*-A4G82Xa, and *cyclo*-A4G82Xb in a dose-dependent manner. Moreover, HT-1080 cell attachment to the cyclic peptides was much more sensitive to heparin than that of the linear peptides. EDTA did not inhibit HT-1080 cell attachment to these peptides (data not shown), indicating that integrins, one of the major receptors for laminins, are not involved in this interaction. The results suggest that these peptides interact with membrane-associated heparin/heparan sulfate

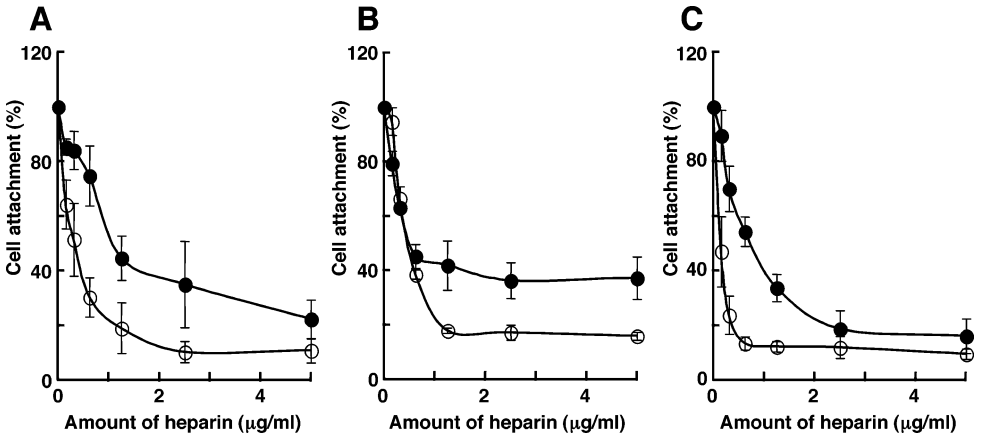

FIGURE 5: Effect of heparin on HT-1080 cell attachment to the peptides (A) A4G82X and *cyclo*-A4G82X, (B) A4G82Xa and *cyclo*-A4G82Xa, and (C) A4G82Xb and *cyclo*-A4G82Xb. Filled and open circles in panels indicate linear and cyclic peptides, respectively. Ninety-six-well plates were coated with 5 μg of peptides. Various concentrations of heparin were added to the wells, and then HT-1080 human fibrosarcoma cells were added. After a 30-min incubation, attached cells were measured as described previously. Each value represents the mean of three separate determinations ± SD. Triplicate experiments indicate similar results.

proteoglycans and that the cyclic peptides bind to the cell-surface proteoglycans better than the linear peptides.

Syndecan-2 or Glypican-1 Overexpressing 293T Cell Attachment Activity to the Peptides. Since HT-1080 cell attachment to the peptides was significantly inhibited by heparin, we anticipated that these peptides interacted with the cells through heparin/heparan sulfate proteoglycans, such as syndecans and glypicans. The expression of syndecans and glypicans in HT-1080 cells was examined by reverse transcriptase PCR and immunoblotting analysis as described previously (*18*). Syndecan-2 and glypican-1 were detected by reverse transcriptase PCR and by immunoblotting in HT-1080 cells (data not shown). Thus, it is possible that syndecan-2 may be responsible for HT-1080 cell attachment to the peptides since syndecans are known to bind laminin (*16*). We used 293T cells that overexpress syndecan-2 to test this hypothesis (*18*). We also obtained glypican-1 overexpressing 293T cells to confirm specificity of the peptides to syndecans. 293T cell attachment activity to the cyclic peptides, *cyclo*-A4G82X, *cyclo*-A4G82Xa, and *cyclo*-A4G82Xb, was significantly increased by overexpressing syndecan-2. Nevertheless, overexpression of glypican-1 did not significantly affect cell attachment activity, except for the *cyclo*-A4G82Xb (Figure 6). These results suggested that syndecan-2 is able to function as a cell surface receptor for adhesion to these peptides.

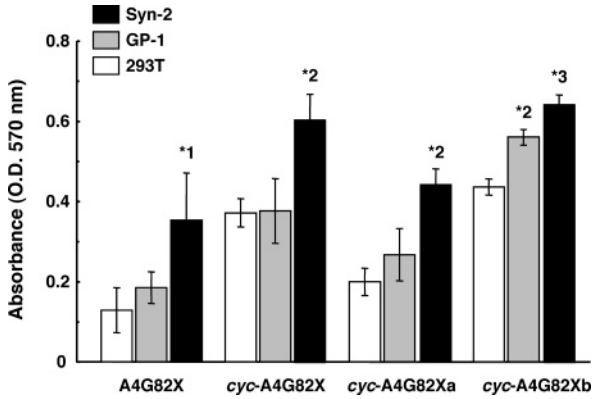

FIGURE 6: Syndecan-2 (Syn-2) or glypican-1 (GP-1) overexpressing 293T cell attachment to the peptides. Peptides (A4G82X, 1.5 μg/well; *cyclo*-A4G82X, 1.5 μg/well; *cyclo*-A4G82Xa, 2.0 μg/well; *cyclo*-A4G82Xb, 1.5 μg/well) were coated on 96-well plates. The cell attachment assay was carried out as described in the legend of Figure 4. Each value represents the mean of three separate determinations ± SD. Triplicate experiments showed similar results. *1 denotes $p < 0.05$; *2 denotes $p < 0.005$; *3 denotes $p < 0.0005$.

DISCUSSION

Previously, we identified the A4G82 peptide as the most potent heparin binding site in the laminin α4 chain LG4 module (*23*). On the basis of a structural-based sequence alignment, A4G82 is located on the connecting loop region between the E and F strands. Homologous peptides from this site showed cell-type-specific activities, indicating that the E−F connecting loop region in the α chain LG4 modules is biologically important (*23, 29*). Since A4G82 is located in the loop region and thought to be exposed on the surface of the protein, we hypothesized that the loop structure of this site is biologically important. Previously, the importance of loop structure for biological activity was demonstrated for other biologically important sites using cyclic peptides. Cyclization of a peptide restricts the conformation so that it may be possible to obtain more biologically active compounds that are conformation-dependent. It is also possible to use this approach to confirm that a loop structure is an active conformation in a protein. For example, RGD peptide, the first characterized cell-adhesive sequence in fibronectin, is located in a loop (*31*), and cyclic RGD peptides showed increased inhibitory activity for cell adhesion to fibronectin substrates (*32*). Additionally, the cyclic EF-1 peptide, located in connecting loop region between the E and F strands of the laminin α1 chain LG4 module, showed increased interaction with α2β1 integrin (*29*). Analysis of syndecan binding using cyclized peptides has not been determined yet. In this report, we focused on the structural importance of the connecting loop region between the E and F strands of the α4 chain LG4 module, which mainly interacts with syndecans. We focused on a syndecan binding peptide, A4G82, located on the α4 chain LG4 module, and synthesized the cyclized form of A4G82X (*cyclo*-A4G82X).

Using various truncated forms of the A4G82X peptide, we identified the minimal active sequence for heparin binding. We also found that the cyclized peptides were more

active than the linear forms for heparin binding using competition for the recombinant protein as well as direct binding assays. Since our attachment data with the peptides showed that the cyclized forms were more active than the linear peptides, we hypothesize that the structure of this region is important for its biological activity.

Previously, it was demonstrated that HT-1080 human fibrosarcoma cells adhered well to rec-α4G (*23*). Thus, we next examined the effect of the peptides on HT-1080 cell attachment to rec-α4G. A4G82X, A4G82Xa, and A4G82Xb showed a strong inhibitory effect on rec-α4G-mediated cell adhesion, but A4G82Xc did not. On the other hand, *cyclo*-A4G82X and *cyclo*-A4G82Xb strongly inhibited HT-1080 cell attachment to rec-α4G. Although our results so far demonstrated that *cyclo*-A4G82Xa strongly bound to heparin and inhibited heparin binding of rec-α4G, *cyclo*-A4G82Xa did not show a very clear effect on rec-α4G cell attachment activity. This may be because the conformation of *cyclo*-A4G82Xa did not allow effective interaction with the cells, resulting in the ineffective inhibitory activity for rec-α4G cell attachment. A4G82X and A4G82Xb showed strong inhibitory activity in rec-α4G cell attachment as effectively as their cyclized form of peptides. We tested inhibitory activity of these peptides in various concentrations, but cyclization did not improve the activity (data not shown). We previously demonstrated that rec-α4G interacts with syndecans, heparan-sulfate proteoglycans (*23*). Recently, it was reported that HT-1080 cell attachment to the recombinant laminin-8 (α4β1γ1) was completely inhibited by antibody against either the α4 chain LG1−3 module, the integrin β1, or integrin α6 subunit (*33*), suggesting that in addition to syndecans, other types of receptors, such as integrins, may be important for interaction of rec-α4G with the cells. Thus, since multiple receptors may cooperatively function in rec-α4G cell attachment, the cyclization of the peptides may not effectively improve the inhibitory effect on rec-α4G cell attachment, but a detailed mechanism is unknown.

We characterized a cell-surface receptor for this domain. Several reports demonstrated that laminin-8, which contains the α4 chain, stimulated cell adhesion mediated by α6β1 and α3β1 integrins (*34, 35*). Previously, we identified several cell binding sequences on the α1 chain that recognize integrins, using EDTA and function-blocking integrin antibodies (*9, 11, 17, 29*). We also identified heparin binding sequences in the α4 chain G domain, one of which was the A4G82 site (*23*). HT-1080 cell attachment to the linear and the cyclic peptides derived from A4G82X was strongly blocked by heparin in a dose-dependent manner. EDTA did not inhibit HT-1080 cell attachment to either the linear or the cyclic peptides derived from A4G82X (data not shown), suggesting that the A4G82 sequence does not interact with integrins. These data indicate that heparin-like molecules on a cell surface play an important role in the adhesion of the A4G82X-derived peptides.

Syndecans, heparan-sulfate proteoglycans, play an important role in cell-matrix adhesion, cell motility, and focal adhesion formation (*36*−*39*). Previously, we demonstrated that the laminin α3 chain LG4 module showed strong cell attachment activity mediated by syndecan-2 or -4 in a cell-type-specific manner and that the A3G75aR sequence (NSFMALYLSKGR, human laminin α3 chain, residues 1412−1423) is mainly responsible for this interaction (*18*). We also determined that the EF-4 peptide (DFMTLFLAHGRLVFMFNVG, mouse laminin α4 chain, residues 1511−1529), which is located on the connecting loop region between the E and F strands of the α4 chain LG4 module and contains the A4G82 sequence, showed strong adhesive activity to human fibroblast cells mediated by syndecan-2 (*29*). Our data suggest that the linear and cyclic peptides derived from A4G82X bind to syndecans on HT-1080 cells. Since we found that syndecan-2 and glypican-1, heparan-sulfate proteoglycan family, are expressed in HT-1080 cells, we hypothesized that these heparan-sulfate proteoglycans are important for the interaction of the peptides with the cells. To determine whether these interactions occur, we used 293T cells that overexpress either syndecan-2 or glypican-1. The cyclized peptides were more adhesive for the syndecan-2 overexpressing cells compared with either the glypican-1 overexpressing cells or the parental 293T cells. This suggests that syndecan-2 is a cell-surface receptor for these peptides and that heparan-sulfate chains are important but not sufficient for the cell adhesive activity of these peptides. It is possible that the specificity of the interaction may depend on a syndecan core protein. Recently, we reported that the A3G756 peptide (KNSFMALYLSKGRLVFALG, human laminin α3 chain, residues 1411−1429), located in the homologous region to the A4G82 site, activated p38 mitogen-activated protein kinase (MAPK) in keratinocytes, leading to increasing the expression of matrix metalloproteinase-1 (*40*). Since the A4G82 sequence is located on the homologous region to the A3G756 site, it is highly possible that the A4G82 sequence also activates MAPK signaling via syndecans, but we have not determined that yet.

In summary, we demonstrate that cyclizing the peptides derived from A4G82X, located on the connecting E−F loop region of the laminin α4 chain LG4 module, enhanced biological activity and that HT-1080 cell attachment activity to these peptides was possibly mediated through syndecan-2. These results indicate that the biological activity of the E−F connecting loop region in the α4 chain LG4 module is dependent on a strict loop conformation. Cyclizing peptides increasing their biological activities could be very useful not only for future research on laminin but also for therapeutic application of biologically active peptides in the future.

REFERENCES

1. Burgeson, R. E., Chiquet, M., Deutzmann, R., Ekblom, P., Engel, J., Kleinman, H. K., Martin, G. R., Meneguzzi, G., Paulson, M., Sanes, J., Timpl, R., Tryggvason, K., Yamada, Y., and Yurchenco, P. D. (1994) A new nomenclature for the laminins, *Matrix Biol. 14*, 209−211.
2. Miner, J. H., Patton, B. L., Lentz, S. I., Gilbert, D. J., Snider, W. D., Jenkins, N. A., Copeland, N. G., and Sanes J. R. (1997) The laminin alpha chains: expression, developmental transitions, and chromosomal locations of alpha1-5, identification of heterotrimeric laminins 8-11, and cloning of a novel alpha3 isoform, *J. Cell Biol. 137*, 685−701.
3. Iivanainen, A., Morita, T., and Tryggvason, K. (1999) Molecular cloning and tissue-specific expression of a novel murine laminin gamma3 chain, *J. Biol. Chem. 274*, 14107−14111.
4. Libby, R. T., Champliaud, M.-F., Claudepierre, T., Xu, Y., Gibbons, E. P., Koch, M., Burgeson, R. E., Hunter, D. D., and Brunken, W. J. (2000) Laminin expression in adult and developing retinae: evidence of two novel CNS laminins, *J. Neurosi. 20*, 6517−6528.
5. Colognato, H., and Yurchenco, P. D. (2000) Form and function: the laminin family of heterotrimers, *Dev. Dyn. 218*, 213−234.

6. Powell, S. K., and Kleinman, H. K. (1997) Neuronal laminins and their cellular receptors, *Int. J. Biochem. Cell Biol. 29*, 401−414.
7. Yamada, Y., and Kleinman, H. K. (1992) Functional domains of cell adhesion molecules, *Curr. Opin. Cell Biol. 4*, 819−823.
8. Yamada, K. M. (1991) Adhesive recognition sequences, *J. Biol. Chem. 266*, 12809−12912.
9. Nomizu, M., Kim, W. H., Yamamura, K., Utani, A., Song, S. Y., Otaka, A., Roller, P. P., Kleinman, H. K., and Yamada, Y. (1995) Identification of cell binding sites in the laminin alpha1 chain carboxyl-terminal globular domain by systematic screening of synthetic peptides, *J. Biol. Chem. 270*, 20583−20590.
10. Nomizu, M., Kuratomi, Y., Song, S. Y., Ponce L. M., Hoffman, M. P., Powell, S. K., Miyoshi, K., Otaka, A., Kleinman, H. K., and Yamada, Y. (1997) Identification of cell binding sequences in mouse laminin gamma1 chain by systematic peptide screening, *J. Biol. Chem. 272*, 32198−32205.
11. Nomizu, M., Kuratomi, Y., Malinda, M. K., Song, S. Y., Miyoshi, K., Otaka, A., Powel, S. K., Hoffman M. P., Kleinman, H. K., and Yamada, Y. (1998) Cell binding sequences in mouse laminin alpha1 chain, *J. Biol. Chem. 273*, 32491−32499.
12. Nomizu, M., Kuratomi, Y., Ponce, L. M., Song, S. Y., Miyoshi, K., Otaka, A., Powell, S. K., Hoffman, M. P., Kleinman, H. K., and Yamada, Y. (2000) Cell adhesive sequences in mouse laminin beta1 chain, *Arch. Biochem. Biophys. 378*, 311−320.
13. Makino, M., Okazaki, I., Nishi, N., and Nomizu, M. (1999) Biologically active sequences in laminin, a multifunctional extracellular matrix protein, *Connect. Tissue 31*, 227−234.
14. Hoffman, M. P., Engbring, J. A., Nielsen, P. K., Vargas, J., Steinberg, Z., Karmand, A. J., Nomizu, M., Yamada, Y., and Kleinman, H. K. (2001) 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. 276*, 22077−22085.
15. Tashiro, K., Sephel, G. C., Greatorex, D., Sasaki, M., Shiraishi, N., Martin, G. R., Kleinman, H. K., and Yamada, Y. (1991) The RGD containing site of the mouse laminin A chain is active for cell attachment, spreading, migration and neurite outgrowth, *J. Cell. Physiol. 146*, 451−459.
16. Hoffman, M. P., Nomizu, M., Roque, E., Lee, S., Jung, D. W., Yamada, Y., and Kleinman, H. K. (1998) 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. 273*, 28633−28641.
17. Ponce, M. L., Nomizu, M., and Kleinman, H. K. (2001) An angiogenic laminin site and its antagonist bind through the alpha(v)beta3 and alpha5beta1 integrins, *FASEB J. 15*, 1389−1397.
18. Utani, A., Nomizu, M., Matsuura, H., Kato, K., Kobayashi, T., Takeda, U., Aota, S., Nielsen, P. K., and Shinkai, H. (2001) 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. 276*, 28779−28788.
19. Tokida, Y., Aratani, Y., Morita, A., and Kitagawa, Y. (1990) Production of two variant laminin forms by endothelial cells and shift of their relative levels by angiostatic steroids, *J. Biol. Chem. 265*, 18123−18129.
20. Sorokin, L. M., Pausch, F., Frieser, M., Kröger, S., Ohage, E., and Deutzmann, R. (1997) Developmental regulation of the laminin alpha5 chain suggests a role in epithelial and endothelial cell maturation, *Dev. Biol. 189*, 285−300.
21. Niime, T., Kumagai, C., Okano, M., and Kitagawa, Y. (1997) Differentiation-dependent expression of laminin-8 (alpha 4 beta 1 gamma 1) mRNAs in mouse 3T3-L1 adipocytes, *Matrix Biol. 16*, 223−230.
22. Iivanainen, A., Kortesmaa, J., Sahlberg, C., Morita, T., Bergmann, U., Thesleff, I., and Tryggvason, K. (1997) Primary structure, developmental expression, and immunolocalization of the murine laminin alpha4 chain, *J. Biol. Chem. 272*, 27862−27868.
23. Okazaki, I., Suzuki, N., Nishi, N., Utani, A., Matsuura, H., Shinkai, H., Yamashita, H., Kitagawa, Y., and Nomizu, M. (2002) Identification of biologically active sequences in the laminin alpha 4 chain G domain, *J. Biol. Chem. 277*, 37070−37078.
24. Matsuura, H., Momota, Y., Murata, K., Matsushima, H., Suzuki, N., Nomizu, M., Shinkai, H., and Utani, A. (2004) Localization of the laminin alpha4 chain in the skin and identification of a heparin-dependent cell adhesion site within the laminin alpha4 chain C-terminal LG4 module, *J. Invest. Dermatol. 122*, 614−620.
25. Yamaguchi, H., Yamashita, H., Mori, H., Okazaki, I., Nomizu, M., Beck, K., and Kitagawa, Y. (2000) High and low affinity heparin-binding sites in the G domain of the mouse laminin alpha 4 chain, *J. Biol. Chem. 275*, 29458−29465.
26. Yamashita, H., Beck, K., and Kitagawa, Y. (2004) Heparin binds to the laminin alpha4 chain LG4 domain at a site different from that found for other laminins, *J. Mol. Biol. 335*, 1145−1149.
27. Hohenester, E., Tisi, D., Talts, J. F., and Timpl, R. (1999) The crystal structure of a laminin G-like module reveals the molecular basis of alpha-dystroglycan binding to laminins, perlecan, and agrin, *Mol. Cell 4*, 783−792.
28. Timpl, R., Tisi, D., Talts, J. F., Andac, Z., Sasaki, T., and Hohenester, E. (2000) Structure and function of laminin LG modules, *Matrix Biol. 19*, 309−317.
29. Suzuki, N., Nakatsuka, H., Mochizuki, M., Nishi, N., Kadoya, Y., Utani, A., Oishi, S., Fujii, N., Kleinman, H. K., and Nomizu, M. (2003) Biological activities of homologous loop regions in the laminin alpha chain G domains, *J. Biol. Chem. 278*, 45697−45705.
30. Suzuki, N., Ichikawa, N., Kasai, S., Yamada, M., Nishi, N., Morioka, H., Yamashita, H., Kitagawa, Y., Utani, A., Hoffman, M. P., and Nomizu, M. (2003) Syndecan binding sites in the laminin alpha 1 chain G domain, *Biochemistry 42*, 12625−12633.
31. Main, A. L., Harvey, T. S., Baron, M., Boyd, J., and Campbell, I. D. (1992) The three-dimensional structure of the tenth type III module of fibronection: an insight into RGD-mediated interactions, *Cell 71*, 671−678.
32. Aumailley, M., Gurrath, M., Müller, G., Calvete, J., Timpl, R., and Kessler, H. (1991) Arg-Gly-Asp constrained within cyclic pentapeptides. Strong and selective inhibitors of cell adhesion to vitronectin and laminin fragment P1, *FEBS Lett. 291*, 50−54.
33. Talts, J. F., Sasaki, T., Miosge, N., Göhring, W., Mann, K., Mayne, R., and Timpl, R. (2000) Structural and functional analysis of the recombinant G domain of the laminin alpha4 chain and its proteolytic processing in tissues, *J. Biol. Chem. 275*, 35192−35199.
34. Kortesmaa, J., Yurchenco, P., and Tryggvason, K. (2000) Recombinant laminin-8 (alpha(4)beta(1)gamma(1)). Production, purification, and interactions with integrins, *J. Biol. Chem. 275*, 14853−14859.
35. Fujiwara, H., Kikkawa, Y., Sanzen, N., and Sekiguchi, K. (2001) Purification and characterization of human laminin-8. Laminin-8 stimulates cell adhesion and migration through alpha3beta1 and alpha6beta1 integrins, *J. Biol. Chem. 276*, 17550−17558.
36. Rapraeger, A. C. (1993) The coordinated regulation of heparan sulfate, syndecans and cell behavior, *Curr. Opin. Cell Biol. 5*, 844−853.
37. Lebakken, C. S., and Rapraeger, A. C. (1996) Syndecan-1 mediates cell spreading in transfected human lymphoblastoid (Raji) cells, *J. Cell Biol. 132*, 1209−1221.
38. Longley, R. L., Woods, A., Fleetwood, A., Cowling, G. J., Gallagher, J. T., and Couchman, J. R. (1999) Control of morphology, cytoskeleton and migration by syndecan-4, *J. Cell Sci. 112*, 3421−3431.
39. Couchman, J. R., and Woods, A. (1999) Syndecan-4 and integrins: combinatorial signaling in cell adhesion, *J. Cell Sci. 112*, 3415−3420.
40. Utani, A., Momota, Y., Endo, H., Kasuya, Y., Beck, K., Suzuki, N., Nomizu, M., and Shinkai, H. (2003) Laminin alpha 3 LG4 module induces matrix metalloproteinase-1 through mitogen-activated protein kinase signaling, *J. Biol. Chem. 278*, 34483−34490.

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