Identification of Biologically Active Sequences in the Laminin α4 Chain G Domain*

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Laminins are a family of trimeric extracellular matrix proteins consisting of α, β, and γ chains. So far, five different laminin α chains have been identified. The laminin α4 chain, which is present in laminin-8/9, is expressed in cells of mesenchymal origin, such as endothelial cells and adipocytes. Previously, we identified heparin-binding sites in the C-terminal globular domain (G domain) of the laminin α4 chain. Here we have focused on the biological functions of the laminin α4 chain G domain and screened active sites using a recombinant protein and synthetic peptides. The rec-α4G protein, comprising the entire G domain, promoted cell attachment activity. The cell attachment activity of rec-α4G was completely blocked by heparin and partially inhibited by EDTA. We synthesized 116 overlapping peptides covering the entire G domain and tested their cell attachment activity. Twenty peptides showed cell attachment activity, and 16 bound to heparin. We further tested the effect of the 20 active peptides in competition assays for cell attachment and heparin binding to rec-α4G protein. A4G6 (LAIKNNDLVVY), A4G20 (DVISLYNKHIY), A4G82 (TLFLAHGRLVFM), and A4G83 (LVFMNVGHIKL), which promoted cell attachment and heparin binding, significantly inhibited both cell attachment and heparin binding to rec-α4G. These results suggest that the four active sites are involved in the biological functions of the laminin α4 chain G domain. Furthermore, rec-α4G, A4G6, and A4G20 were found to interact with syndecan-4. These active peptides may be useful for defining of the molecular mechanism laminin-receptor interactions and laminin-mediated cellular signaling pathways.

Laminins, a family of extracellular matrix proteins, consist of three different subunits, α, β, and γ chains. So far, five α, three β, and three γ chains have been identified, and at least 15 isoforms (laminin1–15) are formed by various combinations of each subunit (1–4). Laminins have diverse biological activities including promotion of cell adhesion, migration, neurite outgrowth, angiogenesis, and tumor metastasis (5). More than 20 receptors have been reported for these laminin molecules (6). Several active sites on laminin-1 have been identified using proteolytic fragments, recombinant proteins, and synthetic peptides (7, 8). Previously, we screened for cell adhesion sequences on laminin-1 using 673 overlapping synthetic peptides covering the entire protein (9–12). Most of the active peptides were localized in the globular domains and found to play a critical role in binding to cell surface receptors in a peptide- and cell type-specific manner (13, 14). Several peptides were found to interact with integrins and syndecans (15–18). Some of the peptides promoted neurite outgrowth, angiogenesis, and tumor metastasis (19–22).

The laminin α chains are generally large ($M_r = 400,000$) and contain a C-terminal globular domain consisting of five globular modules LG1–LG5. The laminin α4 chain lacks the N-terminal short arm and is expressed in cells of mesenchymal origin, such as endothelial cells and adipocytes (23–25). Laminin α4 chain expression is mainly localized to mesenchymal cells present in the lung and cardiac and skeletal muscles fibers (26). The α4 chain is also weakly expressed in other adult tissues, such as brain, spleen, liver, kidney, and testis (26). The laminin α4 chain may play a critical biological role in the tissues. Proteolytic processing of the laminin α4 chain G domain was confirmed with cultured endothelial and Schwannomas cells, where the C-terminal α4 LG4–5 module was released (27). The LG4–5 fragment was detected in cell cultures but not in tissues by immunostaining. Laminin-8 containing the α4 chain bound to α6β1 and ααβ1 integrins (28, 29). The α4 chain G domain bound to heparin, sulfatides, and fibulins but has relatively low affinity for α-dystroglycan receptors compared with other laminin α chains (27). By using recombinant proteins, we previously showed that the laminin α4 chain G domain bound to heparin, and this affinity was stronger than that of the α1 chain G domain (30). Recently, we have identified the heparin-binding sites on the laminin α1, α3, and α5 chains LG4 modules (14, 16, 18, 31). These peptides interacted with syndecan-1 or -2, a membrane-associated proteoglycan, and promoted cell attachment. Heparin binding may be important for the biological activity of the laminin α4 chain.

In this paper, we describe the systematic screening for biologically active sequences in the laminin α4 chain G domain (mouse laminin α4 chain 852–1816) using a recombinant protein and a large set of overlapping peptides. The laminin α4 chain G domain recombinant protein promoted cell attachment and heparin binding. For the initial stage of screening for identification of active sequences, we evaluated the cell attachment activities of 104 different peptides using peptide-conjugated Sepharose beads and peptide-coated plates. We also examined the effect of these peptides on heparin binding to the recombinant protein. Four sequences were identified that were active in all of the assays and were also evaluated for additional biological activities. Two were found to interact with syndecan-4.

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FIG. 1. Attachment of HT-1080 cells to the rec-α4G protein. A, 96-well plates were coated with various amounts of rec-α4G. HT-1080 cells were added and incubated for 1 h. Then the number of attached cells was assessed by crystal violet staining. Data are expressed as the mean of triplicate results ± S.D. Triplicate experiments gave similar results. B, inhibition of HT-1080 cell attachment to rec-α4G by heparin and by EDTA. 96-well plates were coated with 1.2 μg/well rec-α4G. Cells were preincubated with either 10 μg/ml heparin or 5 mM EDTA, and then cells were added to the plates. After a 30-min incubation, the attached cells were assessed by crystal violet staining. Each value represents the mean of three separate determinations ± S.D. Duplicate experiments gave similar results. *, p < 0.02; **, p < 0.0002.

FIG. 2. Sequences and peptides from the laminin α4 chain G domain. Sequences were derived from the mouse laminin α4 chain (26). Locations of peptides are indicated by arrows. Active peptide sequences in peptide-coated plate and peptide-conjugated bead assays are shown by dark arrows. Insoluble peptides are indicated by parentheses.
Active Sequences in the Laminin α4 Chain G Domain

**Fig. 3.** Attachment of HT-1080 cells to peptide-coated plates. 96-Well plates were coated with various amounts of peptides, and HT-1080 cells were added. After 1 h of incubation, the number of attached cells was assessed by crystal violet staining (A and B). Data are expressed as the mean of triplicate results. Triplicate experiments gave similar results. A4G83 showed cell toxicity at more than 2.5 μg/well.

**TABLE I**

| Peptide | Sequence | Cell attachment<sup>a</sup> | rec-α4G inhibition | Solid phase heparin binding<sup>b</sup> |
|---------|----------|-----------------------------|---------------------|-----------------------------------------|
| A4G4    | FVLYLGSKNAKK | + + + | ++ (10 μM) | + |
| A4G6    | LAIKNDNLYYYV | + + | ++ + (150 μM) | + + |
| A4G10   | AYSFIVKIEVRG | ++ + | ++ + (5 μM) | + + |
| A4G20   | DVISLYNFKIH | ++ + | ++ + | + + |
| A4G24   | FFDGGSSYAVVRD | ++ + | ++ + | + + |
| A4G25   | VVRDITRPGKFG | ++ + | ++ + | + + |
| A4G26   | GKPQGVTRFDIE | ++ + | ++ + | + + |
| A4G31   | LHVPYDGFSGNG | ++ + | ++ + | + + |
| A4G32   | EDSLISRRAYPN | ++ + | ++ + | + + |
| A4G47   | RAFYNGQSFIA | ++ + | ++ + | + + |
| A4G59   | SRLRGNKPTKRG | ++ + | ++ + | + + |
| A4G66   | LHKKGNKSSKPR | ++ + | ++ + | + + |
| A4G78   | GEKSQFSRLK | ++ + | ++ + | + + |
| A4G79   | RLKTRSSHGMIF | ++ + | ++ + | + + |
| A4G82   | TLFIAHGLRVMF | ++ + | ++ + | + + |
| A4G83   | LVFMFNVGHKL | ++ + | ++ + | + + |
| A4G84   | HKKLRQSEKQY | ++ + | ++ + | + + |
| A4G90   | GAWKIKGPTY | ++ + | ++ + | + + |
| A4G102  | HSNVGLNLYNH | ++ + | ++ + | + + |
| A4G107  | VIRIDSVQYLDV | ++ + | ++ + | + + |
| AG73    | RKRLQVQLSIRT | ++ + | ++ + | N/D |

<sup>a</sup>For cell attachment assays, various amounts of peptides were coated on 96-well plates as described under "Materials and Methods." In all cases, the biological activities of the peptides were quantitated and evaluated relative to those observed with AG73. Cell attachment was evaluated on the following subjective scale: ++++, adhesion comparable to those on AG73; ++, weak adhesion compared with that on AG73; +, very weak adhesion compared with that on AG73; –, no adhesion. Triplicate experiments gave similar results.

<sup>b</sup>For rec-α4G cell attachment inhibition assays, wells were coated with rec-α4G (1.2 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 rec-α4G; +, moderate inhibited rec-α4G; –, no effect on rec-α4G. N/D, not determined. Triplicate experiments gave similar results.

<sup>c</sup>For rec-α4G heparin binding inhibition assays, heparin binding was evaluated as shown in Fig. 7. +++, completely inhibited rec-α4G; +, moderate inhibited rec-α4G; –, no inhibition. N/D, not determined. IC<sub>50</sub> values (Fig. 7) are indicated by parentheses. Triplicate experiments gave similar results.

<sup>d</sup>For solid phase heparin binding assay, various amounts of peptides were coated on 96-well plates as described under "Materials and Methods." In all cases, the biological activities of the peptides were quantitated and evaluated relative to those observed with AG73 as shown in Fig. 8. Heparin binding activities were evaluated on the following subjective scale: ++++, binding comparable to that on AG73; ++, weak binding compared with that on AG73; +, very weak binding compared with that on AG73; –, no binding. Triplicate experiments gave similar results.

**MATERIALS AND METHODS**

**Recombinant Protein (rec-α4G)—**A recombinant protein (rec-α4G), containing the mouse laminin α4 chain G domain (residues 852–1816) with the c-Myc sequence at the C terminus, was expressed using Chinese hamster ovary cells as described previously (30). Conditioned medium of rec-α4G expressing cells was collected, and the rec-α4G protein was purified using a heparin affinity column (HiTrap, Amersham Biosciences). Purity was confirmed by SDS-PAGE, and by Western blotting analysis. Protein concentration was determined with the BCA assay (Fierce) with BSA<sup>a</sup> as standard.

**Synthetic Peptides—**All peptides were synthesized manually using the 9-fluorenylmethoxycarbonyl (Fmoc) based solid phase strategy and prepared in the C-terminal amide form as described previously (11). The respective amino acids were condensed manually in a stepwise manner using 4-2'4'-dimethoxyphenyl-Fmoc-aminomethyl-phenoxy resin (Novabiochem). Dimethylformamide was used during the synthesis as a solvent. For condensation, diisopropylcarbodiimide was employed, and for deprotection of N-Fmoc groups, 20% piperidine in dimethylformamide was employed. The following side chain protecting groups were used: Asn, Gln, His, trityl; Asp, Glu, Ser, Thr, and Tyr, t-butyl; Arg, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; and Lys, t-butylcarbonyl. The resulting protected peptide resins were deprotected and cleaved from the resin using trifluoroacetic acid/thioanisole/m-cresol/ethanedithiol/H<sub>2</sub>O (80:5:5:5, v/v) at 20 °C for 3 h. Crude peptides were precipitated, washed with ethyl ether, and then purified by reverse phase-high performance liquid chromatography (HPLC) using a Vydac 518 column with a gradient of water/acetoni triate containing 0.1% trifluoroacetic acid. The purity and identity of the peptides were confirmed by HPLC and by fast atom bombardment mass spectrometer.

<sup>1</sup>The abbreviations used are: BSA, bovine serum albumin; Fmoc, 9-fluorenylmethoxycarbonyl; HPLC, high performance liquid chromatography; FBS, fetal bovine serum; ELISA, enzyme-linked immunosorbent assay; DMEM, Dulbecco's modified Eagle's medium.
at the GC-MS & NMR Laboratory, Graduate School of Agriculture, Hokkaido University (Sapporo, Japan). Twelve peptides (A4G3, A4G53, A4G57, A4G80, A4G81, A4G92, A4G94, A4G95, A4G96, A4G98, A4G99, A4G115, and A4G116) were not soluble in aqueous solutions and could not be purified by reverse phase-HPLC.

**Cells and Culture**—HT-1080 human fibrosarcoma cells (32) were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) containing 10% fetal bovine serum (Invitrogen), 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen).

**Cell Attachment Assay Using Recombinant Protein and Synthetic Peptides**—Cell attachment to substrate-coated plates was assayed in 96-well plates (Nunc Inc., Naperville, IL). Plates were coated with various amounts of rec-a4G in Milli-Q H2O (50 μl) at 4 °C overnight. Peptides were also coated on the plates by drying overnight.

**Peptide-coated plates were blocked by addition of 0.1% BSA in 10 mM Tris-HCl (pH 7.4), 100 μM EDTA, or peptide (0.1 μg/ml) in DMEM containing 0.1% BSA. Cells, detached by 0.02% EDTA in phosphate-buffered saline and resuspended in DMEM containing 0.1% BSA, were added (20,000 cells/100 μl) to each well and incubated at 37 °C for 1 h in 5% CO2. The attached cells were stained with 0.2% crystal violet aqueous solution in 20% methanol for 10 min. After removal of the unattached cells, 1% SDS (200 μl) was used to dissolve the attached cells, and the absorbance at 570 nm was measured in a model 550 Microplate reader (Bio-Rad).

In heparin, EDTA, and peptide inhibition experiments, HT-1080 cells were preincubated with heparin (10 μg/ml), EDTA (5 μm), or peptide (0.1 mg/ml) at 37 °C for 10 min and then plated. After 30-min, the attached cells were measured as described above. All assays were carried out in triplicate, and each experiment was repeated at least three times.

**Cell Attachment Assay Using Peptide-conjugated Sepharose Beads**—The 104 soluble peptides were coupled to cyanogen bromide (CNBr)-activated Sepharose 4B (Amersham Biosciences) as described previously (33). Peptide solutions (200 μg, 1 mg/ml in Milli-Q H2O) were mixed with activated Sepharose beads (30 mg). Ethanolamine-coupled beads were prepared as a control. The amount of coupled peptide was determined by amino acid analysis (10–20 μmol peptides per 1 g of Sepharose beads) (33).

Cell attachment to the peptide-Sepharose beads was assayed in 48-well plates (Iwaki, Tokyo, Japan). HT-1080 cells were detached as described above. The cells (100,000 cells/200 μl of DMEM containing 0.1% BSA) were incubated with 200 μl of the bead solution at 37 °C for 1 h in 5% CO2. The cells attached to the beads were stained with 0.2% crystal violet aqueous solution in 20% methanol for 10 min. After removal of unattached cells, attached cells were analyzed under a microscope.

**Inhibition of Heparin Binding of rec-a4G by Synthetic Peptides**—The effect of peptides on the binding of rec-a4G to heparin was tested using heparin-Sepharose beads as described previously (30). The rec-a4G protein (6 μg, 0.68 μM) was incubated with heparin-Sepharose beads (1 mg, Amersham Biosciences) in 50 mM Tris-HCl buffer (75 μl, pH 7.4) in the presence of peptide (20 μg, 176 μM) 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 10 mM Tris-HCl (pH 7.4), containing 100 mM NaCl. The rec-a4G protein bound to the beads was extracted with SDS-PAGE sample buffer. The sample was analyzed by SDS-PAGE in 8% acrylamide gels under reducing conditions.

**Solid Phase Heparin Binding Assay on Peptide-coated Plates**—Heparin binding to peptide-coated plates was tested using biotinylated heparin (Celsius Laboratories Inc, Cincinnati, OH). Various amounts of peptides were coated onto 96-well ELISA plates (Iwaki, Tokyo, Japan) and dried overnight at room temperature. The wells were washed twice with 0.05% Tween 20 in phosphate-buffered saline (buffer B) and then blocked by addition of 150 μl of 3% BSA in buffer B for 2 h. After removal of the supernatant and washing twice with buffer B, 10 ng of biotinylated heparin in buffer B was added to the wells. Following a 1-h incubation at 37 °C, the supernatant was removed and then the wells were washed three times with buffer B. Streptavidin-conjugated horseradish peroxidase (10 ng, Sigma) in buffer B was added to the wells and then incubated at 37 °C for 1 h to detect the biotinylated heparin-bound peptides. After removal of the supernatant and washing three times with buffer B, TMB solution (100 μl, Sigma) was added and incubated for 10 min. After quenching with 1 x H2SO4 (100 μl), the absorbance at 450 nm was measured in a model 550 Microplate reader.

**Solid Phase Syndecan-4 Binding Assay to rec-a4G- and Peptide-coated Plates**—Syndecan-4 binding to rec-a4G- and peptide-coated plates was examined using HT-1080 cell lysate. Cell lysate was prepared as described previously (18). Various amounts of rec-a4G in buffer A (50 μl) were coated on 96-well ELISA plates at 4 °C overnight. Various amounts of peptides were also coated by drying overnight. The wells were blocked with 3% BSA at room temperature for 2 h. HT-1080 cell lysate (5 μl) in 50 μl of 3% BSA was added to the wells and incubated at 4 °C overnight. After washing with buffer B, anti-syndecan-4 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in 3% BSA (1:1000) was added and incubated at 37 °C for 1 h. The wells were washed with buffer B, and then biotinylated anti-mouse IgG antibody (Vector Laboratories, Inc., Burlingame, CA) in 3% BSA (1:2500) was added and incubated at 37 °C for 1 h. After washing with buffer B, streptavidin-conjugated horseradish peroxidase in buffer B (1:2500) was added and incubated at 37 °C for 1 h. After washing with buffer B, TMB solution (50 μl) was added and incubated for 10 min. After addition of 1 M H2SO4 (50 μl), the absorbance at 450 nm was measured using a model 550 Microplate reader.

In an inhibition experiment using heparin, rec-a4G (0.75 μg/well), A4G6 (1 μg/well), and A4G20 (2 μg/well) were coated on 96-well ELISA plates. After blocking with 1% BSA block solution (Celsus Laboratories Inc, Cincinnati, OH). Various amounts of HT-1080 cell lysate and heparin (10 μg/ml) were added. Syndecan-4 bound to the substrate-coated plates was detected as described above.

**RESULTS**

**Cell Attachment and Heparin Binding Activities of a Recombinant Laminin α4 Chain G Domain Protein**—A recombinant protein (rec-a4G) containing the mouse laminin α4 chain G domain and the c-Myc sequence at the C terminus was expressed using Chinese hamster ovary cells and purified using a heparin column as described previously (30). First, we examined the ability of the rec-a4G protein to promote HT-1080 human fibrosarcoma cell attachment. The rec-a4G protein promoted cell attachment in a dose-dependent manner (Fig. 1A). Cell attachment on rec-a4G was completely inhibited by heparin and partially inhibited by EDTA (Fig. 1B). These results suggested that cell attachment to rec-a4G was mediated by heparin and was cation-dependent. Heparin inhibited the G domain-mediated cell attachment to rec-a4G more effectively when compared with EDTA, suggesting that heparin-like molecules are mainly involved in the cell attachment to the laminin α4 chain G domain.

**Cell Attachment Activity of Synthetic Peptides Derived from the Laminin α4 Chain G Domain**—We prepared 116 overlapping synthetic peptides from the laminin α4 chain G domain to screen for biologically active sequences (Fig. 2). One hundred and four peptides were dissolved in aqueous solutions, but 12 peptides (30 kilodaltons (kDa)). In an inhibition experiment using heparin, rec-a4G (0.75 μg/well), A4G6 (1 μg/well), and A4G20 (2 μg/well) were coated on 96-well ELISA plates. After blocking with 1% BSA block solution (Celsus Laboratories Inc, Cincinnati, OH). Various amounts of HT-1080 cell lysate and heparin (10 μg/ml) were added. Syndecan-4 bound to the substrate-coated plates was detected as described above.

**Solid Phase Heparin Binding Assay on Peptide-coated Plates**—Heparin binding to peptide-coated plates was tested using biotinylated heparin (Celsius Laboratories Inc, Cincinnati, OH). Various amounts of peptides were coated onto 96-well ELISA plates (Iwaki, Tokyo, Japan) and dried overnight at room temperature. The wells were washed twice with 0.05% Tween 20 in phosphate-buffered saline (buffer B) and then blocked by addition of 150 μl of 3% BSA in buffer B for 2 h. After removal of the supernatant and washing twice with buffer B, 10 ng of biotinylated heparin in buffer B was added to the wells. Following a 1-h incubation at 37 °C, the supernatant was removed and then the wells were washed three times with buffer B. Streptavidin-conjugated horseradish peroxidase (10 ng, Sigma) in buffer B was added to the wells and then incubated at 37 °C for 1 h to detect the biotinylated heparin-bound peptides. After removal of the supernatant and washing three times with buffer B, TMB solution (100 μl, Sigma) was added and incubated for 10 min. After quenching with 1 x H2SO4 (100 μl), the absorbance at 450 nm was measured in a model 550 Microplate reader.

In an inhibition experiment using heparin, rec-a4G (0.75 μg/well), A4G6 (1 μg/well), and A4G20 (2 μg/well) were coated on 96-well ELISA plates. After blocking with 1% BSA block solution (Celsus Laboratories Inc, Cincinnati, OH). Various amounts of HT-1080 cell lysate and heparin (10 μg/ml) were added. Syndecan-4 bound to the substrate-coated plates was detected as described above.
AG73. Five peptides (A4G10, A4G47, A4G82, A4G83, and A4G90) showed moderate cell attachment activity but weaker than that of AG73 (Fig. 3B). Five peptides (A4G4, A4G25, A4G26, A4G78, and A4G102) showed weak cell attachment activity (Table I). None of the other peptides was active.

The 104 soluble peptides were coupled to CNBr-activated Sepharose beads and tested for HT-1080 cell attachment. AG73-conjugated Sepharose bead was used as a positive control (33). HT-1080 cells strongly attached and spread on the AG73-conjugated Sepharose bead, whereas ethanolamine-coupled control beads did not show cell attachment activity (Fig. 4). Five peptides (A4G47, A4G59, A4G69, A4G84, and A4G90) showed strong cell attachment and spreading activity comparable with that of AG73 (Fig. 4 and Table I). Five additional peptides (A4G6, A4G20, A4G79, A4G82, and A4G83) showed moderate cell attachment activity in the bead assay (Table I). Four peptides (A4G24, A4G31, A4G46, and A4G107), which showed strong activity in the plate assay, did not support cell attachment to the beads. The remainder of the peptide beads did not have significant cell attachment activity. Based on the two separate cell attachment assays, we found 20 potentially active peptides (Table I).

Effects of Peptides on Cell Attachment to rec-α4G—Next we examined the effect of the 20 cell adhesive peptides on HT-1080 cell attachment to rec-α4G (Fig. 5). Cell attachment to rec-α4G was strongly inhibited by A4G6, A4G20, A4G46, A4G47, A4G78, A4G82, A4G83, and A4G90. Either 5 mM EDTA or 10 μg/ml heparin were added to the cell suspensions and incubated for 10 min. Then the cells were added to the plates. After 30 min, the attached cells were assessed by crystal violet staining. Each value represents the mean of three separate determinations ± S.D. Duplicate experiments gave similar results. *, p < 0.02; **, p < 0.0002.

Effects of EDTA and Heparin on Cell Attachment to Laminin Peptides—The effect of EDTA on HT-1080 cell attachment to peptide-coated plates was examined to determine the role of cations. Eleven peptides, which showed strong or moderate cell attachment activity in the plate assay, were examined (Fig. 6). AG73 was used as a control (9). Attachment to A4G24 was partially inhibited by 5 mM EDTA, but none of the other peptides showed reduced attachment activity in the presence of 5 mM EDTA.

Because cell attachment to rec-α4G was completely inhibited by 10 μg/ml heparin (Fig. 1B), we tested the effects of heparin on HT-1080 cell attachment to 11 peptide-coated plates (Fig. 6). Cell attachment to all of the peptides was inhibited in the presence of 5 mM EDTA. These results suggest that the nine active sequences are important for cell attachment of rec-α4G.

Effects of Peptides on Heparin Binding to rec-α4G—The rec-α4G protein strongly bound to the heparin-Sepharose bead. We
evaluated the effect of 104 soluble peptides on binding of rec-a4G to heparin-Sepharose beads. Heparin-Sepharose beads (1 mg), peptide (20 μg), and rec-a4G (6 μg, 0.68 μM) were mixed in 75 μl of 50 mM Tris-HCl buffer (pH 7.4) containing 100 mM NaCl, and then the mixtures were incubated at 4 °C for 1 h. Following removal of the supernatant and washing of the beads, rec-a4G bound to heparin-Sepharose bead was extracted with SDS-PAGE sample buffer, and then the samples were electrophoresed in SDS-PAGE. Duplicate experiments gave similar results. The relative amount (%) of rec-a4G bound to heparin-Sepharose beads was assessed from the band on SDS-PAGE using NIH image 1.62 software. Duplicate experiments gave similar results.

**FIG. 7. Effect of peptides on heparin binding to rec-a4G.** A, effect of the 104 soluble peptides on the binding of rec-a4G to heparin-Sepharose beads. Heparin-Sepharose beads (1 mg), peptide (20 μg, 178 μM), and rec-a4G (6 μg, 0.68 μM) were mixed in 75 μl of 50 mM Tris-HCl buffer (pH 7.4) containing 100 mM NaCl, and then the mixtures were incubated at 4 °C for 1 h. Following removal of the supernatant and washing of the beads, rec-a4G bound to heparin-Sepharose bead was extracted with SDS-PAGE sample buffer, and then the samples were electrophoresed in SDS-PAGE. Duplicate experiments gave similar results. The relative amount (%) of rec-a4G bound to heparin-Sepharose beads was assessed from the band on SDS-PAGE using NIH image 1.62 software. Duplicate experiments gave similar results.

**FIG. 8. Binding of biotinylated heparin to peptide-coated plates.** 96-Well ELISA plates were coated with various amounts of peptides. Biotinylated heparin (10 ng/well) was added after blocking peptide-coated plates with 3% BSA. Following incubation at 37 °C for 1 h, the biotinylated heparin bound to peptide was detected by streptavidin-conjugated horseradish peroxidase. Data are expressed as mean of triplicate results. Triplicate experiments gave similar results.
Table II
Cell attachment activity of N- and C-terminal truncated peptides of A4G6, A4G20, A4G82

| Peptide | Sequence | Plate | Bead |
|---------|----------|-------|------|
| A4G6    | LAIKDNLVVY | +++  | ++  |
| A4G6a   | A1KDNLVVY  | +++  | ++  |
| A4G6b   | KNDNNLVVY  | +++  | ++  |
| A4G6c   | KNDNNLVVY  | +++  | ++  |
| A4G6d   | NDLLVYY    | -    | -   |
| A4G6e   | DNLVYY     | -    | -   |
| A4G6g   | LAIKDNLVVY | +++  | ++  |
| A4G6h   | LAIKDNLVY  | +    | -   |
| A4G6i   | LAIKDNLV   | -    | -   |
| A4G6j   | LAIKNDL    | -    | -   |
| A4G6k   | LAIKNUN    | -    | -   |
| A4G6s   | NLLVNYAKYVDI | - | -  |
| A4G20   | DVISLYNFHYY | +++ | ++  |
| A4G20a  | VISLYNFHYY | ++  | ++  |
| A4G20b  | ISLYNFHYY  | +   | ++  |
| A4G20c  | ILYNFHYY   | +   | +   |
| A4G20d  | LYNFHYY    | +   | +   |
| A4G20e  | YNFHYY     | -   | -   |
| A4G20g  | DVISLYNFHII | +++ | ++  |
| A4G20h  | DVISLYNFH  | ++  | +   |
| A4G20i  | DVISLYNF   | ++  | +   |
| A4G20j  | DVSLYNF    | -   | -   |
| A4G20k  | DVSLYN     | -   | -   |
| A4G20s  | LLYVIKYFIHSD | - | -  |
| A4G82   | TLFLAHGRLVFM | + + | + + |
| A4G82X  | TLFLAHGRVF (X = Nle) | + + | + + |
| A4G82Xa | TLFLAHGRVFX | + + | + + |
| A4G82Xb | FLAHRGRLVF | ++  | +   |
| A4G82Xc | LAGRLVFX   | -   | -   |
| A4G82Xd | AHRGVLFX   | -   | -   |
| A4G82Xe | HGRVLFX    | -   | -   |
| A4G82g  | TLFLAHGRVF | -   | -   |
| A4G82h  | TLFLAHGRLV | -   | -   |
| A4G82i  | TLFLAHGRL  | -   | -   |
| A4G82j  | TLFLAHGR   | -   | -   |
| A4G82k  | TLFLAHG    | -   | -   |
| A4G83   | LVMFNYNFGKIKL | ++ | ++  |
| A4G823  | TLFLAHGRVFNMVFNGKIKL | +++ | +++ |
| A4G82R  | LVIHMALRGFLF | - | -   |
| AG73    | RKLQVQQLSIRT | +++ | +++ |

a Sequences of the synthetic peptides are given in the single-letter code. All peptides have C-terminal amides.

b Activity was scored on the following subjective scale: +++, adhesion comparable with that on AG73; +, weak adhesion compared with that on AG73; −, no adhesion. Active core sequences are written in boldface.

that of A4G82. A4G82Xb, with two amino acids deleted from the N-terminal of A4G82X, had similar cell attachment activity to A4G82X, but the phenylalanine residue deleted peptide A4G82Xc had no activity. C-terminal deletion peptide A4G82g (TLFLAHGRLVF) showed no cell attachment activity in both assays. These results indicate that at least the 10-amino acid sequence is important for A4G82 cell attachment activity.

A4G83, overlapped with the A4G82 sequence, and both peptides promoted cell attachment and inhibited the cell attachment and heparin binding to rec-α4G (Table I). Therefore, we prepared a longer peptide, A4G83, which contained both A4G82 and A4G83. A4G82 showed strong cell attachment activity that was more potent than either A4G82 or A4G83 in both plate and bead assays (Table II). Additionally, A4G823 showed stronger heparin binding activity than that of A4G82 or A4G83. Furthermore, the inhibitory effect of A4G823 on cell attachment to rec-α4G was much higher than that of either A4G82 or A4G83 (Fig. 5). These results suggest that the active core sequence of A4G823 is a potent active site.

Syndecan-4 Binding to rec-α4G and Peptides—Next, the expression of syndecans in HT-1080 cells was examined by reverse transcriptase-PCR and Western blotting analysis as described previously (18). Syndecan-4 was detected by reverse transcriptase-PCR as well as Western blotting and found to be mainly expressed in HT-1080 cells (data not shown). Western blotting with anti-syndecan-4 antibody showed an ~40-kDa single molecule after heparitinase digestion of HT-1080 cells (data not shown). The binding of rec-α4G and the most active peptides (A4G6 and A4G20) to syndecan-4 were determined in a solid phase assay using HT-1080 cell lysate. The rec-α4G protein bound to syndecan-4 in a dose-dependent manner (Fig. 9A). The A4G6 and A4G20 peptides also showed syndecan-4 binding activity, whereas the scrambled peptides A4G6S and A4G20S did not show activity (Fig. 9A). Heparin (10 μg/ml) significantly inhibited syndecan-4 binding to the rec-α4G protein and peptides A4G6 and A4G20, indicating that the substrates bind to synde-
can-4 with a heparin-dependent manner (Fig. 9B). These results suggest that syndecan-4 plays a critical role for rec-α4G- mediated HT-1080 cell binding and that the A4G6 and A4G20 sites have the potential to be involved in this interaction.

DISCUSSION

We have found that the laminin α4 chain G domain recombinant protein promoted cell attachment and that inhibited by heparin and partially inhibited by EDTA. These results suggest that heparin-like binding is involved in the cell attachment activity of the laminin α4 chain G domain. We identified cell binding and heparin-binding sites in the laminin α4 chain G domain by a systematic peptide screening. Twenty cell adhesive peptides were identified using peptide-coated plate and peptide-conjugated bead assays. Fourteen peptides promoted cell attachment activity in either the plate or bead assays, and six peptides were active in both assays. Four of the active peptides were not active when coated on the plates but were active in the bead assay, whereas 10 peptides were not active in the bead assay but were active in the plate assay. These results indicate that both assays should be employed when testing for active peptides. It is likely that the differential activities are due to conformational changes and/or poor coating efficiencies on the plates. For example, A4G59, A4G69, A4G79, and A4G84 showed strong cell attachment activity in the peptide-conjugated Sepharose bead assay, but these peptides were not active in the peptide-coated plate assay. We previously showed (33) that a 12-mer peptide containing the RGD sequence was active in the peptide-conjugated Sepharose bead assay but not active in the peptide-coated plate assay (33). The similarity of A4G59, A4G69, A4G79, and A4G84 and the RGD-containing peptide suggests that the cell binding of these peptides required an active conformation or higher coating efficiency to the plate. In contrast, A4G4, A4G10, A4G24, A4G25, A4G26, A4G31, A4G46, A4G78, A4G102, and A4G107 showed cell attachment activity in the peptide-coated plate assay, but these peptides were not active in the peptide-conjugated Sepharose bead assay. We showed previously (35) that a 12-mer peptide containing the active IKVAV sequence from the laminin α1 chain was active in...
the peptide-coated plate assay but was not active in the peptide-conjugated Sepharose bead assay (11). These α4 chain G domain peptides may behave similarly to the IKAV peptide and require a specific conformation for activity.

We sought to partially characterize the cellular receptors for the most active peptides. Previously, we identified several cell binding sequences on the α1 chain that recognize integrins (9, 11, 17). EDTA partially inhibited cell attachment to A4G24 but did not affect the rest of the active peptides, suggesting that it has the potential to interact with integrins. Cell attachment to all of the active α4 chain G domain peptides was inhibited by heparin. Cell surface heparin-like molecules are likely important for cell attachment to these peptides. Furthermore, the heparin binding of rec-A4G4 is blocked by A4G6, A4G10, A4G20, A4G82, and A4G83 in a dose-dependent manner. In solid phase binding assays, biotinylated heparin also bound to these peptides. These data suggest that heparin-like cell surface molecules are important in α4 chain G domain-mediated cell attachment. Recently, we found that AG73, a laminin α1 chain LG4 module peptide, interacts with syndecan-1 (14, 16), and A3G75, a laminin α3 chain LG4 module peptide, binds to syndecan-2 (18). Here we showed the rec-A4G4 protein and the most active peptides A4G6 and A4G20 bound to syndecan-4. Taken together, the active peptides in the laminin α4 chain G domain have a potential to be involved in syndecan-mediated cell binding.

Active sequences are mainly located on the LG1 and LG4 modules (Fig. 10A). Previously, we identified the AG73 and A4G75 peptides in the laminin α1 and α3 chains LG4 module. AG73 binds to syndecan-1 and promotes various biological activities including cell adhesion, neurite outgrowth, acinar cell differentiation, and liver metastasis (9, 11, 16, 19, 20). A3G75 also promotes cell adhesion and binds to syndecan-2 (18). We have focused on homologous sites of the AG73 and A3G75 sequences in the α4 chain G domain (Fig. 10). Active sequences are mapped using a crystal structure-based sequential alignment of the LG modules reported previously (36) (Fig. 10). The AG73 sequence is located on the β-strand C region, and the A3G75 sequence is located on the loop region between the β-strands E and F in the LG4 module. A4G6 and A4G82–83, which showed the strongest cell attachment and heparin binding activities, are located in the homologous region of A3G75 in the α4 chain LG1 and LG4 modules. A4G6 and A4G82–83 would be extruded at the opposite edge of the 14-stranded β-sheet sandwich structure where a calcium ion binds. These results suggest that the active sites in the α3 and α4 chains are conserved at the loop region between the β-strands E and F and that the region plays a critical role in the biological functions of the laminin α4 chain G domain. In contrast, A4G78 was found to locate on the homologous site of AG73 in the α3 chain LG4 module. The cell attachment activity of A4G78 was much lower than that of AG73. The AG73 site was previously found to be chain-specific and to promote cell type-specific activity (14). A4G20 located on the loop region between the β-strand L to N in the α4 chain LG1 module is not found on any other previously identified active sequences. These chain-specific active sites in the G domain may promote cell type-specific biological activities.

Inhibition of cell attachment on the rec-A4G4 protein substrate by A4G6, A4G20, A4G24, A4G31, A4G78, A4G82, A4G83, A4G90, and A4G107 suggests that these active sites are available on the intact molecule. These results also confirm the importance of conformation for cell interactions. However, not all active sites function in the intact molecule. These data suggest that proteolytic fragments of laminin-5 generated by matrix metalloproteinase-2 was found to induce cell migration (37). Proteolytic fragments of other laminin chains may also contain activity that is cryptic in the intact molecule but is revealed after proteolysis. Fragments of plasminogen and collagen XVIII, designated angiostatin and endostatin, were found to have important functions in regulating angiogenesis and tumor growth (38, 39). It is possible that the active peptide fragments of laminin play a critical role in its biological activity.