Syndecan Binding Sites in the Laminin α1 Chain G Domain†

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ABSTRACT: The laminin α1 chain G domain has multiple biological activities. Previously, we identified cell binding sequences in the laminin α1 chain G domain by screening 113 synthetic peptide-polystyrene beads for cell attachment activity. Here, we have used a recombinant protein of the laminin α1 G domain (rec-α1G) and a large set of synthetic peptides to further identify and characterize heparin, cell, and syndecan-4 binding sites in the laminin α1 chain G domain. The rec-α1G protein promoted both cell attachment and heparin binding (K_D = 19 nM). Cell attachment to the rec-α1G protein was inhibited 60% by heparin and 30% by EDTA. The heparin binding sites were identified by competing heparin binding to the rec-α1G protein with 110 synthetic peptides in solution. Only two peptides, AG73 (IC_{50} = 147 μM) and AG75 (IC_{50} = 206 μM), inhibited heparin binding to rec-α1G. When the peptides were compared in a solid-phase heparin binding assay, AG73 showed more heparin binding than AG75. AG73 also inhibited fibroblast attachment to the rec-α1G protein, but AG75 did not. Cell attachment to the peptides was studied using peptide-coated plates and peptide-conjugated sepharose beads. AG73 promoted cell attachment in both assays, but AG75 only showed cell attachment activity in the bead assay. Additionally, AG73, but not AG75, inhibited branching morphogenesis of mouse submandibular glands in organ culture. Furthermore, the rec-α1G protein bound syndecan-4, and both AG73 and AG75 inhibited this binding. These results suggest that the AG73 and AG75 sites are important for heparin and syndecan-4 binding in the laminin α1 chain G domain. These sites may play a critical role in the diverse biological activities involving heparin and syndecan-4 binding.

Laminin-1 (M_r = 900 000), first identified from the mouse Engelbreth−Holm−Swarm tumor and the most extensively characterized laminin, consists of α1, β1, and γ1 chains that assemble into a triple-stranded coiled−coil structure to form a cross-like structure (*1*). Presently, 15 isoforms of laminin have been identified (laminin-1−15), which are formed with combinations of five α, three β, and three γ chains (*2*−*5*). Laminin-1 has diverse biological activities including promotion of cell adhesion, migration, neurite outgrowth, tumor metastasis, and angiogenesis (*1*). Various receptors have been reported for the laminin-1 molecule (*6*). Several active sites in laminin-1 have been identified using proteolytic fragments, recombinant proteins, and synthetic peptides (*7*, *8*). We have screened cell adhesive sequences in 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*). Several peptides were found to interact with both integrins and syndecan-1 (*14*−*17*). Some of the peptides promoted neurite outgrowth, angiogenesis, and tumor metastasis (*18*−*21*).

The C-terminal globular domain (G domain) of the laminin α1 chain consists of five laminin G domain-like modules (LG1-5) and plays a critical role in the biological functions of laminin-1. Several studies have focused on the biological activities of the G domain. E8, a proteolytic fragment containing the LG1-3 modules, possesses major cell binding activity mediated through the α6β1 integrin (*22*, *23*). Recombinant and reconstitution experiments have suggested that this activity is dependent on protein conformation (*24*, *25*). Several synthetic peptides derived from the G domain promote heparin binding, cell adhesion, neurite outgrowth, and inhibit lung alveolar formation (*26*−*28*). Previously, we screened cell binding sites in the G domain using a large set of synthetic peptide-polystyrene beads and identified several active sequences (*9*). Some of the peptides interact with integrins (*9*). AG73 (RKRLQVQLSIRT, mouse laminin α1 chain 2719−2730) promotes various biological activities and binds syndecan-1, a membrane-associated proteoglycan (*9*, *15*, *16*, *19*, *29*).

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Laminins are heparin binding glycoproteins found in basement membranes. Heparin, the most negatively charged glycosaminoglycan (GAG), has been used to study GAG interactions with cells. GAGs with various patterns of sulfation are found in different cells and tissues at various developmental stages. The variation of the sulfation pattern allows for protein- and cell-specific interactions (*30*, *31*). Interactions between laminin isoforms and cell surface receptors provide mechanisms for regulating the broad range of biological activities of laminins. Several heparin binding sites have been identified in laminin isoforms. The α1 chain proteolytic fragment E3 containing the LG4-5 modules has been found to bind to heparin (*32*, *33*). Recently, other heparin binding sites in the homologous LG4-5 modules of laminin α1-5 chains have been identified (*34*−*38*). Several heparin binding sequences are chain-specific, and some are homologous to each other. The heparin binding sites interact with heparan sulfate proteoglycans, such as syndecans (*15*, *16*, *38*). A3G75aR (NSFMALYLSKGR, human laminin α3 chain 1412−1423) was identified as a syndecan-2 and -4 binding site in the α3 chain G domain (*38*).

In this paper, we have used a recombinant protein of laminin α1 chain G domain, rec-α1G, combined with a synthetic peptide screening strategy to identify and characterize heparin, cell, and syndecan-4 binding sites in the laminin α1 chain G domain. Of the 110 overlapping soluble peptides tested from the α1 chain G domain, only two inhibited rec-α1G binding to heparin. We compared the biological activities of these two peptides in a number of assays and identified syndecan-4 as a potential cell surface ligand that may mediate some of the biological activities of the peptides.

MATERIALS AND METHODS

Recombinant Protein. A recombinant protein (rec-α1G), including the mouse laminin α1 chain (2111−3060) with a c-myc sequence at the C-terminus, was expressed using *dhfr*-deficient CHO DG44 cells as previously described (*35*). The culture medium containing the rec-α1G protein was harvested and centrifuged to remove cell debris. The supernatant was adjusted to 0.1 mM phenylmethylsulfonyl fluoride and was applied to a heparin affinity column (HiTrap, Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated in 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, and 0.5 mM *N*-ethylmaleimide (buffer A). The protein was eluted with buffer A in the presence of 300 mM NaCl. The rec-α1G protein was further purified using a gel filtration column (Superdex 200, Amersham Pharmacia Biotech) equilibrated in buffer A. Purity was monitored by 8% SDS−PAGE under reducing conditions. Fractions containing purified rec-α1G were combined, and the protein concentration was determined with the BCA assay (Pierce, Rockford, IL).

Synthetic Peptides. All peptides were synthesized manually using the 9-fluorenylmethoxycarbonyl (Fmoc)-based solid-phase strategy and prepared in the C-terminal amide form as previously described (*11*). Amino acid derivatives and resins were purchased from Watanabe Chemical, Hiroshima, Japan and Novabiochem, La Jolla, CA. The respective amino acids were condensed manually in a stepwise manner using 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy resin. Dimethylformamide (DMF) was used during the synthesis as a solvent. For condensation, diisopropylcarbodiimide/*N*-hydroxybenzotriazole was employed, and for deprotection of N^{α}-Fmoc groups, 20% piperidine in DMF was employed. The following side chain protecting groups were used: Asn, Gln, and His, trityl; Asp, Glu, Ser, Thr, and Tyr, *tert*-butyl; Arg, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; and Lys, *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) at 20 °C for 3 h. The crude peptides were precipitated and washed with ethyl ether, then purified by reverse-phase high performance liquid chromatography (HPLC) using a Vydac 5C18 column with a gradient of water/acetonitrile containing 0.1% trifluoroacetic acid. Purity of the peptides was confirmed by analytical HPLC. The identity of the synthetic peptides was confirmed by fast atom bombardment mass spectral analysis at the GC-MS and NMR Laboratory, Graduate School of Agriculture, Hokkaido University. Three peptides (AG2, AG24, and AG94) were insoluble in aqueous solutions and could not be purified by reverse-phase HPLC.

Cells and Culture. Human neonatal dermal fibroblasts (Iwaki Co. Ltd., Tokyo, Japan) were cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Rockville, MD) containing 10% fetal bovine serum (FBS, Life Technologies), 100 units/mL penicillin, and 100 μg/mL streptomycin (Life Technologies). The cells were maintained at 37 °C in a humidified 5% CO_2, 95% air atmosphere.

Inhibition of Heparin Binding to rec-α1G. The effect of peptides on the heparin binding of rec-α1G was tested using heparin-sepharose beads as previously described (*35*) with some modifications. The rec-α1G protein (3 μg), heparin-sepharose bead (1 mg, Amersham Pharmacia Biotech), and peptide (20 μg) were mixed in 70 μL of 10 mM Tris-HCl (pH 7.4), containing 100 mM NaCl (buffer B). After a 1 h incubation, the beads were pelleted by centrifugation. The supernatant was removed, and the beads were washed twice with buffer B. The rec-α1G protein bound to the beads was extracted with SDS−PAGE sample buffer, analyzed by 8% SDS−PAGE under reducing conditions, and stained with Coomassie brilliant blue.

Solid-Phase Heparin Binding Assay using Peptide-Coated Plates. The heparin binding assay to peptide-coated plates was carried out using biotinylated heparin (Celsus Laboratories Inc., Cincinnati, OH). Various amounts of peptides in Milli-Q water (50 μL) were coated onto 96-well ELISA plates (Iwaki Co. Ltd.) and dried overnight at room temperature. The wells were washed with 0.05% Tween 20 in PBS (buffer C) and then blocked with 3% bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, MO) in PBS (buffer D) at room temperature for 2 h. After washing with buffer C, 10 ng of biotinylated heparin in 75 μL of buffer C was added to the wells and incubated at 37 °C for 1 h. After washing three times with buffer C, 10 ng of streptavidin-conjugated horseradish peroxidase (Sigma) in buffer C (100 μL) was added to the wells and incubated at 37 °C for 1 h. After washing three times with buffer C, the 3,3′,5,5′-tetramethyl-benzidine (TMB) solution (100 μL, Sigma) was added to the wells and incubated for 10 min. After addition of 1 N H_2SO_4, the optical density at 450 nm was measured using a Model 550 Microplate Reader (Bio-Rad Laboratories, Hercules, CA).

Surface Plasmon Resonance Analysis. Heparin binding was also assayed by surface plasmon resonance analysis using biotinylated heparin as previously described (*16*) with slight modifications. Surface plasmon resonance analysis was performed using a BIAcore 2000 instrument (BIAcore AB, Uppsala, Sweden). Biotinylated heparin was immobilized on a streptavidin-conjugated sensor chip (Sensor Chip SA, BIAcore AB). Various amounts of rec-α1G in 25 mM Tris-HCl (pH 7.5), containing 150 mM NaCl and 0.005% Tween 20, were injected into the biotinylated heparin-immobilized flow cell with a flow rate of 30 μL/min. After each run, 50 mM NaOH (30 μL) was injected twice to regenerate the biotinylated heparin-immobilized surfaces at 30 μL/min for 1 min. Each sample was also analyzed using a flow cell without biotinylated heparin, and the sensorgram was used as background. A dissociation constant (K_D) was determined by a nonlinear fitting method using BIAevaluation 3.1 software (BIAcore AB).

Cell Attachment Assay using rec-α1G Protein- or Peptide-Coated Plastic Plates. Cell attachment assays were performed in 96-well plates (Nunc, Inc., Naperville, IL) coated with various amounts of the rec-α1G protein or synthetic peptides. For rec-α1G coating, various amounts of rec-α1G in 50 μL of buffer A were added to the wells and incubated overnight at 4 °C. 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. 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. Human fibroblasts were detached with 0.02% trypsin-EDTA (Life Technologies) at 37 °C for 10 min and incubated with DMEM containing 10% FBS at 37 °C for 20 min. After washing three times with DMEM containing 0.1% BSA, cells (2×10^4 cells/well) 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 washing 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.

For inhibition of cell attachment, human fibroblasts and the substrate-coated wells were preincubated with either 10 μg/mL heparin, 5 mM EDTA, or 100 μg/mL peptide, for 10 min at 37 °C. Then, the cells (5×10^4 cells/well) were added to the wells and incubated for 30 min at 37 °C. Attached cells were measured as described previously.

Note: We examined the effect of different recovery times (5, 20, and 60 min) after trypsin treatment in DMEM containing 10% FBS. All fibroblasts attached in a manner similar to the AG73 peptide (data not shown). Additionally, attachment was significantly inhibited by 10 μg/mL heparin (data not shown). These results suggest that more than a 5 min recovery is enough for cell adhesion assays. Therefore, we used trypsin-EDTA detachment of human fibroblasts and a 20 min recovery in DMEM containing 10% FBS in the assay.

Cell Attachment Assay using Peptide-Conjugated Sepharose Beads. The synthetic peptides were coupled to cyanogen bromide (CNBr)-activated sepharose 4B (Amersham Pharmacia Biotech AB) as previously described (*39*). The peptide solutions (0.2 mL, 1 mg/mL in Milli-Q H_2O) were mixed with 20 mg of the CNBr-activated sepharose beads. Cell attachment assays to peptide-conjugated sepharose beads were performed in 48-well plates (Nunc, Inc.). Peptide beads were added to the 48-well dishes. Human fibroblast cells were detached with 0.02% trypsin-EDTA as described previously, and 1×10^5 cells (200 μL) in DMEM containing 0.1% BSA were incubated with peptide beads at 37 °C for 1 h. Attached cells on the beads were stained with 0.2% crystal violet aqueous solution in 20% methanol and observed under the microscope.

For inhibition of cell attachment to the beads, human fibroblasts and the peptide beads were preincubated with either 10 μg/mL heparin, 5 mM EDTA, or 100 μg/mL peptide at 37 °C for 10 min and then mixed. After a 30 min incubation, the attached cells were measured as described previously.

Ex Vivo Salivary Gland Organ Culture. Submandibular/sublingual salivary gland rudiments dissected from embryonic day 13 (E13) ICR mice were cultured on Whatman Nucleopore Track-etch filters (13 mm, 0.1 μm pore size, VWR, Buffalo Grove, IL) at the air/medium interface (*16*). The filters were floated on 240 μL of DMEM/F-12 in 50 mm glass-bottom microwell dishes (MatTek, Ashland, MA). The medium was supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin, 150 μg/mL vitamin C, and 50 μg/mL transferrin. Six E13 gland rudiments were cultured on each filter at 37 °C in a humidified 5% CO_2, 95% air atmosphere. Glands were photographed after ∼2, 20, and 40 h, and the number of end buds was counted at each time point. Various peptide concentrations were added to the medium at the beginning of the experiment.

Solid-Phase Syndecan-4 Binding Assay using Human Fibroblast Cell Lysates. Syndecan-4 binding to rec-α1G-coated plates was examined using a human neonatal dermal fibroblast cell lysate. The cell lysate was prepared as described previously (*38*). Various amounts of rec-α1G in buffer A (50 μL) were coated on 96-well ELISA plates at 4 °C overnight. The wells were blocked with buffer D at room temperature for 2 h. The cell lysate in buffer D (50 μL) was added to the wells and incubated at 4 °C overnight. After washing with buffer C, anti-syndecan-4 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in buffer D (1:1000) was added and incubated at 37 °C for 1 h. The wells were washed with buffer C, and then biotinylated anti-mouse IgG antibody (Vector Laboratories, Inc., Burlingame, CA) in buffer D (1:2500) was added and incubated at 37 °C for 1 h. After washing with buffer C, streptavidin-conjugated horseradish peroxidase in buffer D (1:2500) was added and incubated at 37 °C for 1 h. After washing with buffer C, TMB solution (50 μL) was added and incubated for 30 min. After addition of 1 M H_2SO_4 (50 μL), the optical density at 450 nm was measured using a Model 550 Microplate reader.

In heparin and peptide inhibition experiments, rec-α1G (2 μg/well) was coated on 96-well ELISA plates. After blocking with buffer D, the rec-α1G-coated wells and human fibroblast lysate were preincubated with various concentrations of heparin and peptides, respectively. Syndecan-4 bound to the substrate-coated plates was detected as described previously.

RESULTS

Heparin Binding and Cell Attachment Activity of the rec-α1G Protein. A recombinant protein (rec-α1G) containing

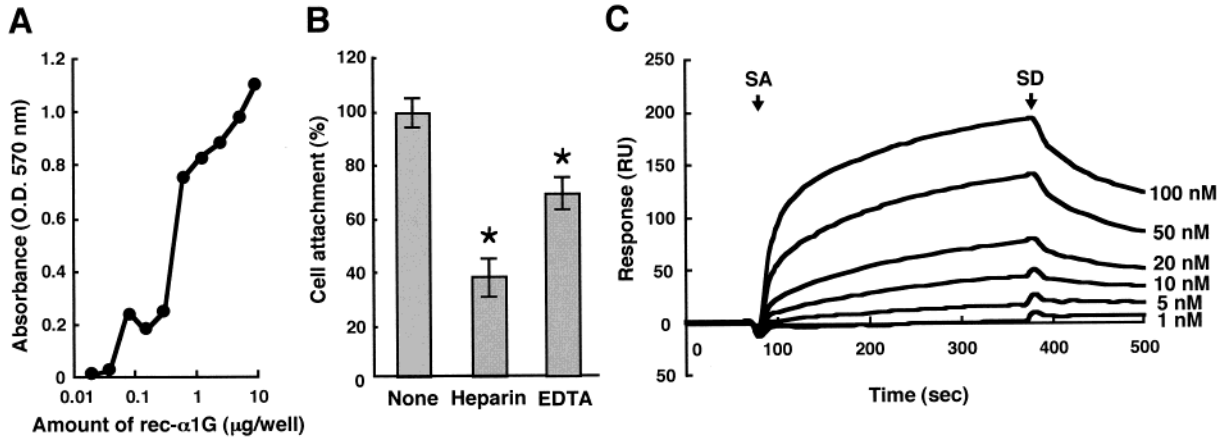

FIGURE 1: Cell attachment and heparin binding activity of rec-α1G. **(**A) Human fibroblasts attach to the rec-α1G protein. Increasing amounts of rec-α1G were coated on the 96-well plate. After blocking with 1% BSA, the cells were added and incubated for 1 h. Following staining with crystal violet, the attached cells were lysed, and the OD was measured. (B) Effect of heparin and EDTA on the fibroblast attachment to rec-α1G. The cells and substrate-coated wells were preincubated with 10 μg/mL heparin or 5 mM EDTA. Then, the cells were added to the wells and incubated for 30 min. The attached cells were stained with crystal violet and measured. Each value represents the mean of six separate determinations ± SD. *$p < 0.0001$. (C) Surface plasmon resonance analysis of heparin binding of the rec-α1G protein. Various concentrations of rec-α1G were examined using a BIAcore 2000 with a biotinylated heparin-immobilized sensor chip. Arrow SA represents the start of the association. Arrow SD represents the start of the dissociation. Triplicate experiments gave similar results.

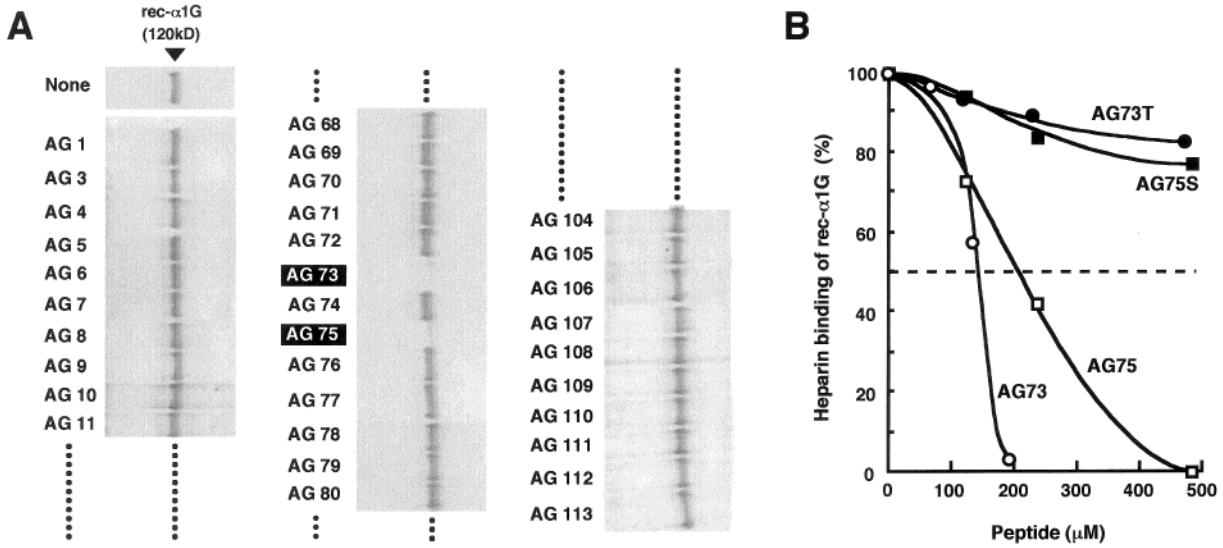

FIGURE 2: Effect of peptides on the heparin binding to the rec-α1G protein. (A) Heparin-sepharose beads (1 mg), peptide (20 μg), and the rec-α1G protein (3 μg) were incubated in 70 μL of 10 mM Tris buffer (pH 7.4) at 4 °C for 1 h. After washing the beads, the rec-α1G protein bound to the heparin-sepharose beads was analyzed by SDS−PAGE, followed by staining with Coomassie brilliant blue. Active peptides are shown by black boxes. (B) Various amounts of peptides were tested. The relative amount (%) of rec-α1G bound to heparin-sepharose beads was assessed by SDS−PAGE using NIH image 1.62 software. IC_{50} values of AG73 and AG75 are 147 and 206 μM, respectively. Duplicate experiments gave similar results.

the C-terminal 950 amino acids (mouse laminin α1 chain 2111−3060) was expressed using the *dhfr*-deficient CHO DG44 cells and purified as previously described (*35*). First, we examined the cell attachment activity of rec-α1G using human fibroblasts. Fibroblasts attached to rec-α1G-coated plates in a dose-dependent manner (Figure 1A). We also examined the effect of heparin and EDTA on the fibroblast attachment to rec-α1G. The 10 μg/mL heparin significantly inhibited (60%) fibroblast attachment to rec-α1G, while 5 mM EDTA partially inhibited (30%) attachment (Figure 1B). These results suggest that the rec-α1G protein promotes cell attachment in both a heparin- and a cation-dependent manner.

Previously, the rec-α1G protein was eluted from a heparin-affinity column with 300 mM NaCl, and the half-maximal concentration (14 nM) for heparin binding was determined in a solid-phase binding assay with immobilized heparin (*35*). We determined a dissociation constant (K_D) of the heparin binding of the rec-α1G protein by surface plasmon resonance analysis (Figure 1C). Sensorgrams with various amounts of rec-α1G were generated using a BIAcore 2000 instrument with a biotinylated heparin-immobilized sensor chip. The K_D value was determined by a nonlinear fitting method using BIAevaluation 3.1 software. The K_D value of the heparin binding of the rec-α1G protein was 19 nM.

Effect of Peptides on the Heparin Binding of the rec-α1G Protein. One hundred and thirteen overlapping peptides covering the laminin α1 chain G domain were prepared as previously described (*9*). One hundred and ten peptides were soluble, but three peptides were not (Table 1). The effect of the 110 soluble peptides on the interaction between rec-α1G

Table 1: Synthetic Laminin α1 Chain G Domain Peptides and Their Inhibitory Effect on the rec-α1G Heparin Binding

peptide	sequence[a]	inhibition[b]	peptide	sequence	inhibition	peptide	sequence	inhibition
AG1	IRAYQPQTSSTN	−	**AG39**	SKAVRKGVSSRS	−	**AG77**	LQLQEGRLHFMF	−
AG2	SSTNYNTLILNV	NS	**AG40**	SSRSYVG	−	**AG78**	HFMFDLGKGRTK	−
AG3	ILNVKTQEPDNL	−	**AG41**	IKNLEISRSTFDL	−	**AG79**	GRTKVSHPALLS	−
AG4	PDNLLFYLGSSS	−	**AG42**	TFDLLRNSYGVRK	−	**AG80**	ALLSDGKWHTVK	−
AG5	GSSSSSDFLAVE	−	**AG43**	ALEPIQSVSFLR	−	**AG81**	HTVKTEYIKRKA	−
AG6	LAVEMRRGKVAF	−	**AG44**	SFLRGGYVEMPP	−	**AG82**	KRKAFMTVDGQE	−
AG7	KVAFLWDLGSGS	−	**AG45**	VEMPPKSLSPESS	−	**AG83**	DGQESPSVTVVG	−
AG8	GSGSTRLEFPEV	−	**AG46**	PESSLLATFATK	−	**AG84**	TVVGNATTLDVE	−
AG9	FPEVSINNNRWH	−	**AG47**	FATKNSSGILLV	−	**AG85**	LDVERKLYLGGL	−
AG10	NRWHSIYITRFG	−	**AG48**	ILLVALGKDAEE	−	**AG86**	LGGLPSHYRARNI	−
AG11	TRFGNMGSLSVK	−	**AG49**	DAEEAGGAQAHV	−	**AG87**	ARNIGTITHSIPA	−
AG12	LSVKEASAAENP	−	**AG50**	QAHVPFFSIMLL	−	**AG88**	IGEIMVNGQQL	−
AG13	AENPPVRTSKSP	−	**AG51**	IMLLEGRIEVHV	−	**AG89**	GQQLDKDRPLS	−
AG14	SKSPGPSKVLDI	−	**AG52**	IEVHVNSGDGTSL	−	**AG90**	RPLSASAVDR	−
AG15	VLDINNSTLMFV	−	**AG53**	GTSLRKALLHAP	−	**AG91**	YVVAQEGTFFEG	−
AG16	LMFVGGLGGQIK	−	**AG54**	LHAPTGSYSDGQ	−	**AG92**	FFEGSGYAALVK	−
AG17	GQIKKSPAVKVT	−	**AG55**	SDGQEHSISLVR	−	**AG93**	ALVKEGYKVRLD	−
AG18	VKVTHFKG	−	**AG56**	SLVRNRRVITIQ	−	**AG94**	VRLDLNITLEFR	NS
AG19	MGEAFLNGKSIGL	−	**AG57**	ITIQVDENSPVE	−	**AG95**	LEFRTTSKNGVL	−
AG20	SIGLWNYIEREGK	−	**AG58**	SPVEMKLGPLTE	−	**AG96**	NGVLLGISSAKV	−
AG21	FGSSQNEDSSFH	−	**AG59**	PLTEGKTIDISN	−	**AG97**	SAKVDAIGLEIV	−
AG22	SSFHFDGSGYAM	−	**AG60**	DISNLYIGGLPE	−	**AG98**	LEIVDGKVLFHV	−
AG23	GYAMVEKTLRPT	−	**AG61**	GLPEDKATPMLK	−	**AG99**	LFHVNNGAGRIT	−
AG24	LRPTVTQIVILF	NS	**AG62**	PMLMRTSFHG	−	**AG100**	GRITATYQPRAA	−
AG25	VILFSTFSPNGL	−	**AG63**	IKNVVLDAQLLD	−	**AG101**	PRAARAL	−
AG26	PNGLLFYLASNG	−	**AG64**	AQLLDFTHATGSE	−	**AG102**	DGKWHTLQAHKS	−
AG27	ASNGTKDFLSIE	−	**AG65**	TGSEQVELDT	−	**AG103**	AHKSKHRIVLTV	−
AG28	LSIELVRGRVKV	−	**AG66**	LLAEEPMQSLHR	−	**AG104**	VLTVDGNSVRAE	−
AG29	RVKVMVDLGSGP	−	**AG67**	SLHREHGELPPE	−	**AG105**	VRAESPHTHSTS	−
AG30	GSGPLTLMTDRR	−	**AG68**	LPPEPPTLPQPEL	−	**AG106**	HSTSADTNDPIY	−
AG31	TDRRYNNGTWYK	−	**AG69**	AVDTAPGYVAGA	−	**AG107**	DPIYVGGYPAHI	−
AG32	TWYKIAFQRNRK	−	**AG70**	VAGAHQFGLSQN	−	**AG108**	PAHIKQN	−
AG33	RNRKQGLLAVFD	−	**AG71**	LSQNSHLVLPLN	−	**AG109**	LSSRASFRG	−
AG34	AVFDAYDTSDKE	−	**AG72**	LPLNQSDVRKRL	−	**AG110**	VRNLRLSRGSQV	−
AG35	SDKETKQGETPG	−	**AG73**	RKRLQVQLSIRT	+ (147 μM)[c]	**AG111**	GSQVQSLDLSRAF	−
AG36	GETPGAASDLNRL	−	**AG74**	SIRTFASSGLIY	−	**AG112**	SRAFDLQGVFPHS	−
AG37	LNRLEKDLIYVG	−	**AG75**	GLIYYVAHQNQM	+ (206 μM)[c]	**AG113**	PGPEP	−
AG38	IYVGGLPHSKAV	−	**AG76**	HQNQMDYATLQLQ	−			

[a] Seqences were derived from the mouse laminin α1 chain (2111−3060). Peptides were generally 12 amino acids in length and overlapped with neighboring peptides by four amino acids. If the N-terminal amino acid was either glutamine or glutamic acid, one amino acid was extended at the N-terminus to avoid pyro-glutamine formation. Cysteine residues were omitted. [b] Inhibitory effect of synthetic peptides was evaluated as described in Materials and Methods and Figure 2. [c] IC_{50} values (μM) of synthetic peptides were determined (Figure 2B). +, inhibition; −, no inhibition. Duplicate experiments gave similar results. NS, not soluble in aqueous solutions.

and the heparin-sepharose bead was evaluated (Figure 2A). AG73 and AG75 significantly inhibited the heparin binding of rec-α1G, while the rest of the soluble peptides did not (Figure 2A). The AG73 and AG75 peptides showed a dose-dependent inhibition, and their IC_{50} values were 147 and 206 μM, respectively (Figure 2B). AG73T (LQQRRSVLRTKI) and AG75S (IQAHYGVMYQNL), scrambled peptides of AG73 and AG75, were also prepared and tested (Figure 2B). However, the scrambled peptides did not inhibit the heparin binding of rec-α1G. These results indicate that the inhibitory effects of AG73 and AG75 on rec-α1G binding to heparin are due to their specific sequences.

Heparin Binding Activity of Peptides. We examined the heparin binding activity of the peptides using peptide-coated plates. In a solid-phase binding assay, biotinylated heparin was added to the AG73- and AG75-coated plates. AG73 showed strong heparin binding activity in a dose-dependent manner (Figure 3). AG75 showed weaker heparin binding activity (Figure 3). The scrambled peptides, AG73T and AG75S, did not show activity in this assay (Figure 3).

Effect of Peptides on Cell Attachment to the rec-α1G Protein. Next, we examined the effect of AG73 and AG75 on fibroblast attachment to rec-α1G (Figure 4). AG73 inhibited fibroblast attachment to rec-α1G, while AG75 did not. AG73T and AG75S did not affect fibroblast attachment

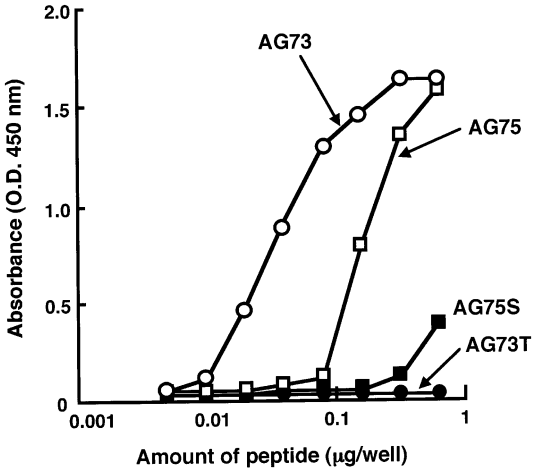

FIGURE 3: Heparin binding of peptides using biotinylated heparin. Binding of biotinylated heparin to peptide-coated plates. 96-well ELISA plates were coated with various amounts of peptides. After blocking with 3% BSA, 10 ng of biotinylated heparin (75 μL) was added to peptide-coated wells. After a 1 h incubation, the biotinylated heparin bound to the peptides was detected by streptavidin-conjugated horseradish peroxidase. Triplicate experiments gave similar results.

to rec-α1G. These data show that the AG73 sequence competes ∼75% cell attachment to rec-α1G, and AG75

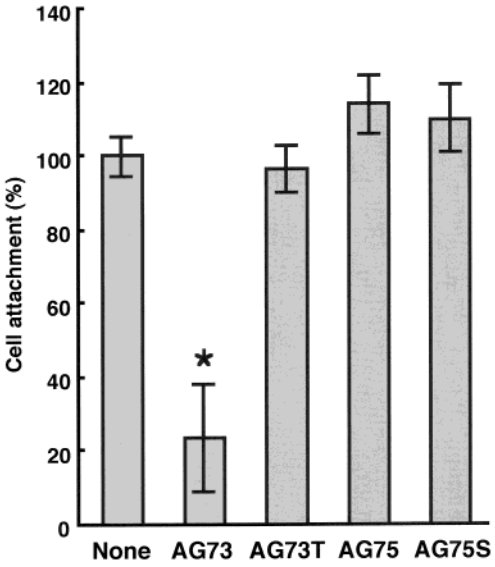

FIGURE 4: Effect of peptides on cell attachment to the rec-α1G protein. 96-well plates were coated with rec-α1G (2 μg/well). Human fibroblasts were preincubated with 100 μg/mL peptide at 37 °C for 10 min, then added to the wells, and incubated for 30 min. Following staining with crystal violet, the attached cells were lysed, and the OD was measured. Each value represents the mean of three separate determinations ± SD. Triplicate experiments gave similar results. *$p < 0.002$.

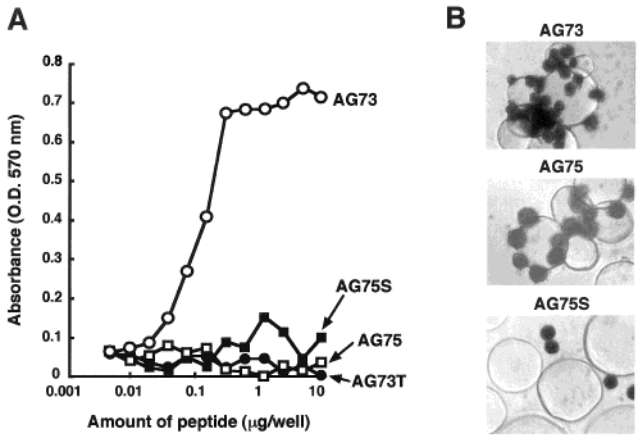

FIGURE 5: Cell attachment activity of peptides. (A) Cell attachment to peptide-coated plates. 96-well plates were coated with various amounts of peptides, and human fibroblasts were added to the wells for 1 h. Following staining with crystal violet, the attached cells were lysed, and the OD was measured. (B) Cell attachment to peptide-conjugated sepharose beads. Human fibroblasts were allowed to attach to peptide-conjugated sepharose beads for 1 h and then stained with crystal violet. Triplicate experiments gave similar results.

cannot inhibit cell attachment. These data suggest that the AG73 peptide can inhibit cell surface heparan sulfate-containing receptors from binding to both the AG73 and the AG75 sites in the rec-α1G. The data also suggest that the AG75 peptide does not inhibit cell adhesion to the AG73 site.

Cell Attachment Activity of Peptides. We tested the cell attachment activity of AG73 and AG75 using peptide-coated plates and peptide-conjugated sepharose beads. In the plate assay, AG73 promoted strong cell attachment activity as previously described (*9*), while AG75 did not show activity (Figure 5A). In the bead assay, both peptides showed strong cell attachment activity (Figure 5B). These results suggest that fibroblast attachment to AG75 may be dependent on the way the peptide is presented to the cell or on the conformation of the peptide.

Additionally, we examined the effect of heparin and EDTA on cell attachment to AG73-coated plates and AG73- and AG75-conjugated sepharose beads (Figure 6A,B). Fibroblast attachment to the AG73-coated plate was significantly inhibited by 10 μg/mL heparin, while the attachment was slightly inhibited by 5 mM EDTA (Figure 6A). Fibroblast attachment to AG73- and AG75-conjugated sepharose beads was also inhibited by 10 μg/mL heparin but not by 5 mM EDTA (Figure 6B). These results suggest that the heparin interaction is critical for the cell attachment activity to both AG73 and AG75.

Effect of Peptides on Branching Morphogenesis of Mouse Submandibular Glands. The heparin binding peptides were tested in a more complex biological assay for their ability to inhibit branching morphogenesis of embryonic mouse salivary glands in organ culture. The peptides bind cell surface heparan sulfate proteoglycans and may disrupt interactions between these molecules and the laminin-1-containing basement membrane. AG73 inhibited branching morphogenesis of mouse submandibular glands as previously described (*16*), whereas AG75 and the scrambled peptides AG73T and AG75S did not inhibit branching (Figure 7). These data suggest that cell surface ligands for AG75 may not be critical in this model system. The data also suggest that in different biological systems the two peptides may bind to different heparan sulfate-containing receptors or different regions of a heparan sulfate chain.

Syndecan-4 Binding to the rec-α1G Protein and Effect of Peptides on Binding. Previously, we demonstrated that AG73 bound to syndecan-1 using an HSG cell lysate (*15*). Human dermal fibroblasts express syndecan-2 and -4 by RT-PCR analysis, while no expression of syndecan-1 was observed (*38*). Therefore, we examined syndecan-4 binding to rec-α1G in a solid-phase assay using a human fibroblast cell lysate. Syndecan-4 bound to rec-α1G in a dose-dependent manner (Figure 8A). Additionally, the syndecan-4 binding to rec-α1G was inhibited by heparin dose dependently (Figure 8B). Further, the binding was significantly inhibited by AG73 and moderately inhibited by AG75, while AG73T did not have an effect (Figure 8B). Taken together, the interaction between syndecan-4 and rec-α1G may be mediated by the heparan sulfate glycosaminoglycan chains of syndecan-4 and the AG73 and AG75 sites in the rec-α1G protein.

DISCUSSION

Previously, the biological functions of the G domain have been identified using enzymatically digested laminin-1 fragments. The E8 fragment containing the LG1-3 modules promoted α6β1 integrin-mediated cell attachment (*22*, *23*), and E3, containing the LG4-5 modules, exhibited heparin and α-dystroglycan binding activities (*32*, *33*, *40*). Syndecan-1, a transmembrane heparan sulfate proteoglycan, is also a receptor for the α1 chain LG4 module and interacts with the AG73 peptide (*15*). In this study, the recombinant α1 chain G domain (rec-α1G) promoted human fibroblast attachment in a heparin- and EDTA-dependent manner.

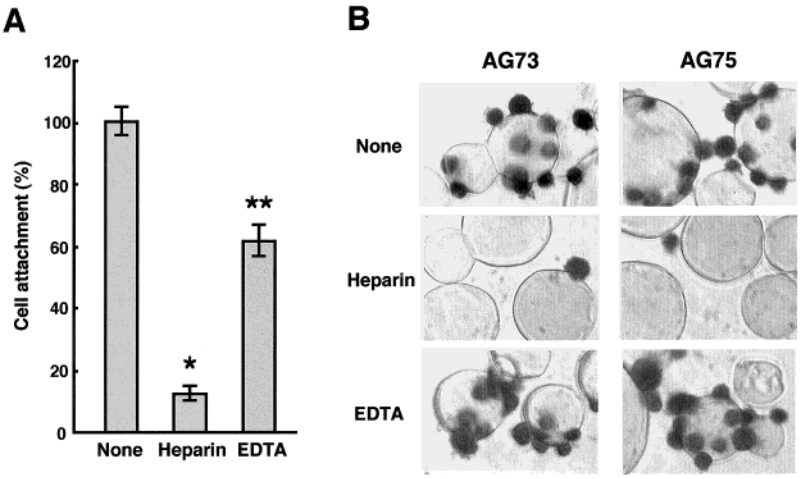

FIGURE 6: Effect of heparin and EDTA on peptide-mediated cell attachment. (A) Effect of heparin and EDTA on the cell attachment to the AG73-coated plate. 96-well plates were coated with AG73 (1 μg/well). The AG73-coated well and fibroblasts were preincubated with 10 μg/mL heparin or 5 mM EDTA for 10 min, and then the cells were added to the wells for 30 min. Following staining with crystal violet, the attached cells were lysed, and the OD was measured. Each value represents the mean of three separate determinations ± SD. *p < 0.0001; **p < 0.001. (B) Effect of heparin on cell attachment to the peptide-conjugated sepharose beads. 10 μg/mL heparin or 5 mM EDTA was added to cell suspensions and peptide-conjugated sepharose beads. After a 10 min incubation, the cells were allowed to attach to the peptide-conjugated sepharose beads and were incubated for 30 min. The attached cells were observed by crystal violet staining under the microscope. Triplicate experiments gave similar results.

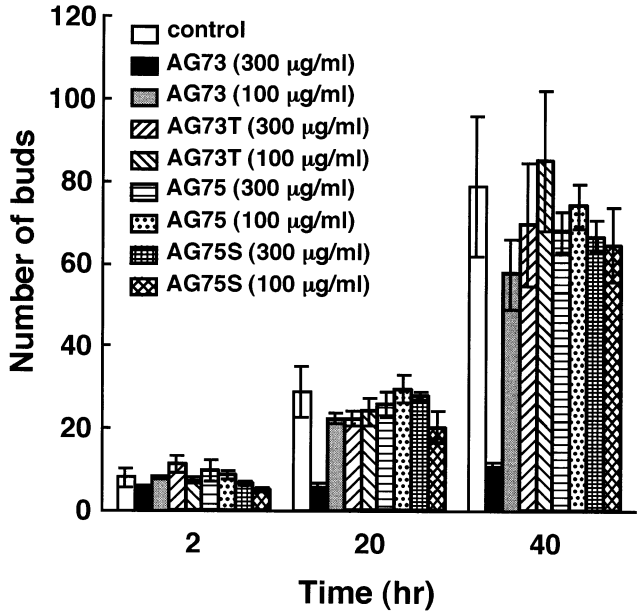

FIGURE 7: AG73 inhibits branching morphogenesis of E13 mouse submandibular glands in culture. Salivary gland organ culture is described under Materials and Methods. Peptides were added to the culture media, and the glands were cultured for 40 h. AG73 (300 μg/well) inhibits branching morphogenesis, whereas AG75 and the scrambled peptides AG73T and AG75S do not. Branching morphogenesis is quantitated by counting the number of terminal epithelial buds after ~ 2, 20, and 40 h. Each value represents the mean of five separate determinations ± SD. Duplicate experiments gave similar results.

Furthermore, rec-α1G bound syndecan-4 from a fibroblast cell lysate. Taken together, these data suggest that syndecan-4 and possibly other cation-dependent molecules, such as integrins and α-dystroglycan, may be cell surface receptors for the α1 chain G domain. A dissociation constant of heparin binding to rec-α1G was determined (K_D = 19 nM) by surface plasmon resonance analysis. The dissociation constant of AG73 binding to heparin (K_D = 6.4 μM) was previously reported (*16*). The dissociation constant for rec-α1G binding to heparin (K_D = 19 nM) suggests a higher affinity interaction with the recombinant protein as compared to the interaction with AG73 (K_D = 6.4 μM). These data suggest either multiple interactions between the recombinant protein and heparin or a conformation-dependent interaction that increases the affinity of the interaction.

Previously, we screened cell binding sites in the α1 chain G domain using 113 synthetic peptide-polystyrene beads in which peptides were not purified (*9*). AG73 had the greatest cell attachment activity in the screening analysis and showed diverse biological activities (*9*, *15*, *16*, *19*, *29*). Here, we used a recombinant α1 chain G domain and screened 110 soluble purified synthetic peptides to identify heparin binding sites. Only two peptides, AG73 and AG75, inhibited rec-α1G binding to heparin, suggesting that the AG73 and AG75 sites may play a critical role in heparin binding of the α1 chain G domain. AG75 was not active for cell attachment when coated on a plate and did not inhibit cell attachment to rec-α1G or inhibit branching morphogenesis. The biological activity of AG73 was dependent on the type of assay used and was cell-type specific (*9*, *15*, *16*, *19*, *29*). Therefore, additional activities for AG75 may be identified in other assays with other cell types.

Our results show a higher affinity binding of heparin to rec-α1G (19 nM) than to AG73 (6.4 μM), which suggests that there are additional heparin binding sites that may act in a synergistic manner or that a particular conformation of the recombinant molecule is required for optimal heparin binding. Potentially, the AG75 site could serve as a synergy site for the AG73 site or for other sites in the G domain. The conformation of the AG75 sequence may be critical for the heparin binding activity. It is also possible that the peptides may interact with specific heparan sulfate chains in a cell-specific manner.

Laminin-1, composed of α1, β1, and γ1 chains, has various biological activities involving heparin binding (*1*, *41*). AG73 partially inhibited heparin binding (data not shown) and cell attachment (*9*, *15*) to laminin-1, suggesting

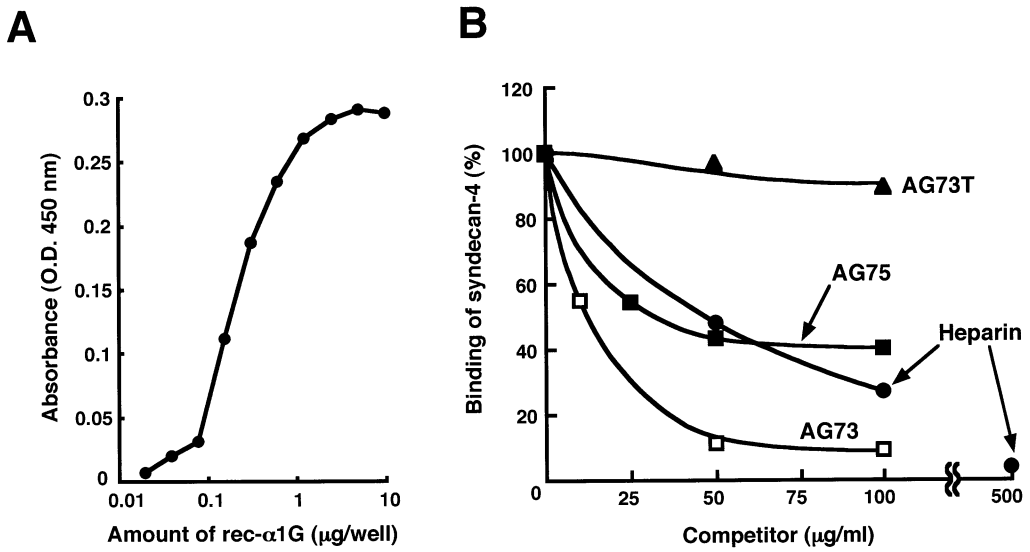

FIGURE 8: Syndecan-4 binding to rec-α1G using human neonatal dermal fibroblast cell lysates. (A) Various amounts of rec-α1G were coated on the 96-well ELISA plate. Human neonatal dermal fibroblast cell lysate was added after blocking with 3% BSA. Syndecan-4 bound to the rec-α1G-coated plates was detected by anti-syndecan-4 antibody, biotinylated anti-mouse IgG antibody, and streptavidin-conjugated horseradish peroxidase. (B) Effect of heparin and peptide on syndecan-4 binding to rec-α1G. Wells were coated with rec-α1G (2 μg/well). The fibroblast cell lysate was added with various concentrations of heparin or peptides. Triplicate experiments gave similar results.

that the AG73 site functions as a heparin/heparan sulfate binding and cell attachment site in laminin-1 although laminin-1 has multiple cell attachment sites. Other heparin binding sites in laminin-1 have been identified (*1*, *24*, *33*, *42*, *43*). These sites are important for interactions between laminin-1 and cell surface and extracellular heparan sulfate proteoglycans, such as syndecans and perlecan.

Recently, we have identified heparin binding sites in the laminin α3, α4, and α5 chain G domains using recombinant proteins and synthetic peptides. A3G75aR (NSFMALYL-SKGR, human laminin α3 chain 1412−1423), A4G82 (TLFLAHGRLVFM, mouse laminin α4 chain 1514−1525), and A5G81 (F4, AGQWHRVSVRWG, mouse laminin α5 chain 3337−3348) inhibited heparin binding to the G domain recombinant proteins (*35*, *37*, *38*). Interestingly, these heparin binding sites and the AG73 and AG75 sites are located in the LG4 module, but these sites are not homologous sites based on alignment of the LG4 sequences (*44*, *45*). A3G75aR and A4G82 are located in the connecting loop in the E and F strands, whereas A5G81 is located on the H strand. AG73 and AG75 are located in the C strand and the connecting loop in the D and E strands, respectively. The different localization of these sites may provide a rationale for specificity in laminin isoform function and/or for the cell type- and tissue-specific activity.

Syndecans, a family of heparan sulfate proteoglycans, have multiple functions and are involved in cell−extracellular matrix interactions, cell motility, and focal adhesion assembly (*46*−*49*). Previously, syndecan-1 was identified as a receptor for AG73 (*15*). In this study, AG73 and AG75 inhibited syndecan-4 binding of rec-α1G. Taken together, heparan sulfate chains of syndecans may interact with the AG73 and AG75 sites in the α1 chain G domain. Recently, syndecan-2 and -4 were identified as cell surface receptors recognizing the connecting loop region in the E and F strands of the α3 chain (*38*). When a homologous peptide of the α1 chain G domain (DYATLQLQEGRLHFMFDLG, mouse laminin α1 chain 2747−2765) was prepared and tested for cell attachment activity, the peptide strongly promoted α2β1 integrin-mediated fibroblast attachment (*50*). These data suggest that the connecting loop in the E and F strands of the α1 chain LG4 module has also a potential to be involved in cell binding.

In conclusion, we systematically screened the laminin α1 chain G domain for heparin binding sites using a recombinant protein and 110 soluble synthetic peptides. Both AG73 and AG75, a newly identified heparin binding peptide, were able to complete the binding of rec-α1G to heparin. AG73 and AG75 both interact with syndecan-4. These peptides are useful tools to investigate the heparin-mediated biological activities of the laminin α1 chain G domain.

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