BIOCHEMISTRY
including biophysical chemistry & molecular biology

Article

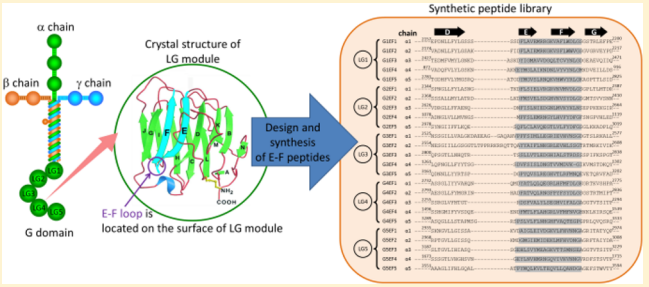
pubs.acs.org/biochemistry

Biological Activities of the Homologous Loop Regions in the Laminin α Chain LG Modules

Fumihiko Katagiri, Toshihiro Hara, Yuji Yamada, Shunsuke Urushibata, Kentaro Hozumi, Yamato Kikkawa, and Motoyoshi Nomizu*

Laboratory of Clinical Biochemistry, School of Pharmacy, Tokyo University of Pharmacy and Life Sciences, Hachioji, Tokyo 192-0392, Japan

ABSTRACT: Each laminin α chain (α1−α5 chains) has chain-specific diverse biological functions. The C-terminal globular domain of the α chain consists of five laminin-like globular (LG1−5) modules and plays a critical role in biological activities. The LG modules consist of a 14-stranded β-sheet (A−N) sandwich structure. Previously, we described the chain-specific biological activities of the loop regions between the E and F strands in the LG4 modules using five homologous peptides (G4EF1−G4EF5). Here, we further analyze the biological activities of the E−F strands loop regions in the rest of LG modules. We designed 20 homologous peptides (approximately 20 amino acid length), and 17 soluble peptides were used for the cell attachment assay. Thirteen peptides promoted cell attachment activity with different cell morphologies. Cell attachment to peptides G1EF1, G1EF2, G2EF1, G3EF4, and G5EF4 was inhibited by heparin, and peptides G1EF1, G1EF2, and G2EF1 specifically bound to syndecan-overexpressing cells. Cell attachment to peptides G2EF3, G3EF1, G3EF3, G5EF1, G5EF3, and G5EF5 was inhibited EDTA. Further, cell attachment to peptides G3EF3, G5EF1, and G5EF5 was inhibited by both anti-integrin α2 and β1 antibodies, whereas cell attachment to peptide G5EF3 was inhibited by only anti-integrin β1 antibody. Cell attachment to peptides G1EF4, G3EF4, and G5EF4 was inhibited by both heparin and EDTA and was not inhibited by anti-integrin antibodies. The active peptide sequence alignments suggest that the syndecan-binding peptides contain a "basic amino acid (BAA)-Gly-BAA" motif in the middle of the molecule and that the integrin-binding peptides contain an "acidic amino acid (AAA)"-Gly-BAA motif. Core-switched peptide analyses suggested that the "BAA-Gly-BAA" motif is critical for binding to syndecans and that the "AAA-Gly-BAA" motif has potential to recognize integrins. These findings are useful for understanding chain-specific biological activities of laminins and to evaluate receptor-specific binding mechanisms.



Laminins are hetero-trimeric glycoproteins consisting of three different subunits, α, β, and γ chains. Laminins have diverse biological activities, including promotion of cell adhesion, migration, neurite outgrowth, tumor metastasis, and angiogenesis.[1] So far, five α, three β, and three γ chains have been identified, and at least 15 isoforms are formed by various combinations of each subunit.[2−5] Each laminin isoform has tissue- and developmental stage-specific biological activities, and the α chains are mainly involved in the specificity. The C-terminal globular domains (G domains) of the α chains consist of five laminin-like globular (LG1—5) modules that play a critical role in the biological functions of laminins.[6] The crystal structure of the laminin α2 chain LG5 module and the sequence alignment analysis suggest that the LG modules consist of a 14 β strand (A−N) sandwich structure.[6,7]

Previously, we evaluated the biological activities of the loop regions of the E and F strands in the LG4 modules using five homologous peptides from each of the mouse laminin α chains (G4EF1, G4EF2, G4EF3, G4EF4, and G4EF5: the sequences are shown in Figure 1).[8] These homologous peptides showed chain-specific biological activities. Cell attachment to G4EF2 and G4EF4 was mediated by syndecan-2.[8] In contrast, G4EF1 promoted α2β1 integrin-mediated cell attachment.[8] Further, when the active core sequence of G4EF1 (G4EF1Xm: LQLQEGRLHF-Nle-FD) was cyclized, the cyclic peptide significantly enhanced integrin-mediated cell attachment.[8] These results indicate that integrin-mediated cell attachment to the G4EF1 sequence is conformation-dependent and that the loop structure is important for the activity.

Biologically active sites in the G domains of the other α chains have been identified.[9−14] The A3G75 peptide (KNSFM-ALYLSKG, human α3 chain 1411−1422) promoted syndecan-2 and -4 mediated cell attachment and neurite outgrowth.[9,10] The A4G82 sequence (TLFLAHGRLVFM, mouse laminin α4 chain 1514−1525) showed heparin binding and cell attachment activity.[11,12] These active sequences are in the homologous region of the LG4 modules of the laminin α3 and α4 chains. Additionally, mutagenesis analysis using recombinant LG4 proteins with Ser or Ala substitutions in the E−F loop regions

Received: March 29, 2014
Revised: May 13, 2014
Published: May 21, 2014

dx.doi.org/10.1021/bi5003822 | *Biochemistry* 2014, 53, 3699−3708

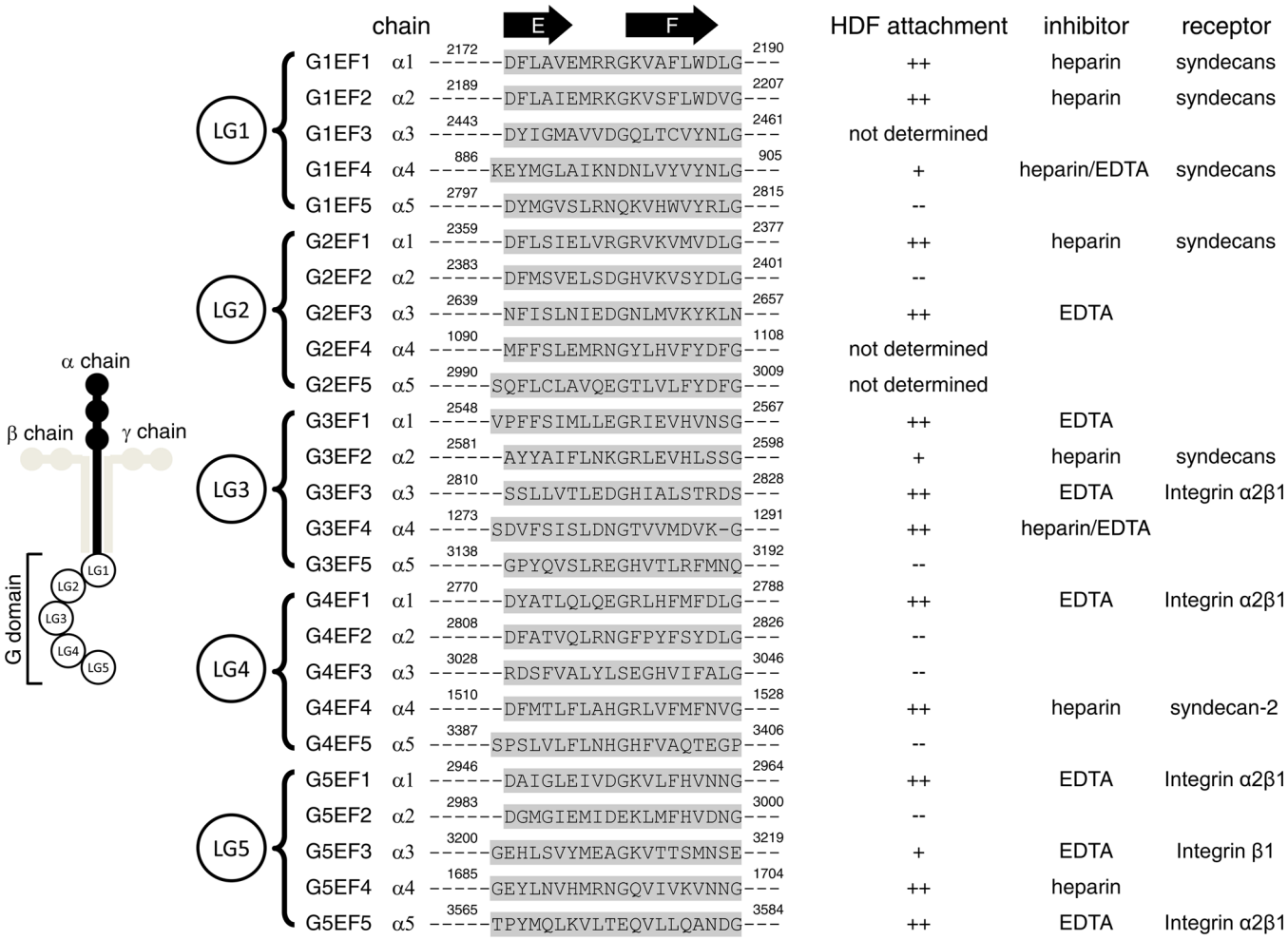


Figure 1. Location and sequences of homologous peptides in the connecting loop regions in the α chain LG modules. Sequences are from mouse laminin α1−α5 chains. Arrows represent β strands. Shaded area denotes the sequences of the synthetic peptide used here.

(Lys[1421] and Arg[1423] in human laminin α3 chain; His[1519] and Arg[1521] in mouse laminin α4 chain) suggested that these regions are critical for the biological activity.[9,11]

In this study, we focused on the E−F loop regions of the laminin α chain LG modules. We designed 20 homologous peptides from the LG1−3 and LG5 modules of the mouse laminin α chains (α1−α5) and evaluated their biological activity. These peptides showed chain-specific cell attachment activity with different receptor interactions, including integrins and syndecans. On the basis of active sequence alignments, active motifs for integrin- and syndecan-bindings are proposed.

MATERIALS AND METHODS

Synthetic Peptides. All peptides were prepared manually using *N*-(9-fluorenyl)methoxycarbonyl (Fmoc)-based solid-phase peptide synthesis with a C-terminal amide. The respective amino acids were condensed manually in a stepwise manner using 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy resin (Novabiochem, San Diego, CA). Dimethylformamide (DMF) was used during the synthesis as a solvent. For condensation, the diisopropylcarbodiimide/*N*-hydroxybenzotriazole method was employed. The resulting protected peptide resins were deprotected and cleaved from the resin using trifluoroacetic acid (TFA)-thioanisole-*m*-cresol-ethanedithiol-H_2O (80:5:5:5:5, v/v) at room temperature for 2 h. The crude peptides were precipitated and washed with diethyl 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% TFA. Peptides G1EF3, G2EF4, and G2EF5 did not dissolve in aqueous solution. Purity and identity of the peptides were confirmed by HPLC and electrospray ionization mass spectrometry at the Central Analysis Center, Tokyo University of Pharmacy and Life Sciences.

Antibodies. Mouse monoclonal antibodies against human integrins α1 (FB12), α2 (P1E6), α3 (P1B5), αV (P3G8), and β1 (6S6) were purchased from Millipore Co. Ltd. (Billerica, MA). Rat monoclonal antibody against human integrin α6 (GoH3) was purchased from AMAC, Westbrook, ME. Mouse monoclonal antibody against human IgG heavy chain (MR36G) was purchased from Cymbus Biotechnology Ltd., Chandlers Ford, UK.

Cells and Culture. Human neonatal dermal fibroblasts (HDFs, AGC Techno Glass Co., Ltd., Chiba, Japan) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin (Invitrogen Life Technologies, Carlsbad, CA). The series of human B-lymphoid cell lines ARH77, which are transfected with heparan sulfate proteoglycans (syndecan-1, −2, −4 and glypican-1),[15,16] were grown in suspension in RPMI-1640 medium (Wako Pure

dx.doi.org/10.1021/bi5003822 | *Biochemistry* 2014, 53, 3699−3708

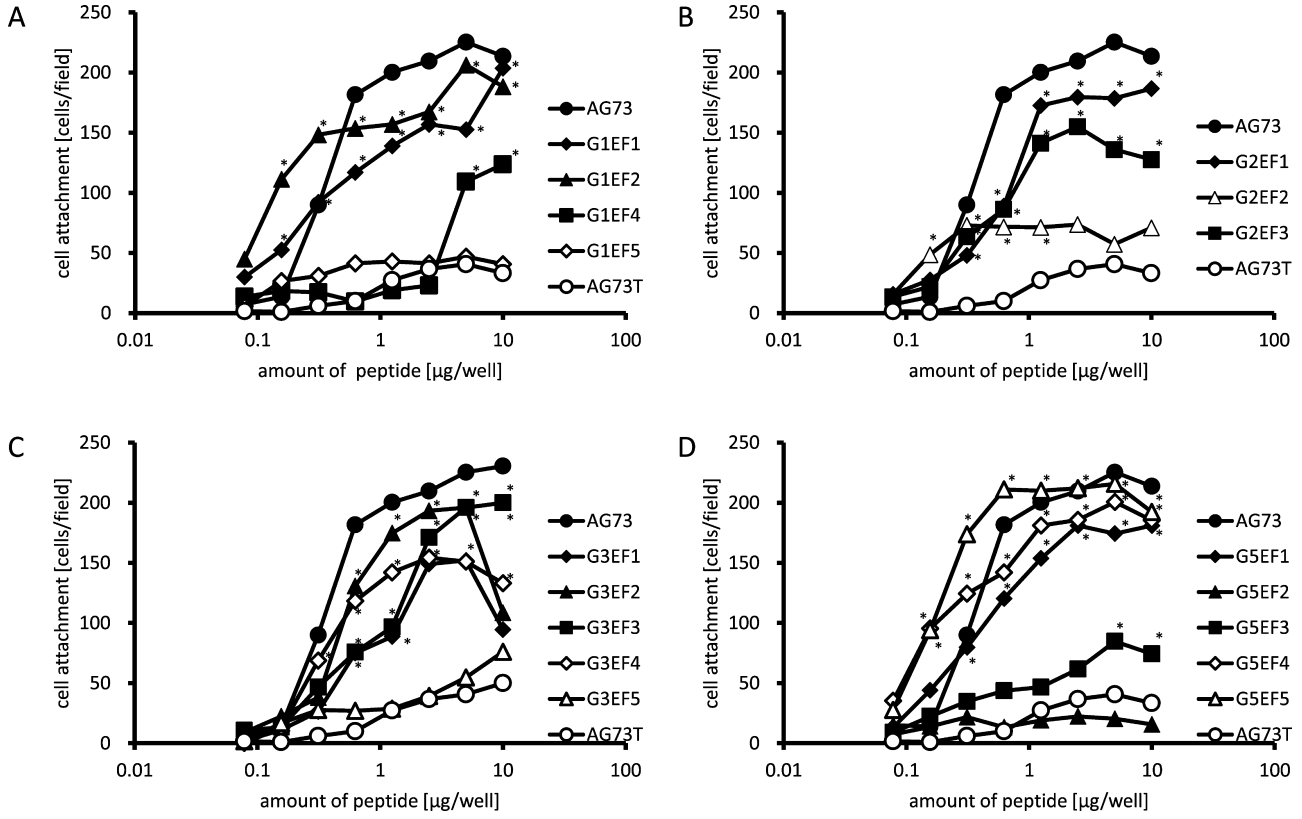


Figure 2. Cell attachment activity of the homologous peptides. A, B, C, and D show HDF attachment activities of peptides derived from the LG1, 2, 3, and 5 modules of laminin $\alpha1-\alpha5$ chain G domain, respectively. Ninety-six-well plates were coated with various amounts of synthetic peptides and examined for cell attachment activity using HDFs. The 20000 cells were added to the wells for 1 h. After the cells were stained with crystal violet, the attached cells in three randomly selected fields (0.67 mm^2/field) were counted under a microscope. Triplicate experiments gave similar results. *$p < 0.01$.

Chemical Industries, Ltd., Osaka, Japan) supplemented with 5% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin. All cells were maintained at 37 °C in an humidified, 5% CO_2, and 95% air atmosphere.

Cell Attachment Assay. Cell attachment assays were performed in 96-well plates (Nunc, Inc., Naperville, IL) coated with various amounts of synthetic peptides. For peptide coating, various amounts of peptides in 50 μL of water were added to the wells and dried overnight at room temperature. The peptide-coated wells were blocked with 150 μL of 1% bovine serum albumin (BSA, Sigma Chemical Co., St. Louis, MO) in DMEM at 37 °C for 1 h and then washed with DMEM containing 0.1% BSA. HDFs were detached with 0.02% trypsin-EDTA (Invitrogen Life Technologies) and recovered with DMEM containing 10% FBS at 37 °C for 20 min. After being washed with DMEM containing 0.1% BSA, cells (20000 cells/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 being washed with water, the attached cells were photographed using a BZ-8000 microscope (Keyence, Osaka, Japan). Images were captured and analyzed using BZ-analyzer software (Keyence). The attached cells in three randomly selected fields (0.67 mm^2) were counted. All assays were carried out in triplicate, and each experiment was repeated at least three times.

For inhibition of cell attachment with heparin, EDTA, HDFs were preincubated in suspension with either 10 μg/mL heparin or 5 mM EDTA for 10 min at room temperature. Then, the cells were added to the wells and incubated for 30 min at 37 °C. For inhibition of cell attachment with anti-integrin antibodies, HDFs were preincubated in suspension with 10 μg/mL of the anti-integrin antibody for 15 min at room temperature. Then, the cells were added to the wells and incubated for 30 min at 37 °C. Attached cells were measured as described above.

Actin Cytoskeleton Formation and Vinculin Localization in Cultured Cells. Various amounts of peptides in 150 μL of water were coated on an 8-well chamber slide (Nunc) and dried overnight at room temperature. The cells were fixed with 4% paraformaldehyde and 5% sucrose in Tris-buffered saline (TBS) for 10 min and permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 10 min. The fixed cells were washed with PBS for 30 min, blocked with 1% BSA in PBS for 1 h, and then incubated with mouse monoclonal antibody against vinculin (clone hVIN-1; 1:100) overnight at 4 °C. After washing with 0.05% Tween 20 in PBS, bound antibody and actin filaments were labeled with a mixture of rhodamine red-labeled donkey antimouse IgG antibody (Jackson Immuno Research Laboratories, West Grove, PA, 1:50) and Allexa Fluoro 488 phalloidin (1 U/mL; Invitrogen, 1:100) for 90 min. Nuclei were labeled with 4,6-diamidino-2-phenylindole (DAPI, Invitrogen, 1:10000). After being washed, cells were mounted with antifade and examined under a FV100D Ix81 fluorescent confocal microscope (Olympus, Tokyo).

Statistics Analysis. Results were expressed as + standard deviation (S.D.). Comparison of mean values was performed using one-way analysis of variance and a homoscedastic *t* test. $p < 0.01$ indicated statistical significance.

dx.doi.org/10.1021/bi5003822 | *Biochemistry* 2014, 53, 3699−3708

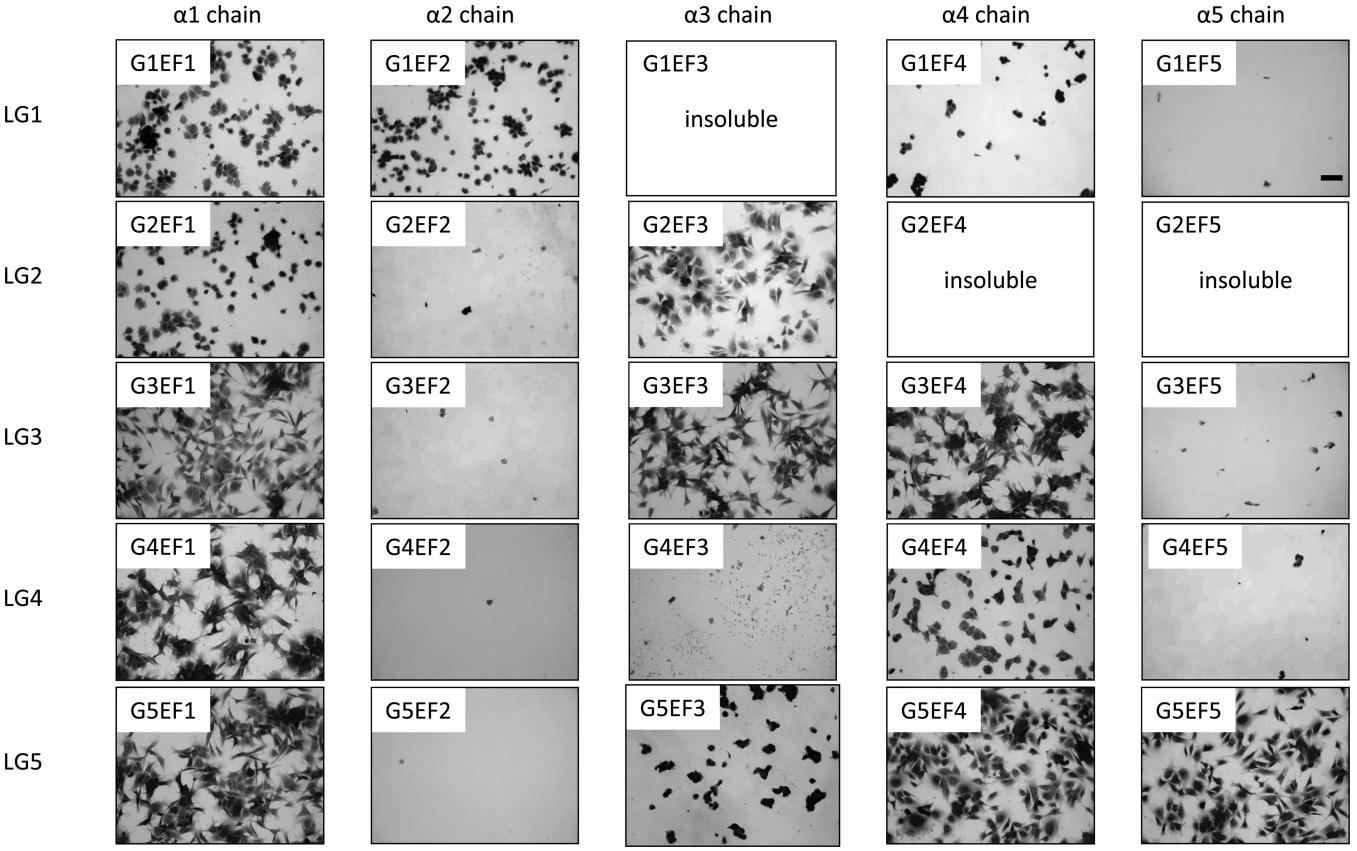


Figure 3. Morphological appearance of HDFs attached to the homologous peptide. Ninety-six-well plates were coated with 10 μg/well of peptides G1EF5, G2EF2, G2EF3, G3EF1, G3EF2, G3EF5, G4EF2, G4EF3, G4EF4, G4EF5, and G5EF2, 5 μg/well of peptides G1EF1, G1EF2, G1EF4, G5EF3, G5EF4, and G5EF5, 2 μg/well of peptide G2EF1, 1 μg/well of peptides G3EF3, and G3EF4, 0.5 μg/well of peptides G4EF1, and G5EF1. HDFs (5000 cells) were added to the wells. After a 2 h incubation, attached cells were stained with crystal violet. Triplicate experiments gave similar results. Bar, 100 μm.

RESULTS

Cell Attachment Activity of the Homologous Peptides. Twenty homologous peptides were designed based on the alignment of the LG module sequences (Figure 1).[6,7] These peptides contained the E and F strands and their connecting loop regions. Three of the peptides (G1EF3, G2EF4, and G2EF5) did not dissolve in an aqueous solution, and the rest of 17 peptides were used for the experiments. Five homologous peptides G4EF1, G4EF2 (DFGTVQLRNGFPFFSYDLG, mouse laminin α2 chain 2808−2826), G4EF3, G4EF4, and G4EF5 from mouse laminin α chain LG4 sequences were also synthesized as a control.[8] First, we tested their cell attachment activity using HDFs (Figure 2). Peptides AG73 (RKRLQVQLSIRT, mouse laminin α1 chain 2719−2730) and AG73T (LQQRRSVLRTKI, scrambled AG73) were used as positive and negative controls, respectively.[17] AG73 has strong cell attachment activity and binds heparin and syndecans, membrane-associated heparan sulfate proteoglycans (HSPG) (Figure 2).[9,18,19] Thirteen peptides promoted HDF attachment in a dose-dependent manner. G4EF1 and G4EF4 promoted HDF attachment as described previously (Figure 2).[8] Four peptides (G1EF5, G3EF5, G5EF2, and G5EF3) did not promote cell attachment (Figure 2).

The morphological appearance of HDFs on the various peptides differed (Figure 3). G4EF1 promoted extensive HDF spreading, while the cells on G4EF4 showed ruffling, as previously described.[8] G4EF2 did not show cell attachment. G3EF1, G3EF3, G3EF4, and G5EF1 promoted extensive HDF spreading similar to that on G4EF1. G2EF3, G5EF4, and G5EF5 promoted cell spreading, but the morphological appearance differed from that on G4EF1. G1EF1, G1EF2, and G2EF1 showed cell attachment with ruffling similar to that on G4EF4. G1EF4, G2EF2, and G3EF2 promoted weak cell attachment without spreading.

Effects of Heparin and EDTA on HDF Attachment to the Homologous Peptides. Next, we evaluated the effects of heparin and EDTA on HDF attachment to the peptides (Figure 4). We used AG73 and G4EF1 as controls for heparin-dependent and cation-dependent cell attachment, respectively. Heparin strongly inhibited attachment on G1EF1, G1EF2, G1EF4, G2EF1, G3EF2, G3EF4, and G5EF4, suggesting that these peptides have the potential to interact with membrane-associated HSPGs. In contrast, EDTA significantly inhibited cell attachment on G1EF4, G2EF3, G3EF1, G3EF3, G3EF4, G5EF1, G5EF3, and G5EF5. HDFs attached to these peptides in a cation-dependent manner, suggesting that the peptides promoted integrin-mediated cell adhesion.

Effect of Anti-Integrin Antibodies on HDF Attachment to the Homologous Peptides. Since HDF attachment to eight peptides (G1EF4, G2EF3, G3EF1, G3EF3, G3EF4, G5EF1, G5EF3 and G5EF5) was divalent cation-dependent and these peptides promoted HDF spreading, integrins are major candidates for their cellular receptor(s). We attempted to identify the cell surface receptors for the active peptides using function-blocking antibodies against integrin subunits. HDF

dx.doi.org/10.1021/bi5003822 | Biochemistry 2014, 53, 3699−3708

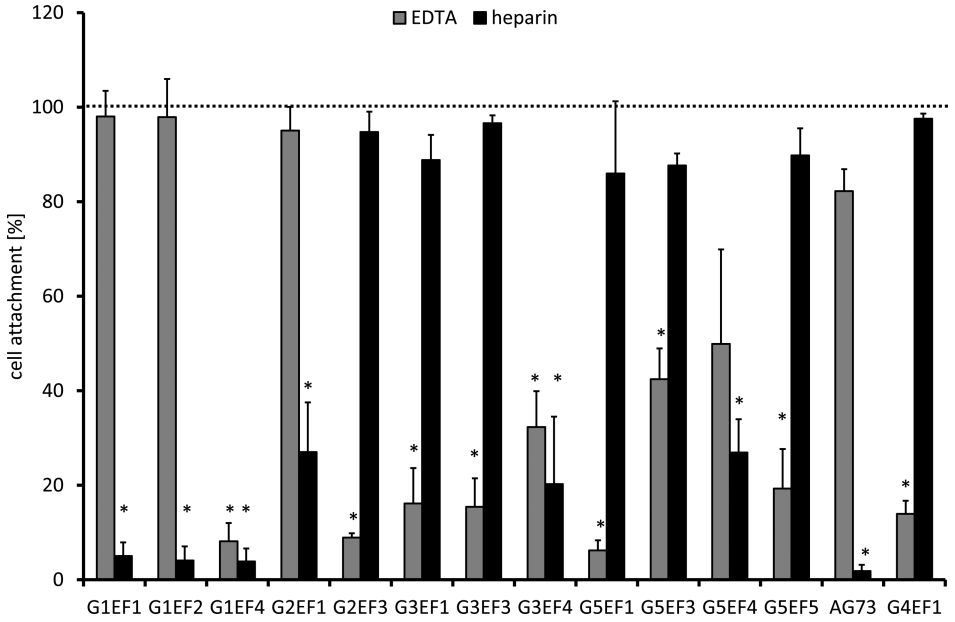


Figure 4. Effect of heparin and EDTA on HDF attachment to peptide-coated plates. HDFs were allowed to attach to the peptide-coated plates in the absence (100%) and presence of either 5 mM EDTA (gray bars) or 10 μg/mL heparin (black bars). Ninety-six-well plates were coated with 10 μg/well of peptides G2EF3, G3EF1, G3EF2, and G4EF4, 5 μg/well of peptides G1EF1, G1EF2, G1EF4, G5EF3, G5EF4, and G5EF5, 2 μg/well of peptide G2EF1, 1 μg/well of peptides G3EF3, and G3EF4, 0.5 μg/well of peptides G4EF1, G5EF1, and AG73. Either 10 μg/mL heparin or 5 mM EDTA was added to the cell suspensions. The cell suspensions in the presence of either 10 μg/mL heparin or 5 mM EDTA were preincubated at room temperature for 10 min. Then, the cells were added to the peptide-coated plates. After a 30 min incubation, the attached cells were stained with crystal violet, and the adherent cells were counted under a microscope in three different fields which were selected at random (0.67 mm^2/field). Each value represents the mean of three separate determinations + SD. Triplicate experiments gave similar results. *p < 0.01.

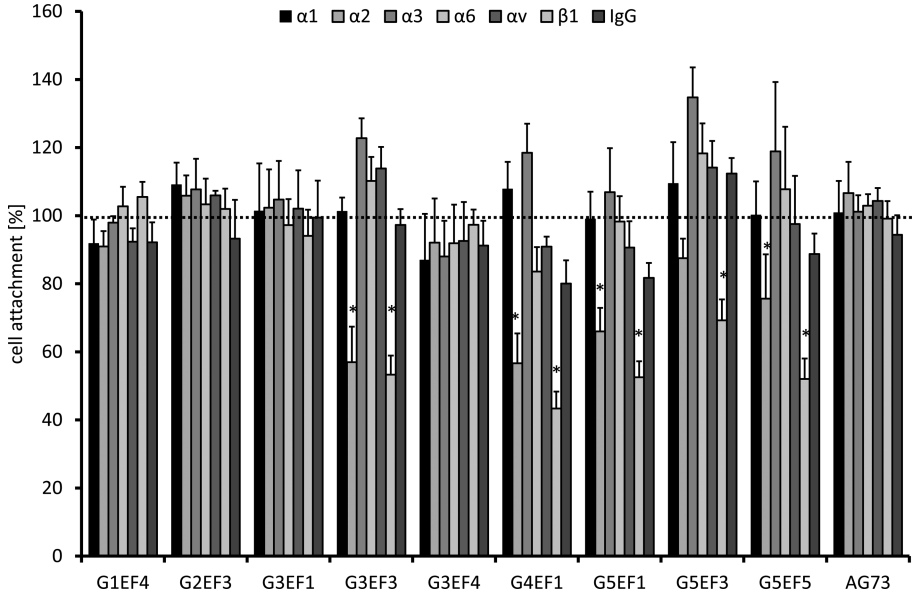


Figure 5. Effect of anti-integrin antibodies on the attachment of HDFs to peptide-coated plates. HDFs were allowed to attach to the peptide-coated plates in the absence (100%) and presence of either 10 μg/mL of anti-integrin α1, α2, α3, α6, αv, or β1 antibody. Ninety-six-well plates were coated with 10 μg/well of peptides G2EF3 and G3EF1, 5 μg/well of peptides G1EF4, G5EF3, and G5EF5, 1 μg/well of peptides G3EF3, and G3EF4, 0.5 μg/well of peptides G4EF1, G5EF1, and AG73. Each 10 μg/mL of anti-integrin antibody was added to the cell suspensions and preincubated at room temperature for 15 min. Then, the cells were added to the wells and incubated for 30 min. After being stained with crystal violet, the attached cells were counted under a microscope in three different fields that were selected at random (0.67 mm^2/field). Each value represents the mean of three separate determination + SD. Triplicate experiments gave similar results. *p < 0.01.

attachment to five peptides (G3EF3, G5EF1, G5EF3, and G5EF5) was inhibited by anti-integrin β1 antibody (Figure 5). In addition, HDF attachment to G3EF3, G5EF1, and G5EF5 was inhibited by anti-integrin α2 antibody. These results suggest that HDF attachment to three peptides (G3EF3, G5EF1, and G5EF5) is mediated by integrin $\alpha 2\beta 1$ similar to that to G4EF1.

Attachment of the Homologous Peptides to Syndecans-1, -2, -4, and to Glypican-1 Overexpressing Lymphoid Cells. Syndecans and glypicans are cell surface

dx.doi.org/10.1021/bi5003822 | *Biochemistry* 2014, 53, 3699−3708

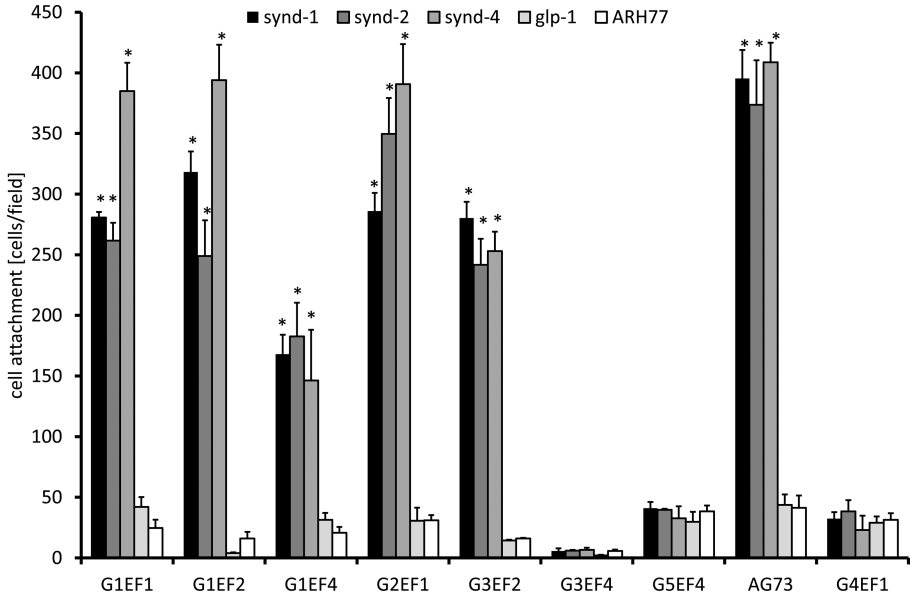


Figure 6. Cell attachment activity of the homologous peptide with various heparan sulfate proteoglycan-overexpressing cell lines. Heparan sulfate proteoglycan-overexpressing cell lines with syndecan-1 (synd-1), syndecan-2 (synd-2), syndecan-4 (synd-4), and glypican-1 (glp-1) and controls without these receptors (ARH77) were used. Ninety-six-well plates were coated with 10 μg/well of peptides G3EF2, 5 μg/well of peptides G1EF1, G1EF2, G1EF4, and G5EF4, 2 μg/well of peptide G2EF1, 1 μg/well of peptide G3EF4, 0.5 μg/well of peptides G4EF1, and AG73. After each plate had been blocked with 1% BSA in DMEM, 20000 cells/well were added to the well and incubated for 1 h. After the cells had been washed, the number of attached cells was assessed by crystal violet staining. Data are shown as the mean + SD of triplicate results. *P < 0.01 vs ARH77.

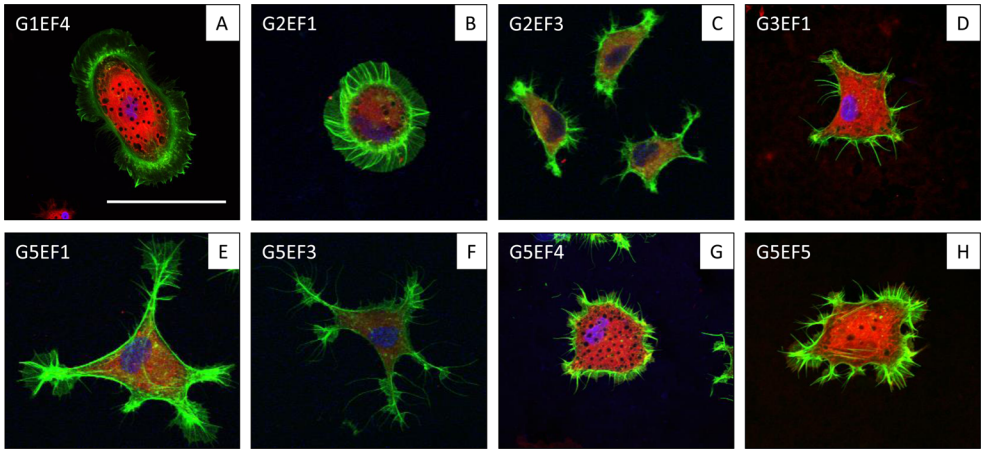


Figure 7. Organization of actin filaments and localization of vinculin in fibroblasts on the peptide-coated plates. Wells of a chamber slide were coated with 20 μg/well of peptides G2EF3 (C) and G3EF1 (D), 10 μg/well of peptides G1EF4 (A), G5EF3 (F), G5EF4 (G), G5EF5 (H), 5 μg/well of G2EF1 (B), and 1 μg/well of peptide G5EF1 (E). HDFs were seeded, and after a 2 h incubation, the attached cells were stained with triple color fluorescence consisting of actin filaments (green), vinculin (red), and nuclei (blue). Bar, 25 μm.

HSPGs. Peptides G1EF1, G1EF2, G1EF4, G2EF1, G3EF2, G3EF4, and G5EF4 strongly promoted heparin-dependent HDF attachment. Binding of these peptides to syndecans and to glypican-1 was examined using syndecan-1-, -2-, -4-, and glypican-1-overexpressing ARH77 B-lymphoid cells (synd-1, synd-2, synd-4, glp-1, respectively) (Figure 6).[15,16] ARH77 cells express low amounts of cell surface heparan sulfate. Peptides AG73 and G4EF1 were used as controls. ARH77 cells did not attach to any peptide. Peptides G1EF1, G1EF2, G1EF4, G2EF1, and G3EF2 bound to the all syndecan-overexpressing cell lines but did not bind to glp-1-overexpressing cells, suggesting that the peptides promote syndecan-mediated cell attachment. In contrast, peptides G3EF4 and G5EF4 did not bind to the cells, suggesting that the peptides promote heparin-dependent HDF attachment through other mechanisms.

Organization of Actin Filaments and Localization of Vinculin. Next, we focused on peptides G1EF1, G2EF1, G2EF3, G3EF1, G5EF1, G5EF3, G5EF4, and G5EF5, which promoted HDF attachment, and examined the organization of actin filaments and localization of vinculin on the peptide-coated plates (Figure 7). On peptides G1EF4 and G2EF1, cells displayed actin filament spikes associated with membrane ruffles and no focal contacts with vinculin were observed (Figure 7A,B). Peptides G5EF1 and G5EF5 induced well-organized actin stress fibers and focal contacts containing vinculin (Figure 7E,H). The HDFs on the other peptides showed spreading but the actin stress fibers did not appear. These results demonstrate that the cellular responses to the peptides are different.

dx.doi.org/10.1021/bi5003822 | *Biochemistry* 2014, 53, 3699−3708

peptide	sequence	central motif	spread	inhibition	receptor
G2EF3 (α3 chain LG2)	NFISLNIE DGN LMVKYKLN	AGX	+	EDTA	
G3EF1 (α1 chain LG3)	VPFFSIMLL EGR IEVHVNSG	AGB	+	EDTA	
G3EF3 (α3 chain LG3)	SSLLVTLE DGH IALSTRDS	AGB	+	EDTA	α2β1 integrin
G4EF1 (α1 chain LG4)	DYATLQLQ EGR LHFMFDLG	AGB	+	EDTA	α2β1 integrin
G5EF1 (α1 chain LG5)	DAIGLEIV DGK VLFHVNNG	AGB	+	EDTA	α2β1 integrin
G5EF3 (α3 chain LG5)	GEHLSVYME AGK VTTSMNSE	XGB	—	EDTA	β1 integrin
G5EF5 (α5 chain LG5)	TPYMQLKVL TEQ VLLQANDG	XAX	+	EDTA	α2β1 integrin
G1EF4 (α4 chain LG1)	KEYMGLAIK NDN LVYVYNLG	XAX	—	EDTA/heparin	syndecans
G3EF4 (α4 chain LG3)	SDVFSISLD NGT VVMDVKG	XGX	+	EDTA/heparin	
G5EF4 (α4 chain LG5)	GEYLNVHMR NGQ VIVKVNNG	XGX	+	EDTA/heparin	
G1EF1 (α1 chain LG1)	DFLAVEMR RGK VAFLWDLG	BGB	—	heparin	syndecans
G1EF2 (α2 chain LG1)	DFLAIEMR KGK VSFLWWVG	BGB	—	heparin	syndecans
G2EF1 (α1 chain LG2)	DFLSIELV RGR VKVMVDLG	BGB	—	heparin	syndecans
G3EF2 (α3 chain LG3)	AYYAIFLN KGR LEVHLSSG	BGB	—	heparin	syndecans
G4EF4 (α4 chain LG4)	DFMTLFLA HGR LVFMFNVG	BGB	—	heparin	syndecan-2

Figure 8. Summary of the active homologous peptides. Central motif of the HDF adhesive peptides inhibited by EDTA or heparin is in the boxes with solid lines or dotted lines, respectively. The alignment of active peptides sequence indicates that the center motif of integrin-mediated HDF adhesive peptides was "acidic amino acid (**A**)-Gly (G)-basic amino acid (**B**)" and that the center motif of syndecan-mediated HDF adhesive peptides was "**BGB**". X, any amino acids.

Cell Attachment Activity of the Core-Switched Peptides. Thirteen peptides showed cell attachment activities. The alignment of these peptides indicated a similar motif in the center of the peptide sequence (Figure 8). The integrin $\alpha 2\beta 1$-mediated cell adhesive peptide (G3EF3, G4EF1, and G5EF1) had an "acidic amino acid (AAA)-Gly-basic amino acid (BAA)" motif, while the syndecan-mediated cell adhesive peptides (G1EF1, G1EF2, G2EF1, G3EF2, and G4EF4) had a "BAA-Gly-BAA" motif (Figure 8). Next, we designed "core-switched" peptides, which were switched the core AAA-Gly-BAA motif (Asp-Gly-His and Asp-Gly-Lys) of the integrin $\alpha 2\beta 1$-mediated cell adhesive peptides (G3EF3 and G5EF1, respectively) to the core BAA-Gly-BAA motif (Arg-Gly-Lys, Lys-Gly-Lys, Lys-Gly-Arg, and Arg-Gly-Arg) of the syndecan-mediated cell adhesive peptides (G1EF1, G1EF2, G3EF2, and G2EF1, respectively) (Figure 9). Peptides AG73 and AG73T were used as positive and negative controls, respectively.[17] The BAA-Gly-BAA motif-switched peptides (G3EF3:RGK, G3EF3:KGK, G5EF1:RGR, and G5EF1:KGR) showed HDF attachment activity. In contrast, the AAA-Gly-BAA motif-switched G3EF2:DGK peptide showed HDF attachment activity but the other peptides (G1EF1:DGH, G1EF2:DGH, and G2EF1:DGK) did not show activity (Figure 10A).

Effects of Heparin and EDTA on HDF Attachment to the Core-Switched Peptides. Next, we evaluated the effects of heparin and EDTA on HDF attachment to the peptides (Figure 10B). Heparin significantly inhibited HDF attachment on all peptides that had the BAA-Gly-BAA motif switch (G3EF3:RGK, G3EF3:KGK, G5EF1:RGR, and G5EF1:KGR). In contrast, EDTA significantly inhibited HDF attachment on G3EF2:DGK, which had the AAA-Gly-BAA motif swtich.

Effect of Anti-Integrin Antibodies on HDF Attachment to the Core-Switched Peptide. We tried to identify the cell surface receptors for the G3EF2:DGK peptides using anti-integrin antibodies. HDF attachment to G3EF2:DGK peptides was inhibited by only anti-integrin $\beta 1$ antibody (Figure 10C).

Attachment of the Core-Switched Peptides to Syndecans-1, -2, -4, and to Glypican-1 Overexpressing Lymphoid Cells. Binding of these peptides to syndecans and to glypican-1 was examined using synd-1, synd-2, synd-4-, and glp-1- overexpressing cells, and ARH77. Peptides G3EF3:RGK, G3EF3:KGK, and G5EF1:RGR strongly promoted synd-1-, synd-2-, and synd-4-overexpressing cell attachment, which suggested that peptides G3EF3:RGK, G3EF3:KGK, and G5EF1:RGR bound syndecans specifically (Figure 10D). Peptide G5EF1:KGR promoted synd-1-, synd-2-, synd-4-, and glp-1-overexpressing cell attachment, which suggested that G5EF1:KGR bound HSPGs nonspecifically.

DISCUSSION

In this study, we evaluated the biological activities of the homologous peptides derived from the laminin LG module E–F loop region sequences, and the sequential requirement for

dx.doi.org/10.1021/bi5003822 | *Biochemistry* 2014, 53, 3699–3708

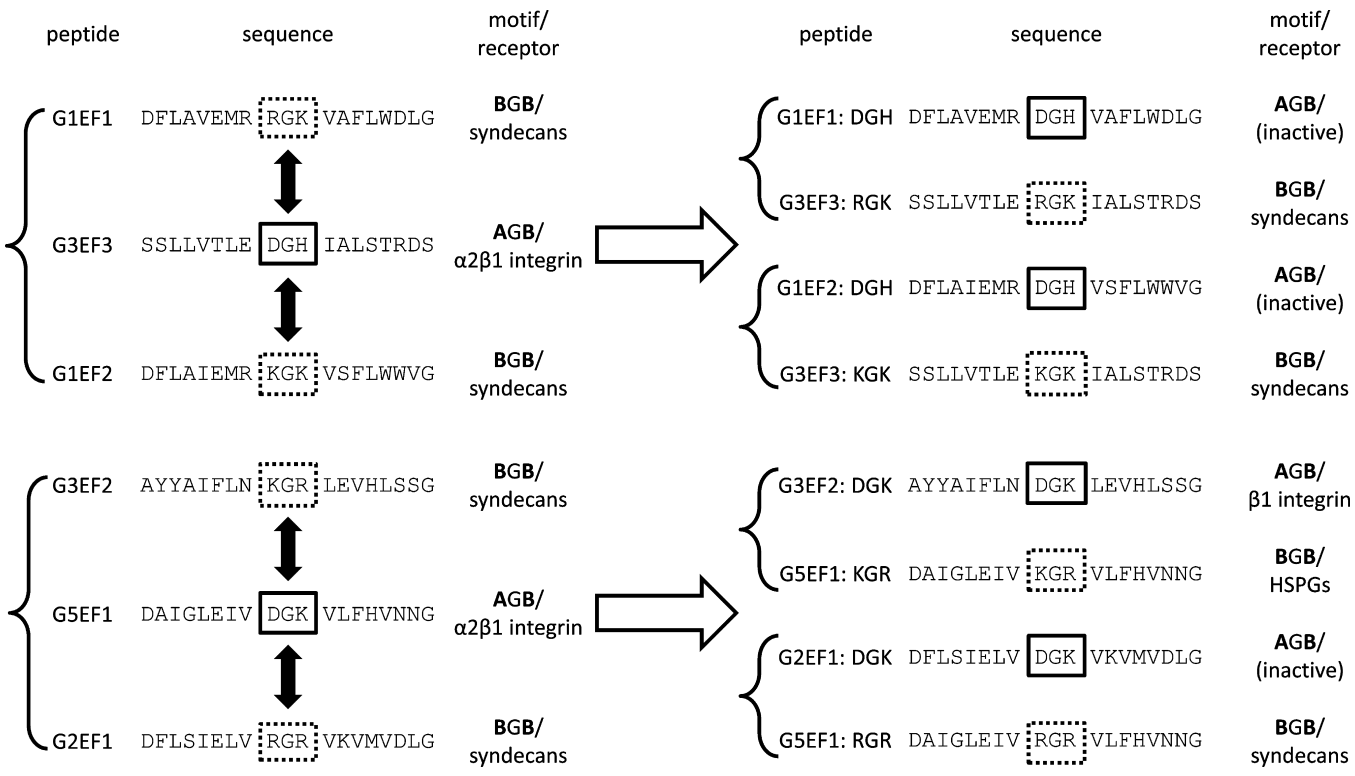


Figure 9. Design of core-switched peptides. Central motif of the HDF adhesive peptides inhibited by EDTA or heparin is in the boxes with solid lines or dotted lines, respectively. The center motif of $\alpha 2\beta 1$ integrin binding peptides (G3EF3 and G5EF1) was switched to the center motif of syndecan binding peptide (G1EF1, G1EF2 (G3EF3), G3EF2, and G2EF1 (G5EF1)). **A**, acidic amino acid; **B**, basic amino acid.

receptor binding. We prepared 22 peptides (Figure 1), and 15 of these peptides promoted cell attachment activity (Figure 3).

Laminins interact with more than 20 types of receptors and are involved in many biological activities.[20] Integrins and syndecans are major laminin receptors. Heparan sulfate proteoglycans (HSPGs) are a diverse family of glycosaminoglycan-bearing protein cores that include syndecans, glypicans, perlecan, and agrin. HSPGs play key roles during normal processes that occur during development, tissue morphogenesis, and wound healing. The morphological appearance of attached HDFs depends on the receptors present and bound to their ligand. The morphological appearance of HDF attached via syndecans shows a round shape, and syndecan-mediated cell attachment is inhibited by heparin. HDFs attached to peptides G1EF1, G1EF2, G1EF4, G2EF1, G3EF2, G4EF4, and G5EF3 showed a round shape (Figure 3), and HDF attachment to peptides G1EF1, G1EF2, G1EF4, G2EF1, G3EF2, and G4EF4 was inhibited by heparin (Figure 4). These six peptides also promoted strong cell attachment with HSPGs-overexpressing cells (Figure 6), which suggested that peptides G1EF1, G1EF2, G1EF4, G2EF1, G3EF2, and G4EF4 bound to syndecans.

Integrins are a family of cell adhesion receptors that are active with the extracellular matrix (ECM). They also play a role in cell signaling and thereby define cell shape and mobility, and regulate the cell cycle.[21] Integrins are obligate heterodimers containing two distinct chains, called α and β subunits. In mammals, 18 α and 8 β subunits have been characterized.[21] To date, 24 integrins have been identified and each integrin has tissue-specific expression and induces various signals.[22,23] When HDFs are attached to integrins, the cells are spread, and focal contacts are induced along with actin cytoskeleton formation. Integrins are activated by divalent cations,[21] and the cell attachment mediated by integrin is inhibited by EDTA. HDFs attached to peptides G2EF3, G3EF1, G3EF3, G3EF4, G4EF1, G5EF1, G5EF4, and G5EF5 were spread (Figure 3), whereas HDF attached to peptides G2EF3, G3EF1, G3EF3, G3EF4, G4EF1, G5EF1, and G5EF5 were inhibited by EDTA (Figure 4). Furthermore, cell attachment to peptides G3EF3, G4EF1, G5EF1, G5EF3, and G5EF5 was inhibited by anti-integrin antibodies (Figure 5), which suggested that these peptides bound to integrins. HDFs attached to peptides G4EF1,[8] G5EF1, and G5EG5 had organized cytoskeleton with localized vinculin, suggesting formation of focal contact (Figure 7E,H).

The active peptide sequence alignments suggest that the syndecan binding peptide contain a "basic amino acid (BAA)-Gly-BAA" motif in the middle of the sequence and that most integrin binding peptides contain an "acidic amino acid (AAA)"-Gly-BAA motif (Figure 8). The BAA-Gly-BAA and AAA-Gly-BAA motifs might contribute to the receptor recognition. Kato et al. also suggests that the Lys-Gly-Arg motif in the A3G75aR (NSFMALYSKGR, human laminin $\alpha 3$ chain 1412−1423), which is located the E-F loop region, is critical for binding to syndecan-2/-4.[9] We designed the "core-switched peptides" where we switched the integrin-binding center motif "AAA-Gly-BAA" with the syndecan-binding center motif "BAA-Gly-BAA" (Figure 9). All integrin-binding peptides with the "BAA-Gly-BAA" motif (G3EF3:RGK, G3EF3:KGK, G5EF1:RGR, and G5EF1:KGR) promoted HDF attachment, which was inhibited by heparin (Figure 10A,B). Furthermore, most of these peptides (G3EF3:RGK, G3EF3:KGK, and G5EF1:RGR) were active with the syndecan-overexpressing cells (Figure 10D). The "BAA-Gly-BAA" motif is thought to be both necessary and sufficient for binding syndecans. On the other hand, most of the syndecan-binding peptides with the

dx.doi.org/10.1021/bi5003822 | *Biochemistry* 2014, 53, 3699−3708

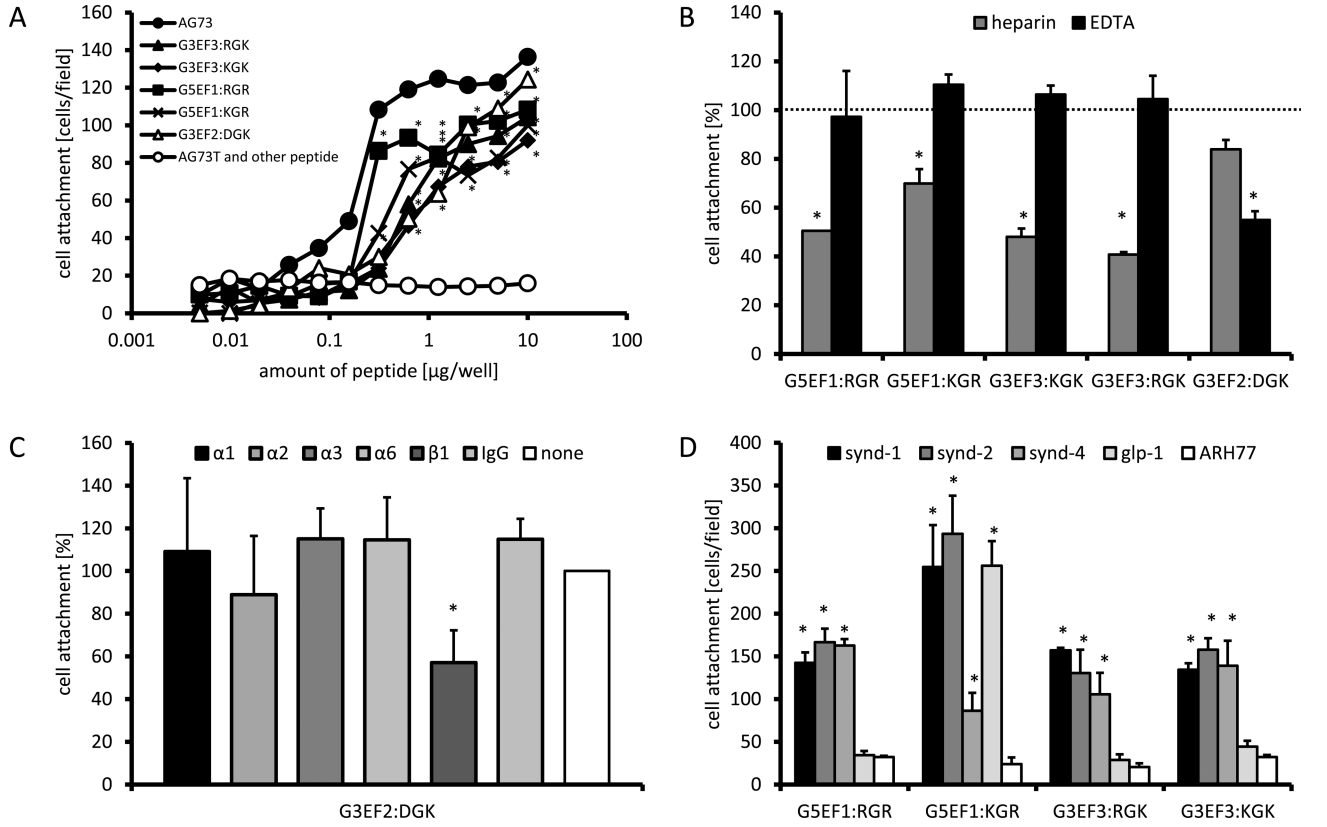


Figure 10. Cell attachment activity of the core-switched peptides. 96-well plates were coated with various amounts of synthetic peptides and examined for cell attachment activity using HDFs (A). HDFs were allowed to attach to the peptide-coated plates in the absence (100%) and presence of either 5 mM EDTA (gray bars), 10 μg/mL heparin (black bars) (B), or 10 μg/mL of anti-integrin α1, α2, α3, α6, or β1 antibodies (C). Heparan sulfate proteoglycan-overexpressing cell lines with syndecan-1 (synd-1), syndecan-2 (synd-2), syndecan-4 (synd-4), and glypican-1 (glp-1) and controls without these receptors (ARH77) were used (D). After the cells were stained with crystal violet, the attached cells in three randomly selected fields (0.67 mm^2/field) were counted under a microscope. Each value represents the mean of three separate determinations + SD. Triplicate experiments gave similar results. *$p < 0.01$ vs controls.

"AAA-Gly-BAA" motif did not promote HDF attachment (Figure 10A). Suzuki et al. reported that the Asp residue in the G4EF1 peptide is important for cell attachment activity using truncated peptides of G4EF1 in attachment assays.[8] For integrin-binding activity, it might be necessary that both the "AAA-Gly-BAA" motif in the center of sequence and another acidic amino acid residue are present.

Previously, we screened for active peptides for HDF attachment in the laminin peptide library, which consisted of 12 amino acid residue peptides, and covered all sequence of each laminin α chain G domain.[24−27] AG28 (LSIELVRGR-VKV, mouse laminin α1 chain, 2361−2372),[25] A2G52 (YAIFLNKGRLEV, mouse laminin α2 chain, 2582−2593),[26] and A4G6 (LAIKNDNLVYV, mouse laminin α4 chain, 891−901),[27] which contain part of G2EF1, G3EF2, and G1EF4, respectively, show heparin-dependent cell attachment. These reports support our hypothesis that the "BAA-Gly-BAA" motif is critical for syndecan-mediated cell adhesion, while AG6 (LAVEMRRGKVAF, mouse laminin α1 chain, 2174−2185),[25] A2G6 (LAIEMRKGKVSF, mouse laminin α2 chain, 2191−2202),[26] AG77 (LQLQEGRLHFMF, mouse laminin α1 chain, 2774−2785),[25] AG98 (LEIVDGKVLFHV, mouse laminin α1 chain, 2950−2961),[25] and MA3G50 (LVTLEDGHIALS, mouse laminin α3 chain, 2813−2824),[24] which contain part of G1EF1, G1EF2, G4EF1, G5EF1, and G3EF3, respectively, do not promote cell attachment in the peptide-coated plate assay. Kato and Suzuki report that the cyclic peptides, which mimic the loop structure, promote improved cell attachment when compared with that of the linear peptides.[8,28] Since the peptides in this study were designed to cover the E−F loop regions, the peptides easily formed the loop structure in solution. For integrin/syndecan-specific binding, it may be important that the peptides form the loop (turn) structure.

In conclusion, homologous peptides derived from the E−F loop region sequence in the laminin α chain G domain showed chain-specific biological activities. These results are important for understanding the biological functions of laminin α chains. Furthermore, we found that formation of the loop structure was critical for interaction with integrin/syndecan. We suggest that the "BAA-Gly-BAA" motif is critical for binding to syndecans and that the "AAA-Gly-BAA" motif has a potential to recognize integrins. These findings are useful for design of receptor-specific binding peptides.

AUTHOR INFORMATION

Corresponding Author

*Phone/Fax: +81-426-76-5662. E-mail: nomizu@toyaku.ac.jp.

Notes

The authors declare no competing financial interest.

REFERENCES

(1) Colognato, H., and Yurchenco, P. D. (2000) Form and function: the laminin family of heterotrimers. *Dev. Dyn. 218*, 213−234.

(2) Burgeson, R. E., Chiquet, M., Deutzmann, R., Ekblom, P., Engel, J., Kleinman, H., Martin, G. R., Meneguzzi, G., Paulsson, M., Sanes, J., et al. (1994) A new nomenclature for the laminins. *Matrix Biol. 14*, 209−211.
(3) 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.
(4) 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.
(5) 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. Neurosci. 20*, 6517−6528.
(6) 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.
(7) 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.
(8) 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.
(9) 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.
(10) Kato, K., Utani, A., Suzuki, N., Mochizuki, M., Yamada, M., Nishi, N., Matsuura, H., Shinkai, H., and Nomizu, M. (2002) Identification of neurite outgrowth promoting sites on the laminin alpha 3 chain G domain. *Biochemistry 41*, 10747−10753.
(11) 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.
(12) 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.
(13) Nielsen, P. K., Gho, Y. S., Hoffman, M. P., Watanabe, H., Makino, M., Nomizu, M., and Yamada, Y. (2000) Identification of a major heparin and cell binding site in the LG4 module of the laminin alpha 5 chain. *J. Biol. Chem. 275*, 14517−14523.
(14) Makino, M., Okazaki, I., Kasai, S., Nishi, N., Bougaeva, M., Weeks, B. S., Otaka, A., Nielsen, P. K., Yamada, Y., and Nomizu, M. (2002) Identification of cell binding sites in the laminin alpha5-chain G domain. *Exp. Cell Res. 277*, 95−106.
(15) Liu, W., Litwack, E. D., Stanley, M. J., Langford, J. K., Lander, A. D., and Sanderson, R. D. (1998) Heparan sulfate proteoglycans as adhesive and anti-invasive molecules. Syndecans and glypican have distinct functions. *J. Biol. Chem. 273*, 22825−22832.
(16) Langford, J. K., Stanley, M. J., Cao, D., and Sanderson, R. D. (1998) Multiple heparan sulfate chains are required for optimal syndecan-1 function. *J. Biol. Chem. 273*, 29965−29971.
(17) Hosokawa, Y., Takahashi, Y., Kadoya, Y., Yamashina, S., Nomizu, M., Yamada, Y., and Nogawa, H. (1999) Significant role of laminin-1 in branching morphogenesis of mouse salivary epithelium cultured in basement membrane matrix. *Dev. Growth Differ, 41*, 207−216.
(18) Kadoya, Y., Nomizu, M., Sorokin, L. M., Yamashina, S., and Yamada, Y. (1998) Laminin alpha1 chain G domain peptide, RKRLQVQLSIRT, inhibits epithelial branching morphogenesis of cultured embryonic mouse submandibular gland. *Dev. Dyn. 212*, 394−402.
(19) Malinda, K. M., Nomizu, M., Chung, M., Delgado, M., Kuratomi, Y., Yamada, Y., Kleinman, H. K., and Ponce, M. L. (1999) Identification of laminin alpha1 and beta1 chain peptides active for endothelial cell adhesion, tube formation, and aortic sprouting. *FASEB J. 13*, 53−62.
(20) Powell, S. K., and Kleinman, H. K. (1997) Neuronal laminins and their cellular receptors. *Int. J. Biochem. Cell Biol. 29*, 401−414.
(21) Hynes, R. O. (2002) Integrins: bidirectional, allosteric signaling machines. *Cell 110*, 673−687.
(22) De Arcangelis, A., and Georges-Labouesse, E. (2000) Integrin and ECM functions: roles in vertebrate development. *Trends in Genetics 16*, 389−395.
(23) Danen, E. H., and Sonnenberg, A. (2003) Integrins in regulation of tissue development and function. *J. Pathol. 201*, 632−641.
(24) Urushibata, S., Katagiri, F., Takaki, S., Yamada, Y., Fujimori, C., Hozumi, K., Kikkawa, Y., Kadoya, Y., and Nomizu, M. (2009) Biologically active sequences in the mouse laminin alpha3 chain G domain. *Biochemistry 48*, 10522−10532.
(25) Hozumi, K., Akizuki, T., Yamada, Y., Hara, T., Urushibata, S., Katagiri, F., Kikkawa, Y., and Nomizu, M. (2010) Cell adhesive peptide screening of the mouse laminin alpha1 chain G domain. *Arch. Biochem. Biophys. 503*, 213−222.
(26) Urushibata, S., Hozumi, K., Ishikawa, M., Katagiri, F., Kikkawa, Y., and Nomizu, M. (2010) Identification of biologically active sequences in the laminin alpha2 chain G domain. *Arch. Biochem. Biophys. 497*, 43−54.
(27) Katagiri, F., Ishikawa, M., Yamada, Y., Hozumi, K., Kikkawa, Y., and Nomizu, M. (2012) Screening of integrin-binding peptides from the laminin alpha4 and alpha5 chain G domain peptide library. *Arch. Biochem. Biophys. 521*, 32−42.
(28) Kato-Takagaki, K., Suzuki, N., Yokoyama, F., Takaki, S., Umezawa, K., Higo, J., Mochizuki, M., Kikkawa, Y., Oishi, S., Utani, A., and Nomizu, M. (2007) Cyclic peptide analysis of the biologically active loop region in the laminin alpha3 chain LG4 module demonstrates the importance of peptide conformation on biological activity. *Biochemistry 46*, 1952−1960.