Bifunctional Peptides Derived from Homologous Loop Regions in the Laminin α Chain LG4 Modules Interact with both α2β1 Integrin and Syndecan-2†

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ABSTRACT: Laminin α chains show diverse biological functions in a chain-specific fashion. The laminin G-like modules (LG modules) of the laminin α chains consist of a 14-stranded β-sheet sandwich structure with biologically active sequences found in the connecting loops. Previously, we reported that connecting loop regions between β-strands E and F in the mouse laminin α chain LG4 modules exhibited chain-specific activities. In this study, we focus on the homologous loop regions in human laminin α chain LG4 modules using five synthetic peptides (hEF-1−hEF-5). These homologous peptides induced chain-specific cellular responses in various cell types. Next, to examine the dual-receptor recognition model, we synthesized chimeras (cEF13A−cEF13E) derived from peptides hEF-1 and hEF-3. All of the chimeric peptides promoted fibroblast attachment as well as the parental peptides. Attachment of fibroblasts to cEF13A and cEF13B was inhibited by anti-integrin α2 and β1 antibodies and by heparin, while cell adhesion to cEF13C, cEF13D, and cEF13E was blocked only by heparin. Actin organization of fibroblasts on cEF13C was not different from that on hEF-3, but cEF13B induced membrane ruffling at the tips of the actin stress fibers. These results suggest that cEF13B had bifunctional effects on cellular behaviors through α2β1 integrin and heparin/heparan sulfate proteoglycan. We conclude that the approach utilizing chimeric peptides is useful for examining cellular mechanisms in dual-receptor systems.

Laminins, multifunctional glycoproteins in the basement membrane, have diverse biological activities, including promotion of cell attachment and migration, neurite outgrowth, tumor metastasis, angiogenesis, and wound healing (*1*). Laminins consist of three distinct subunits: α, β, and γ chains. So far, five α, three β, and three γ chains have been identified, and at least 15 laminin isoforms (laminin-1−15) have been reported with various combinations of each subunit (*2*−*5*). The laminin α chains are generally the largest of the three subunits and are distributed in a tissue-specific manner. The α chains contain the unique C-terminal globular domains, called the G-domain, consisting of five tandem laminin G-like modules (LG1−LG5). The LG modules contain the majority of the sites that bind to cellular receptors, including integrins, dystroglycan, Lutheran glycoprotein, and syndecans (*1*, *6*−*9*).

Various cell surface receptors mediate the biological functions of laminins (*1*, *6*). Integrins are heterodimeric cell surface proteins consisting of α and β subunits. Integrins have been studied well as cellular receptors for extracellular matrix molecules, including laminins, fibronectin, and collagens. The α3β1, α6β1, α6β4, and α7β1 integrins are recognized by the G-domains of laminins (*1*, *6*). The α5β1 integrin is the major receptor for fibronectin (*10*), while the α2β1 integrin is a receptor for collagens (*11*). Syndecans are a family of cell surface haparan sulfate proteoglycans that interact with various extracellular ligands via heparan sulfate chains (*12*). Syndecans function as coreceptors and bind extracellular ligands for other cell surface receptors. There are several reports suggesting that syndecans interact with the G-domains of laminin α chains and are involved in the biological functions of laminins (*9*, *13*−*15*). Furthermore, it is proposed that a balance of signaling from both integrins and syndecans is essential in extensive cell spreading and in focal adhesion formation (*16*).

Previously, we systematically screened biologically active sequences in laminin-1 using a large number of synthetic peptides covering the entire chains (*17*−*20*). Most of the active sequences were located in the globular domains, and the active peptides found in the G-domains were extensively studied (*9*, *13*−*15*, *21*−*26*). The A4G82 sequence (TLFLAH-

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GRLVFM, mouse laminin α4 chain residues 1514−1525) showed strong heparin binding and cell attachment activity (*22*, *27*), and this site was found to be located in the connecting loop region between β strands E and F in the LG4 module (*28*, *29*). Homologous peptides derived from the E−F connecting loop in the other α chains showed distinct chain-specific biological activity via different cell surface receptors, such as integrins and syndecans (*24*). Furthermore, the hA3G75 site (KNSFMALYLSKG, human laminin α3 chain residues 1411−1422), which is also located in the E−F loop region in the LG4 module, showed diverse biological activities, including the promotion of cell attachment, neurite outgrowth, and matrix metalloprotease-1 induction (*14*, *15*, *26*). These findings suggest that the E−F loop regions in the LG4 module play important roles in the biological activity of laminins. The chain-specific biological activity of the homologous loop regions with respect to the hA3G75 site in human laminin has not been determined.

In this study, we first examined the biological activity of E−F connecting loop regions in human laminin α chain LG4 modules using five homologous synthetic peptides: hEF-1, DYAVLQLHGGRLHFMFDLG, α1 chain residues 2762−2780; hEF-2, DFATVQLRNGLPYFSYDLG, α2 chain residues 2812−2830; hEF-3, KNSFMALYLSKGRLVFALG, α3 chain residues 1411−1429; hEF-4, DFMTLFLAHGRLVYMFNVG, α4 chain residues 1511−1529; and hEF-5, SPSLALFLSNGHFVAQMEGL, α5 chain residues 3389−3408. These homologous peptides exhibited chain-specific cell adhesive activity through different cellular receptors, including integrins and syndecans. Further, to propose a dual-receptor recognition model, we designed chimeric peptides derived from hEF-1 and hEF-3, which interacted with α2β1 integrin and syndecan-2, respectively, and evaluated their biological activity. Some of the chimeric peptides interacted with both α2β1 integrin and heparin/heparan sulfate proteoglycans and exhibited unique biological functions.

MATERIALS AND METHODS

Synthetic Peptides. Peptides were manually synthesized using an Fmoc-based solid-phase strategy and prepared in the C-terminal amide form as described previously (*18*). Amino acid derivatives and resins were purchased from Novabiochem (La Jolla, CA). The respective amino acids were condensed manually in a stepwise manner using 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy or NovaSyn TGR resin (Novabiochem). Dimethylformamide was used during the synthesis as a solvent. For condensation, diisopropylcarbodiimide/*N*-hydroxybenzotriazole was employed, and for deprotection of N^{α}-Fmoc groups, 20% piperidine in dimethylformamide was employed. The following side chain protecting groups were used: trityl for asparagine, glutamine, and histidine, *tert*-butyl for aspartic acid, glutamic acid, serine, threonine, and tyrosine, 2,2,5,7,8-pentamethylchroman-6-sulfonyl or 2,2,5,7,8-pentamethyldihydrobenzofuran-5-sulfonyl for arginine, and *tert*-butoxycarbonyl for lysine. The resulting protected peptide resins were deprotected and cleaved from the resin using a trifluoroacetic acid/thioanisole/*m*-cresol/ethanedithiol/H_2O mixture (80:5:5:5:5, v/v/v/v/v) at room temperature for 3 h. The crude peptides were precipitated and washed with ethyl ether, and then purified by reverse-phase high-performance liquid chromatography (HPLC) using a Mightysil RP-18 column (Kanto Chemical Co., Inc., Tokyo, Japan) and a water/acetonitrile gradient containing 0.1% trifluoroacetic acid.

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

Antibodies and Reagents. Rat monoclonal antibody against human integrin α6 (GoH3) was purchased from SantaCruz Biotechnology, Inc. (Santa Cruz, CA). Mouse monoclonal antibodies against human integrin α1 (FB12), α2 (P1E6), α3 (P1B5), αv (P3G8), β1 (6S6), and β4 (ASC-3) and purified mouse IgG were purchased from Chemicon International, Inc. (Temecula, CA).

Cy3-conjugated streptavidin was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA), and fluorescein isothiocyanate (FITC)-conjugated anti-heparan sulfate antibody (10E4) was from Seikagaku Kogyo (Tokyo, Japan).

Cells and Culture. Human neonatal dermal fibroblasts (Iwaki Co. Ltd., Tokyo, Japan), HT-1080 human fibrosarcoma cells (*30*), human submandibular gland (HSG)[1] cells (*31*), and 293T human renal epithelial cells (*32*) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin (Invitrogen). The cells were maintained at 37 °C in a humidified 5% CO_2, 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. Various amounts of peptides in 50 μL of Milli-Q 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) in DMEM at room temperature for 1 h and then washed with DMEM containing 0.1% BSA. Cells were detached with 0.02% trypsin-EDTA (Invitrogen) and recovered with DMEM containing 10% FBS at 37 °C for 20 min. After being washed with DMEM containing 0.1% BSA, cells (2×10^4 cells/100 μL) were added to the wells and incubated at 37 °C for 1 h. The attached cells were stained with a 0.2% crystal violet aqueous solution in 20% methanol for 10 min. After the cells has been washed with Milli-Q water, 1% SDS (150 μL) was used to lyse the stained cells, and the optical density at 570 nm was measured using a model 550 microplate reader (Bio-Rad Laboratories, Hercules, CA). Assays were separately carried out at least three times.

Assays for cell morphology were conducted in eight-well glass slides (Nunc). Fibroblasts (1.5×10^4 cells/200 μL) were added to the wells and incubated at 37 °C for 2 h. After the cells has been stained with crystal violet for 10 min, photographs of the cells were taken with a 400× objective on a microscope. Assays were separately carried out at least three times.

For inhibition of cell attachment with heparin and with EDTA, human fibroblasts were incubated for 30 min at 37

[1] Abbreviations: HPLC, high-performance liquid chromatography; FITC, fluorescein isothiocyanate; HSG, human submandibular gland; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin.

Table 1: Activities of the Homologous Loop Regions in the Human Laminin α Chain LG4 Modules

Peptide	Sequence[a]	Cell attachment[b]			Cytoskeletal organization[c] (fibroblasts)	Inhibitory effect[d] (fibroblasts)	Cellular receptor[e] (fibroblasts)
		Fibroblast	HT-1080	HSG			
hEF-1	DYAVLQLHGGRLHFMFDLG	++	−	+	Actin stress fiber	EDTA	α2β1 integrin
hEF-2	DFATVQLRNGLPYFSYDLG	+	−	−	N. D.[f]	Heparin	N. D.
hEF-3	KNSFMALYLSKGRLVFALG	++	++	++	Membrane ruffling	Heparin	Syndecan-2
hEF-4	DFMTLFLAHGRLVYMFNVG	++	++	+	Membrane ruffling	Heparin	N. D.
hEF-5	SPSLALFLSNGHFVAQMEGL	−	−	−	N. D.	N. D.	N. D.

[a] Sequences of the synthetic peptides are given in the single-letter code. All peptides have C-terminal amides. [b] For cell attachment assays, various amounts of peptides were coated on 96-well plates as described in Materials and Methods. Human neonatal dermal fibroblasts, HT-1080 human fibrosarcoma cells, and HSG cells were used. Cell attachment was evaluated on the following subjective scale: ++, strong adhesion; +, weak adhesion; −, no adhesion. [c] Actin organization and vinculin localization were analyzed in the fibroblasts attached on peptides as described in Materials and Methods. [d] For inhibition assays of cell attachment, human fibroblasts were incubated on peptide-coated plates in the presence of 10 μg/mL heparin or 5 mM EDTA as described in Materials and Methods. [e] Cellular receptors for attachment of fibroblasts to hEF-1 and hEF-3 were analyzed. Anti-α2 and anti-β1 integrin antibodies inhibited cell attachment to hEF-1. hEF-3 colocalized with syndecan-2 on the cell surface of syndecan-2-overexpressing human fibroblasts. [f] N.D., not determined.

°C in the presence of either 10 μg/mL heparin or 5 mM EDTA. For inhibition of cell attachment with anti-integrin antibodies, human fibroblasts were preincubated in a suspension with 10 μg/mL anti-integrin antibodies for 15 min at 37 °C. Then the cells were added to the wells and incubated for 30 min at 37 °C. Attached cells were assessed as described above, and data were shown as the percentage of the OD values without inhibitors. Assays were carried out in triplicate, and each assay was repeated at least three times. The Student's *t*-test was employed for the calculation of *p* values.

Actin Cytoskeleton and Vinculin Localization of Cultured Cells. Various amounts of peptides in 150 μL of Milli-Q water were coated on a eight-well Chamber Slide (Nunc) and dried overnight at room temperature. The wells were blocked by the addition of 1% BSA in DMEM for 1 h and then washed with 0.1% BSA in DMEM. Then, fibroblasts (1.5×10^4 cells) were added and incubated at 37 °C for 2 h. The attached cells were fixed with 10% formalin in PBS, treated with 0.5% Triton X-100 in PBS for 5 min, and incubated with mouse monoclonal antibody against vinculin (clone V284; Cymbus Biotechnology) diluted to 10 μg/mL with 1% BSA in PBS overnight. Bound antibodies and actin filaments were labeled for 1 h with a mixture of FITC-labeled antibody against mouse IgG (Vector Laboratories, Burlingame, CA) and rhodamine-labeled phalloidin (Molecular Probes, Eugene, OR) diluted to 1:100 and 1:200, respectively, with 1% BSA in PBS, which also contained 0.01 μg/mL 4,6-diamidino-2-phenylindole (DAPI) for nuclear staining. After being washed with PBS, the cells were mounted with Permafluore (Shandon, Pittsburgh, PA) and examined under an Axioplan-2 epifluorescence microscopy (Zeiss, Tokyo, Japan). Images were captured with a Quantix CCD camera (Photometrics, Munchen, Germany) and processed using IPLab software (Scanalytics, Fairfax, VA). Deconvolution of the images was performed with HazeBuster software (Vay Tek, Fairfield, IA).

Colocalization of a Peptide with Syndecan-2 on Cultured Cells. A similar procedure was carried out as described previously (*15*). Syndecan-2-overexpressing fibroblasts on a cover glass were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature and blocked with 5% normal donkey serum (Chemicon International, Inc.) and 1% BSA in PBS for 15 min. The cells were incubated with biotinylated hEF-3 peptide (0.1 mg/mL) at 4 °C overnight, followed by the incubation with Cy3-conjugated streptavidin at room temperature for 30 min. For syndecan-2 staining, the cells were incubated with FITC-conjugated anti-heparan sulfate antibody. After the cells were washed, photographs were taken with a Nicon camera attached to a fluorescence microscope. The syndecan-2-overexpressing clones were analyzed by Western blotting with anti-syndecan-2 antibody and immunostaining with FITC-conjugated anti-heparan sulfate antibody as described previously (*15*).

RESULTS

Cell Attachment Activity of Five Homologous Peptides from the Loop Region in Human Laminin α Chain LG4 Modules. Five homologous peptides derived from the loop region between the E and F strands of human laminin α chain LG4 modules were prepared on the basis of sequence alignment data (Table 1) (*24*, *29*). First, we evaluated the cell attachment activity of the homologous peptides, hEF-1−hEF-5, using human neonatal dermal fibroblasts, HT-1080 human fibrosarcoma cells, and HSG cells (Table 1 and Figure 1A−C). hEF-3 (KNSFMALYLSKGRLVFALG, α3 chain residues 1411−1429) and hEF-4 (DFMTLFLAHGRLVYMFNVG, α4 chain residues 1511−1529) significantly promoted the attachment activity of all the cell types. hEF-1 (DYAVLQLHGGRLHFMFDLG, α1 chain residues 2762−2780) showed strong adhesive activity for fibroblasts and weak attachment for HSG cells, whereas no activity was observed for HT-1080 cells. hEF-2 (DFATVQLRNGLPYFSYDLG, α2 chain residues 2812−2830) showed weak cell attachment activity for fibroblasts only. hEF-5 (SPSLALFLSNGHFVAQMEGL, α5 chain residues 3389−3408) had no activity for any of the cells tested in this study.

Next, we assessed the morphological appearance of fibroblasts on the peptides. Fibroblasts on hEF-1 promoted extensive cell spreading (Figure 1D). hEF-3 and hEF-4 promoted a round cellular appearance with membrane ruffling. The morphology of the cells on hEF-2 was also round, similar to that on hEF-3 and hEF-4, but little ruffling was observed. These results demonstrated that the peptides

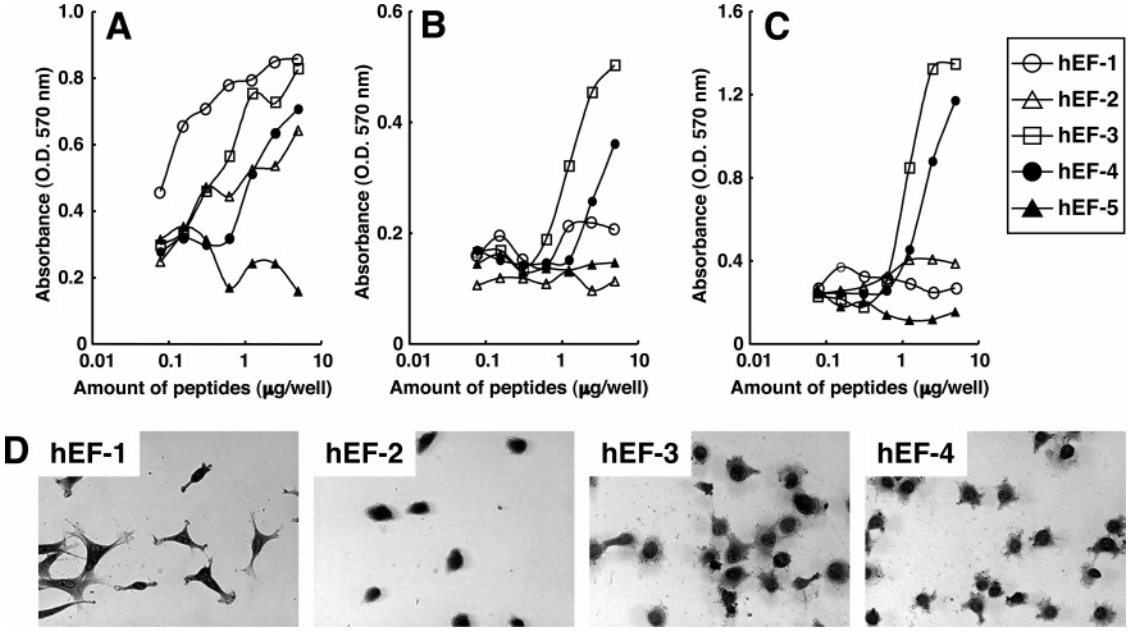

FIGURE 1: Cell attachment activity of the hEF peptides. (A−C) Cell attachment of human fibroblasts (A), HT-1080 cells (B), and HSG cells (C). Ninety-six-well plates were coated with various amounts of peptides, and cells were added to the wells for a 1 h incubation. After being stained with crystal violet, the attached cells were lysed in 1% SDS, and the OD (570 nm) was measured. Triplicate experiments gave similar results. (D) Morphological appearance of fibroblasts on the peptides. Wells of a chamber slide were coated with 5 μg of either hEF-1, hEF-3, or hEF-4 or 10 μg of hEF-2. Fibroblasts were added to the wells. After a 2 h incubation, attached cells were stained with crystal violet. Photographs were taken with a 400× objective on a microscope. Triplicate experiments gave similar results.

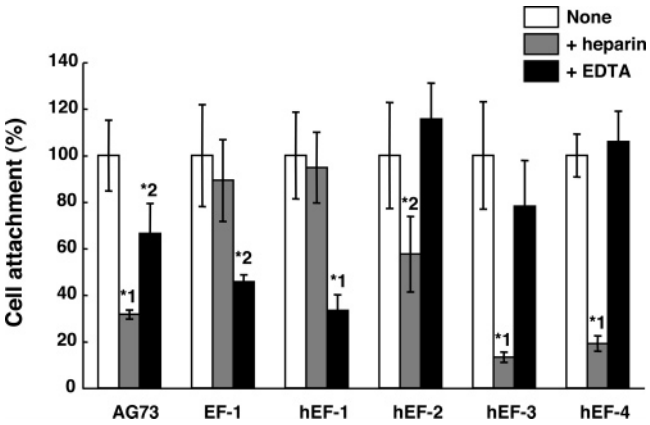

FIGURE 2: Effect of heparin and EDTA on attachment of fibroblasts to peptide-coated plates. Ninety-six-well plates were coated with 0.5 μg of AG73 and EF-1/well, 1.5 μg of hEF-1 and hEF-3/well, and 2.5 μg of hEF-2 and hEF-4/well. Either 10 μg/mL heparin or 5 mM EDTA was added to the cell suspensions, and then the cells were added to the wells. After a 30 min incubation, the attached cells were assessed by crystal violet staining. Each value represents the mean of three separate determinations ± the standard deviation. Triplicate experiments gave similar results. Asterisks denote p values (*1, $p < 0.005$; *2, $p < 0.06$).

interacted with cells in a chain-specific manner and that cellular responses were dependent on the peptides.

Effect of Heparin and EDTA on the Attachment of Fibroblasts to the Homologous Peptides. To determine the cellular ligands for the peptides, we tested the effects of heparin and EDTA on the attachment of fibroblasts to the peptides (Figure 2). We used AG73 (RKRLQVQLSIRT, mouse laminin α1 chain residues 2719−2730) and EF-1 (DYATLQLQEGRLHFMFDLG, mouse laminin α1 chain residues 2747−2765) as positive controls for heparin-dependent and cation-dependent cell attachment, respectively (*24*, *25*). EDTA effectively inhibited the attachment of fibroblasts to hEF-1. No inhibition with EDTA was observed for the attachment to the other peptides. Heparin significantly inhibited cell adhesion to both hEF-3 and hEF-4 and had a moderate inhibitory effect on adhesion to hEF-2. Fibroblasts adhered to hEF-1 in a divalent cation-dependent fashion, suggesting that the cellular interaction with hEF-1 was integrin-mediated. On the other hand, attachment of fibroblasts to hEF-2−hEF-4 was heparin-dependent, indicating that these peptides potentially interact with membrane-associated heparin/heparan sulfate proteoglycans.

Organization of Actin Filaments and Localization of Vinculin on the Homologous Peptides. To analyze the effect of the homologuos peptides on fibroblast cell morphology in more detail, we examined the organization of actin filaments and localization of vinculin on the active peptides by immunostaining (Figure 3). Vinculins are cytoskeletal proteins known to localize on focal adhesions (*33*). hEF-1 induced well-organized actin stress fibers and specific localization of vinculins at the tips of the actin stress fibers, indicating the formation of focal adhesions (Figure 3A). Fibroblasts on hEF-2 were circular in shape with poor actin organization (Figure 3B). Cells on hEF-3 and hEF-4 showed a similar morphology with extensive actin filament spikes and membrane ruffling (Figure 3C,D). These results indicate that the homologous peptides differently affected the organization of the cytoskeleton in fibroblasts.

Cellular Receptor Analysis for hEF-1 and hEF-3. Since the attachment of fibroblasts to hEF-1 was divalent cation-dependent, integrins are strong candidates for its cellular receptor(s). To identify the cell surface receptor(s) for hEF-1, we examined the effect of anti-integrin subunit antibodies on cell attachment (Figure 4A). Attachment of cells to hEF-1 was significantly inhibited by anti-α2 and anti-β1 integrin

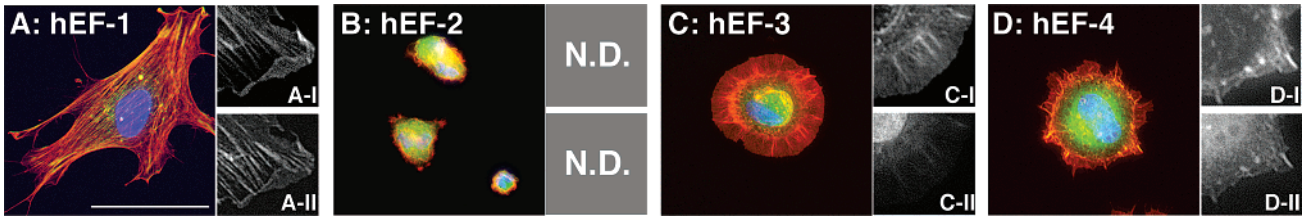

FIGURE 3: Organization of actin filaments and localization of vinculin. Wells of a chamber slide were coated with either 5 μg of hEF-1, hEF-3, or hEF-4 or 10 μg of hEF-2. Fibroblasts were seeded as described in Materials and Methods. After a 2 h incubation, attached cells were fixed with formalin. Triple-color fluorescence microscopy was performed for simultaneous detection of actin filaments, vinculin, and nuclei. The localization of vinculin was examined with a mouse monoclonal antibody against vinculin, followed by detection with a fluorescein isothiocyanate-labeled antibody against mouse IgG. Actin filaments and nuclei were visualized by incubation with rhodamine-labeled phaloidin and DAPI, respectively. Yellow, red, and blue pseudocolors were given for images for fluorescein isothiocyanate, rhodamine, and DAPI, respectively. Merged images are shown in panels A−D. Localization of actin filaments (A-I−D-I) and of vinculin (A-II−D-II) are also shown in separate images. N.D. means not determined. The bar is 50 μm.

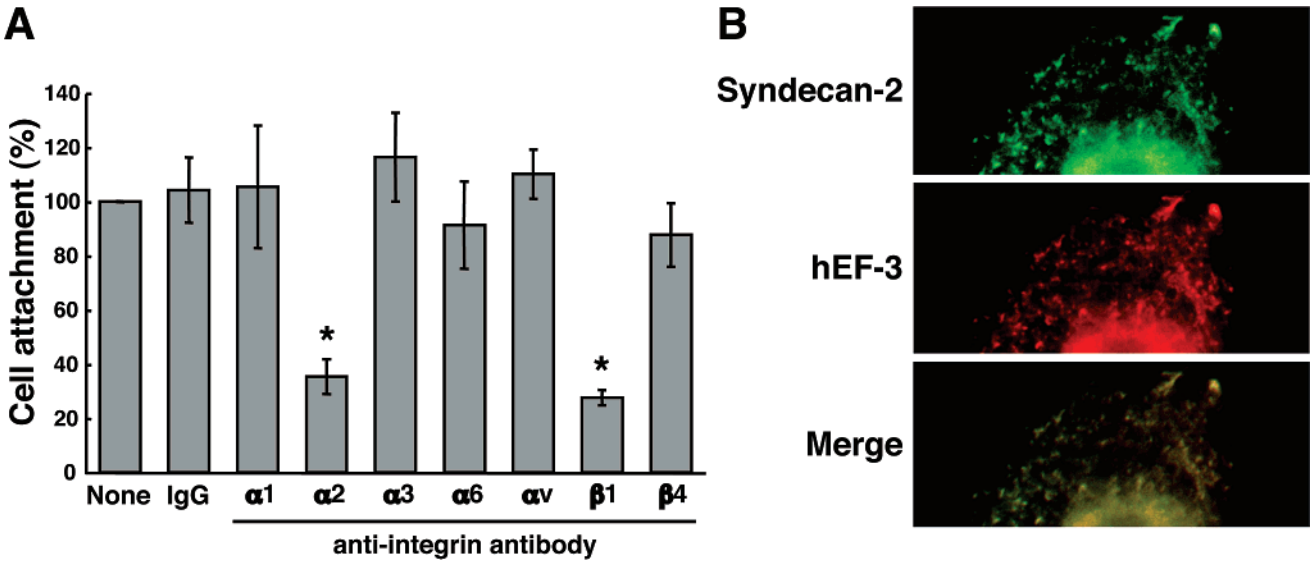

FIGURE 4: Cell surface receptor analysis for hEF-1 and hEF-3. (A) Effect of anti-integrin subunit antibodies on attachment of fibroblasts to the hEF-1-coated plates; 96-well plates were coated with hEF-1 (1.5 μg/well). Human fibroblasts were preincubated with 10 μg/mL integrin antibodies at room temperature for 15 min, and then added to the wells for a 30 min incubation. After being stained with crystal violet, the attached cells were lysed, and the OD (570 nm) was measured. Each value represents the mean of three separate determinations ± the standard deviation. Triplicate experiments gave similar results. An asterisk denotes a p value of <0.001. (B) Colocalization of hEF-3 with syndecan-2 on fibroblasts. Immunostaining analysis of hEF-3 and syndecan-2 in fibroblasts. Dermal fibroblasts overexpressing syndecan-2 were fixed on a cover glass and incubated with 0.1 mg/mL biotinylated hEF-3. The bound hEF-3 and syndecan-2 were visualized with Cy3-conjugated streptavidin and FITC-conjugated anti-heparan sulfate antibody. The original magnification was 400×.

antibodies. Other function-blocking anti-integrin subunit antibodies, anti-α1, -α3, -α6, -αv, and -β4, did not show a significant effect on cell attachment. These antibodies did not inhibit attachment of fibroblasts to hEF-3 (data not shown). These data indicate that hEF-1 promoted fibroblast attachment through α2β1 integrin.

Since hEF-3 exhibited heparin-dependent cell attachment, we examined colocalization of hEF-3 with syndecan-2 on the fibroblast cell surface. Fibroblasts overexpressing syndecan-2 on the cell surface were incubated with biotinylated hEF-3 and anti-heparan sulfate antibody. Double immunostaining showed colocalization of hEF-3 with syndecan-2 on the cell surface (Figure 4B). This colocalization was lost in the presence of heparin (data not shown). Previously, we demonstrated that the recombinant human laminin α3 chain LG4 module colocalized with syndecan-2 and -4 on the fibroblast cell surface (*15*), and that this peptide was able to interact with syndecan-4 as well (*14*). We previously examined the expression of syndecan-1 and -4 in fibroblasts and could not detect them in Western blotting analyses (*14*). Thus, although syndecan-4 can possibly function as the receptor for hEF-3 in other cell types, we conclude that syndecan-2 was the major cellular receptor of fibroblasts for hEF-3 in this study.

Design of Chimeric Peptides Derived from hEF-1 and hEF-3 and Their Cell Attachment Activity. As determined above, hEF-1 interacted with fibroblasts through α2β1 integrin, and hEF-3 can adhere via syndecan-2. We sought to determine if combinations of sequences from hEF-1 and hEF-3 result in "bifunctional" peptides. We designed chimeras (cEF13A−cEF13E) derived from hEF-1 and hEF-3 and tested their biological activities. Amino acid sequences of the chimeric peptides were based on that of hEF-1, and the sequences were replaced systematically with that of hEF-3 from the N-terminus (Table 2). First, we assayed the attachment of fibroblasts to the chimeric peptides (Figure 5A). All of the chimeric peptides, cEF13A−cEF13E, exhibited strong cell attachment activity comparable to that of hEF-1 and hEF-3.

The morphology of the fibroblasts was assessed on the chimeric peptides (Figure 5B). hEF-1 induced extensive cell spreading, while the cells on hEF-3 spread with membrane

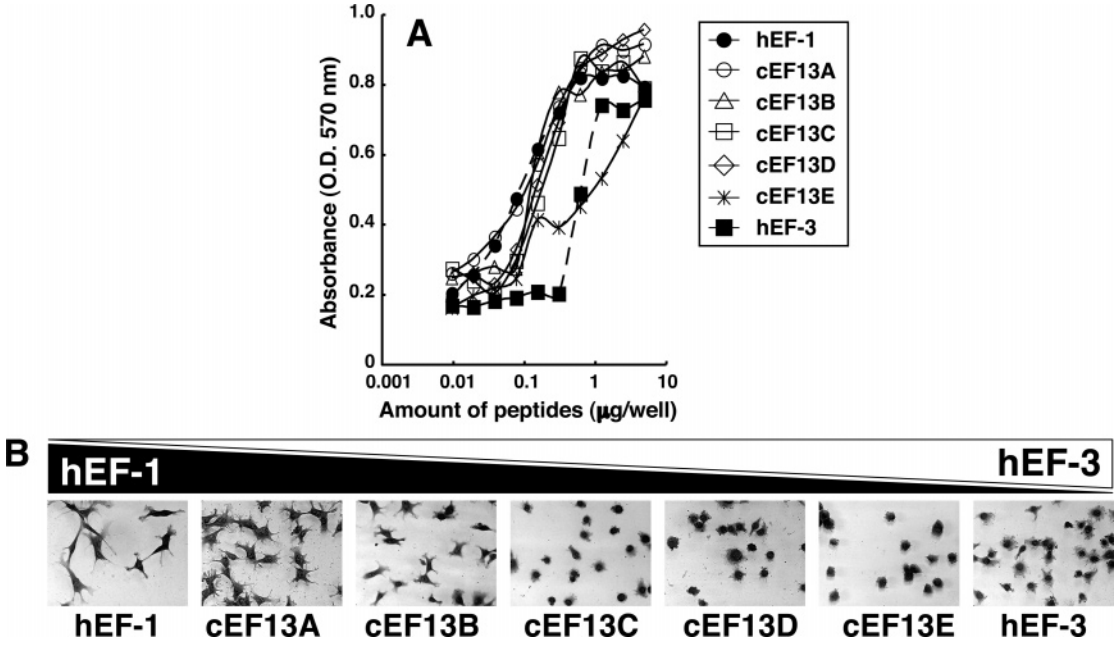

FIGURE 5: Fibroblast attachment activity of chimeric peptides derived from hEF-1 and hEF-3. (A) Ninety-six-well plates were coated with various amounts of peptides. Cell attachment assays were conducted as described in the legend of Figure 1. Triplicate experiments gave similar results. (B) Morphological appearance of fibroblasts on chimeric peptides. Wells of chamber slides were coated with 5 μg of the peptides. The assays were carried out described in the legend of Figure 1D. Triplicate experiments gave similar results.

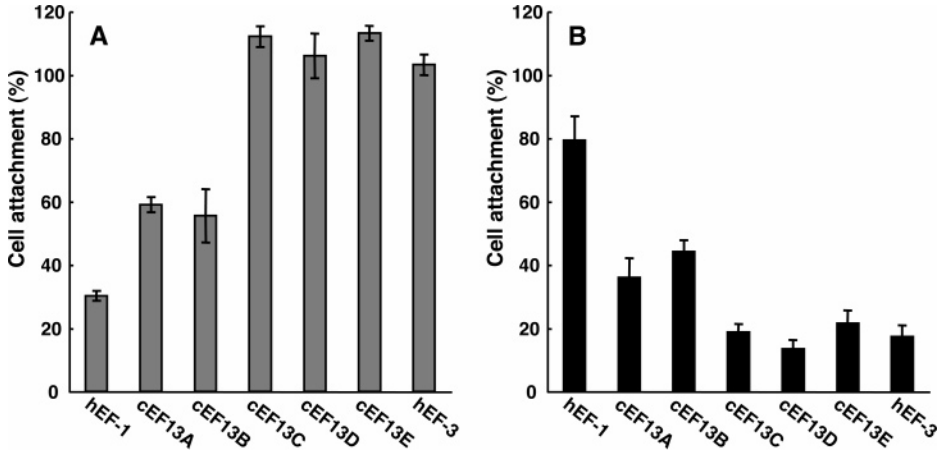

FIGURE 6: Effect of anti-integrin antibodies and heparin on attachment of fibroblasts to the chimeric peptides. Ninety-six-well plates were coated with 0.5 μg of cEF13B/well, 1.0 μg of cEF13C/well, 1.5 μg of hEF-1, hEF-3, cEF13A, and cEF13D/well, and 2.5 μg of cEF13E/well. Either 10 μg/mL anti-integrin antibodies against α2 and β1 subunits in combination (A) or 10 μg/mL heparin (B) was added to the cell suspensions, and then analyzed as described in the legends of Figure 4A and Figure 3, respectively. Each value represents the mean of three separate determinations $\pm$ the standard deviation. Triplicate experiments gave similar results.

ruffling, similar to what is seen in Figures 1D and 3. Fibroblasts on cEF13A and cEF13B showed a morphology similar to the morphology of those on hEF-1, but spreading was less extensive. On the other hand, cEF13C−cEF13E induced cell spreading with membrane ruffling similar to that on hEF-3. In addition, the morphology of the fibroblasts on hEF-1 and hEF-3 mixed wells was a round shape with membrane ruffling similar to that on hEF-3 (data not shown). These results demonstrate that the chimeric peptides derived from hEF-1 and hEF-3 showed cell attachment activity equivalent to that of the original peptides, but the cells had different morphology depending on the peptide.

Effects of Anti-Integrin α2 and β1 Subunit Antibodies and Heparin on the Attachment of Cells to the Chimeric Peptides. The chimeric peptides appear to contain novel activities based on the attachment and spreading data. We therefore hypothesized that cEF13A and cEF13B acquired affinities for both $\alpha2\beta1$ integrin and syndecans since the parental peptides can respectively interact with these receptors. To clarify our hypothesis, we examined the effects of anti-integrin α2 and β1 subunit antibodies and heparin on the attachment of fibroblasts to the chimeric peptides (Figure 6). The anti-integrin antibodies inhibited attachment of cells to hEF-1 (approximately 70%) as expected (Figure 6A). Cell attachment to cEF13A and cEF13B was effectively blocked by the antibodies (approximately 40%), but the inhibitory effect was smaller than that of hEF-1. The antibodies did not affect the attachment of cells to cEF13C, cEF13D, cEF13E, and hEF-3. On the other hand, the addition of heparin led to a strong inhibitory effect on cell attachment to cEF13C, cEF13D, and cEF13E as well as hEF-3 (approximately 80%) (Figure 6B). While heparin did not significantly block the attachment of cells to hEF-1, attachment of fibroblasts to cEF13A and cEF13B was effectively inhibited by heparin

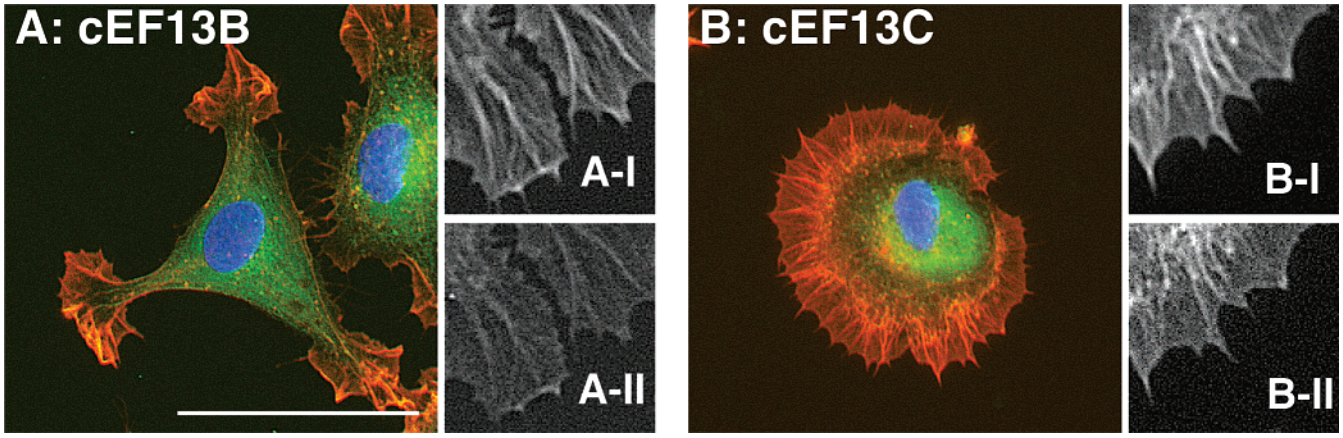

FIGURE 7: Organization of actin filaments and localization of vinculin in fibroblasts attached to chimeric peptides. Wells of a chamber slide were coated with 5 μg of the peptides. Merged images are shown in panels A and B. Localization of actin filaments (A-I and B-I) and of vinculin (A-II and B-II) are also shown in separate images. Assays were carried out as described in the legend of Figure 2. The bar is 50 μm.

(approximately 60%). These results demonstrate that cEF13A and cEF13B are able to interact with cells through both α2β1 integrin and heparin/heparan sulfate proteoglycans, while attachment of fibroblasts to the other chimeric peptides, cEF13C−cEF13E, is mediated only by heparin/heparan sulfate proteoglycans (probably syndecans).

Organization of Actin Filaments and Localization of Vinculin on the Chimeric Peptides. Since the "switch" in the morphology and receptors for the chimeric peptides was between cEF13B and cEF13C, we next examined differences in the organization of actin filaments and localization of vinculin on the cEF13B and cEF13C peptides (Figure 7). cEF13C induced membrane ruffling similar to that on hEF-3 (Figure 7B). In contrast, fibroblasts on cEF13B showed relatively extensive spreading with actin stress fibers. Interestingly, well-developed membrane ruffling was observed at the tips of actin filaments (Figure 7A). hEF-1S (RHDYAVMFLGLFGLLQDGH) and cEF13BS (RHGNLFMMFALGFLHSGDK), scrambled peptides of hEF-1 and cEF13B, respectively, did not promote fibroblast attachment (data not shown). Additionally, we previously reported that the scrambled peptide of hEF-3 had no adhesive activity (*15*). These results demonstrate that cEF13B had effects on cells different from those of the parental peptides, hEF-1 and hEF-3, while cEF13C had almost the same effects as hEF-3.

DISCUSSION

Previously, we localized the biological activity in the connecting loop regions between β-strands E and F in the laminin α chain LG4 module (*15*, *24*, *27*). In this study, we demonstrated that five homologous synthetic peptides from the human laminin α chain LG4 module (hEF-1−hEF-5) showed chain-specific biological activity. Furthermore, two chimeric peptides derived from hEF-1 and hEF-3 were able to induce cellular activity through both α2β1 integrin and heparin/heparan sulfate proteoglycans. We first evaluated the biological activity of five homologous peptides derived from E−F connecting loop regions in the human laminin α chain LG4 modules and compared to that of the homologous E−F peptides from the mouse laminin α chian LG4 modules (EF-1−EF-5), previously described (*24*). EF-1 exhibited α2β1 integrin-mediated attachment. Similarly, hEF-1 from the α1 chain promoted fibroblast attachment via α2β1 integrin and induced well-organized actin stress fibers which colocalized with vinculin. Attachment of cells to recombinant human laminin α chain LG4 module was inhibited by hEF-1 in a dose-dependent manner (data not shown), suggesting the physiological relevance of the hEF-1 sequence. While EF-3 did not show strong activities, hEF-3 from the α3 chain did generate membrane ruffling in fibroblasts through syndecan-2. hEF-2 and hEF-4 exhibited heparin-dependent fibroblast attachment, suggesting that their cellular receptors were heparin/heparan sulfate proteoglycans. EF-2 and EF-4 showed syndecan-2-mediated biological activity and induced membrane ruffling in fibroblast. hEF-4 induced membrane ruffling in fibroblasts, whereas fibroblasts on hEF-2 were round in shape with little ruffling. Although EF-5 showed attachment activity, hEF-5 from the α5 chain did not show any cellular activity. Taken together, these results suggest that the E−F connecting loop regions in the laminin α chain LG4 module are conserved as biologically important sites and are involved in chain-specific biological functions.

Here, we prepared chimeric peptides (cEF13A−cEF13E) derived from hEF-1 and hEF-3 to create bifunctional peptides that can interact with both α2β1 integrin and syndecan-2. In fact, attachment of fibroblasts to cEF13A and cEF13B was partially inhibited by the addition of EDTA; cEF13A and cEF13B had heparin binding activity comparable to that of hEF-3 in a solid-phase assay, whereas hEF-1 showed no activity (data not shown). Moreover, attachment of cells to cEF13A and cEF13B was effectively blocked by either anti-integrin α2 and β1 subunit antibodies or heparin. These data indicate that cEF13A and cEF13B interacted with fibroblasts via both α2β1 integrin and heparin/heparan sulfate proteoglycans, perhaps syndecan-2.

Integrins are heterodimeric cellular receptors for extracellular matrix molecules (*1*, *6*, *10*). So far, several integrin-binding sequences have been identified in extracellular matrix proteins. The well-known tripeptide, Arg-Gly-Asp (RGD), binds to αvβ3 integrin (*34*−*36*). A tetrapeptide, Asp-Gly-Glu-Ala (DGEA), was identified as the recognition sequence for α2β1 integrin in type I collagen (*37*). Attachment of cells to cEF13B (KNSFMALHGGRLHFMFDLG) was inhibited by anti-integrin α2 and β1 antibodies, while the antibodies did not affect attachment of cells to cEF13C (KNSFMALYLSRLHFMFDLG), suggesting that replacement of the

Table 2: Activities of Chimeric Peptides Derived from the hEF-1 and hEF-3 Peptides

Peptide	Sequence[a]	Cell attachment[b]	Cell morphology[c]	% of inhibition ± S. D.		Cytoskeletal organization[f]
				Antibodies[d]	Heparin[e]	
hEF-1	DYAVLQLHGGRLHFMFDLG	+	Extensive spreading	30.2 ± 1.6	79.4 ± 7.5	Actin stress fiber
cEF13A	KNSFLQLHGGRLHFMFDLG	+	Spreading	59.0 ± 2.4	35.9 ± 6.2	N. D.[g]
cEF13B	KNSFMALHGGRLHFMFDLG	+	Spreading	55.5 ± 8.4	44.2 ± 3.6	Membrane ruffling
cEF13C	KNSFMALYLSRLHFMFDLG	+	Round	112.1 ± 3.3	18.7 ± 2.6	Membrane ruffling
cEF13D	KNSFMALYLSKGRFMFDLG	+	Round	106.0 ± 7.0	13.5 ± 2.8	N. D.
cEF13E	KNSFMALYLSKGRLVFDLG	+	Round	113.2 ± 2.4	21.6 ± 4.0	N. D.
hEF-3	KNSFMALYLSKGRLVFALG	+	Round	103.2 ± 3.3	17.3 ± 3.6	Membrane ruffling

[a] Sequences of the synthetic peptides are given in the single-letter code. All peptides have C-terminal amides. Underlined residues are from hEF-3. [b] For cell attachment assays, various amounts of peptides were coated on 96-well plates as described in Materials and Methods. Human neonatal dermal fibroblasts were used for assays. Cell attachment was evaluated on the following subjective scale: +, strong adhesion comparable to that of hEF-1 and hEF-3. [c] The cell morphology of human fibroblasts was evaluated by crystal violet staining as described in Materials and Methods. [d] For inhibition assays of cell attachment, human fibroblasts were incubated on peptide-coated plates in the presence of 10 μg/mL anti-α2 and -β1 integrin antibodies in combination as described in Materials and Methods. [e] For inhibition assays of cell attachment, human fibroblasts were incubated on peptide-coated plates in the presence of 10 μg/mL heparin as described in Materials and Methods. [f] Actin organization and vinculin localization were analyzed in the fibroblasts attached on peptides as described in Materials and Methods. [g] N.D., not determined.

His-Gly-Gly sequence with a Tyr-Leu-Ser sequence caused decreased binding affinity of cEF13C for $\alpha 2\beta 1$ integrin.

Syndecans are transmembrane heparan sulfate proteoglycans which bind many growth factors and extracellular matrix molecules via heparan sulfate chains (*12*). Previously, we identified syndecan-binding sequences in the G-domains of laminins. The AG73 sequence (RKRLQVQLSIRT, mouse laminin α1 chain residues 2719−2730) bound to syndecan-1 (*9*, *13*). The A4G82 sequence (TLFLAHGRLVFM, mouse laminin α4 chain residues 1512−1525) was able to bind to syndecan-2 (*27*). These sites contain positively charged amino acid residues, such as Lys and Arg, that are crucial for interaction with negatively charged heparan sulfate chains on syndecans. cEF13A and cEF13B showed heparin binding activity in a solid-phase assay (data not shown), and cell attachment was blocked by heparin. When the aspartic acid of the hEF-1 sequence was replaced with lysine in cEF13A and cEF13B (Table 2), these chimeric peptides interacted with heparan sulfate proteoglycans.

A better understanding of the signaling pathways of extracellular matrices via syndecans and integrins is evolving with more detail on the biology of the matrix proteins (*16*, *38*−*43*). The mechanisms of receptor-specific intracellular signaling need to be investigated further since extracellular matrix proteins recognize multiple cell surface molecules. Thus, sorting out the multiple biological effects of extracellular matrix proteins on cell behaviors and signaling pathways is very complicated. In contrast, peptides are so simple that individual cell interactions, receptors, and signaling pathways can be defined. Our chimeric peptides that bifunctionally act on cells via both integrin and syndecan are very useful tools for investigating receptor-specific signaling pathways and crosstalk. Further, we will be able to control higher-level cellular behaviors by means of such bifunctional or multifunctional peptides in the future, leading to application for regenerative medicine and tissue engineering.

In summary, we suggest that the E−F loop regions in the human laminin α chain LG4 modules play crucial roles for the biological activity of the LG4 modules in a chain-specific manner based on data using the five homologous synthetic peptides. Further, we demonstrate that the approach utilizing chimeric peptides can be used to investigate cellular mechanisms via dual-receptor systems that may be important in cell biology and in tissue engineering.

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