Biological Activities of Homologous Loop Regions in the Laminin α Chain G Domains*

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Laminin α chains (α1–α5 chains) have diverse chain-specific biological functions. The LG4 modules of laminin α chains consist of a 14-stranded β-sheet (A-N) sandwich structure. Several biologically active sequences have been identified in the connecting loop regions. Here, 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 α chains (EF-1: DYTALQLQEGRLHFMFDLGLG, α1 chain 2747–2765; EF-2: DFTGVLRNGPPFSYDLG, α2 chain 2808–2826; EF-3: RDSFVALYSEGHVFIALG, α3 chain 2266–2284; EF-4: DFMTLFLAHGRLVFMFNVG, α4 chain 1511–1529; EF-5: SPSLVFLNHGHFVAQTEGP, α5 chain 3304–3323). These homologous peptides showed chainspecific cell attachment and neurite outgrowth activities. Well organized actin stress fibers and focal contacts with vinculin accumulation were observed in fibroblasts attached on EF-1, whereas fibroblasts on EF-2 and EF-4 showed filopodia with ruffling. Fibroblast attachment to EF-2 and EF-4 was mediated by syndecan-2. In contrast, EF-1 promoted α5β1 integrin-mediated fibroblast attachment and inhibited fibroblast attachment to a recombinant laminin α1 chain LG4-5. The receptors for EF-3 and EF-5 are unknown. Further, when the active core sequence of EF-1 was cyclized, utilizing two additional cysteine residues at both the N and C termini through a disulfide bridge, the cyclic peptide significantly enhanced integrin-mediated cell attachment. These results indicate that integrin-mediated cell attachment to the EF-1 sequence is conformation-dependent and that the loop structure is important for the activity. The homologous peptides, which promote either integrin- or syndecan-mediated cell attachment, may be useful for understanding the cell type- and chain-specific biological activities of the laminins.

Laminins, multifunctional glycoproteins of basement membranes, have diverse biological activities, including promotion of cell adhesion, migration, neurite outgrowth, tumor metastasis, and angiogenesis (1). Laminins consist of three different subunits, α, β, and γ chains. So far, five α, three β, and three γ chains have been identified, and at least 15 isoforms (laminin-1 to -15) are formed by various combinations of each subunit (2–5). Laminin-1, which is the most extensively characterized laminin isoform, has been analyzed for biological activity using proteolytic fragments, recombinant proteins, and synthetic peptides (6, 7). Previously, we screened for cell-adhesive sequences in laminin-1 (α1β1γ1) using 673 overlapping synthetic peptides covering the entire protein (8–11). 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 (12, 13).

The C-terminal globular domains (G domains) of the laminin α chains consist of five laminin G-domain-like modules (LG1 to -5) that play a critical role in the biological functions of laminins (14). Several studies have focused on the biological activities of the α1 chain G domain. E8, a proteolytic fragment containing the LG1–3 modules of the α1 chain, possesses potent cell binding activity mediated through α1β1 integrin (15, 16). Recombinant and reconstitution experiments with the α1 chain G domain have suggested that this activity is dependent on protein conformation (17, 18). Several synthetic peptides derived from the α1 chain G domain promote heparin binding, cell adhesion, neurite outgrowth, and tumor growth and metastasis (8, 19–21). Some of the peptides promote acinar-like formation of human salivary gland cells in culture and inhibit acinar development and lung alveolar formation in organ culture (22–27). Many of these peptides interact with integrins or syndecans, a membrane-associated heparan sulfate proteoglycan (8, 13, 25, 27, 28).

Biologically active sites in the G domains of the other α chains have begun to be identified (29–34). The A3G75 site (KNSFMALYLSKG, human laminin α1 chain 1411–1422) promoted syndecan-2- and syndecan-4-mediated cell attachment and neurite outgrowth (29, 30). The A4G82 (TLFLAHLRVF, mouse laminin α1 chain 1514–1525) sequence showed heparin binding and cell attachment activity (31, 32). These active sequences are in the homologous region of the LG4 modules of the α3 and α4 chains. The crystal structure of the laminin α5 LG5 module has been prepared and found to consist of 14 β strands (A-N strands) (35). The homologous regions of A3G75 and A4G82 are located on the connecting loop region between the E and F strands (14, 29, 30, 32). Additionally, mutagenesis analysis using recombinant LG4 proteins with serine or alanine substitutions of basic residues (Lys1421 and Arg1425, human laminin α1 chain; His1519 and Arg1521, mouse laminin α4 chain) in the E-F loop regions suggested that the loop regions are critical for the biological activity of the recombinant proteins (29, 31).

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In this study, we focused on the E-F loop regions of laminin α chain LG4 modules. We prepared five homogeneous peptides (EF-1 to EF-5) from the LG4 modules of the 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, and some of the peptides promoted neurite outgrowth. Further, a cyclized form of the peptide, which interacted with αβ2 integrin, enhanced the biological activity.

MATERIALS AND METHODS

Synthetic Peptides—All linear peptides were synthesized manually using an Fmoc-based solid-phase strategy and prepared in the C-terminal amide form as previously described (10). Amino acid derivatizations and resins were purchased from Watanabe Chemical (Hiromicho, Japan) and Novabiochem (La Jolla, CA). The respective amino acids were condensed manually in a stepwise manner using 4-(2-A’-dime-thoxyphenyl)-Fmoc-aminoethyl-phenoxy or NovabioSyn TGR resin (Novabiochem). Dimethyformamide was used during the synthesis as a solvent. For condensation, disopropylcarbodiimide/N-hydroxybenzotriazole was employed, and for protection of N-Fmoc groups, 20% piperidine in dimethylformamide was employed. The following side chain protecting groups were used: Asn, Gln, and His, trityl; Asp, Glu, Ser, Thr, and Tyr, t-butyli; Arg, 2,2,5,7,8-pentamethylthiochroman-6-sulfanyl or 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; and Lys, t-butyloxycarbonyl. The resulting protected peptide resins were deprotected and cleaved from the resin using a trifluoroacetic acid/thiouanol/EDTAns-H3O (90:5:5, v/v/v) at 100 °C for 5 h.

The crude peptides were precipitated and washed with ethyl ether and then purified by reverse-phase high performance liquid chromatography (HPLC) using a Mightysil RP-18 column (Kanto Chemical Co., Inc., Tokyo, Japan) and a gradient of water/acetonitrile containing 0.1% trifluoroacetic acid.

A cyclic peptide, cyc-EF-1Xm (CLQLQEGRLHFV284; Cymbus Biotechnology, UK) diluted to 10^4 M in Milli-Q water was coated on a 24-well plate (Nunc, Inc., Naperville, IL) coated with either various concentrations of an anti-human integrin α5 (GoH3) or murine integrin αv (BP15), α5 (P3G8), β1 (6S6), and β3 (ASC-3) were purchased from Chemicon International, Inc. (Temecula, CA). 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 (Iwaki Co. Ltd., Tokyo, Japan), HT-1080 human fibrosarcoma cells (38), human submandibular glands (HSG) cells (37), and 293T human renal epithelial cells (38) were cultured in DMEM containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). 293T cells stably overexpressing syndecan-2 and glypican-1 were cultured in the presence of 0.4 μg/ml puromycin (Sigma), as previously described (29). PC12 cells were plated onto 96-well plates (Nunc, Inc.) coated with either laminin α1 chain LG4 module (rec-α1LG4-5) or 1 chain LG4-5 module (rec-α1LG4-5) at a density of 10^4 cells/100 μl of Milli-Q water were coated on the wells and dried overnight at room temperature. For inhibition of cell attachment with heparin, EDTA, or peptides, human fibroblasts were incubated for 30 min at 37 °C in the presence of 5 μg/ml heparin, 10 μg/ml EDTA, or various concentrations of peptides. For inhibition of cell attachment with anti-integrin antibodies, human fibroblasts were preincubated in suspension with 10 μg/ml of the anti-integrin antibody 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 measured as described above.

Neurite Outgrowth Assay—For neurite outgrowth assays, a 24-well plate was coated with the indicated amounts of peptides as described above. After priming with nerve growth factor (100 ng/ml) for 24 h, PC12 cells were washed with DMEM and seeded into the wells in serum-free DMEM/F-12 (Invitrogen). The cells were then incubated at 37 °C for 24 h in 4% CO2. The cells were fixed and stained with 1% crystal violet aqueous solution in 10% 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 (Bio-Rad).

For inhibition of cell attachment with heparin, EDTA, and peptides, human fibroblasts were incubated for 30 min at 37 °C in the presence of 5 μg/ml heparin, 10 μg/ml EDTA, or various concentrations of peptides. For inhibition of cell attachment with anti-integrin antibodies, human fibroblasts were preincubated in suspension with 10 μg/ml of the anti-integrin antibody 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 measured as described above.

Actin Cytoskeleton and Vinculin Localization of Cultured Cells—Various amounts of peptide in 120 μl of Milli-Q water were coated on a 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 rinsed with PBS. Then, 10% BSA in DMEM was added and incubated at 37 °C for 2 h. 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, UK) 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 fluorescein isothiocyanate-labeled anti-human IgG (Vector Laboratories, Burlingame, CA) and rhodamine-labeled phalloidin (Molecular Probes, Inc., Eugene, OR) diluted to 1:100 and 1:200, respectively, with 1% BSA in PBS, which also contained 0.1 μg/ml 4,6-diamidino-2-phenylindole (DAPI) for nuclear staining.
RESULTS

Cell Attachment and Neurite Outgrowth Activity of Peptides—Five homologous peptides (EF-1 to EF-5) derived from mouse laminin α chains (α1–α5 chains) were prepared based on the alignment of the LG4 modules (Fig. 1). These peptides contained the E and F β-sheet strands and their connecting loop regions. First, we tested their cell attachment activity using human neonatal dermal fibroblasts, HT-1080 human fibrosarcoma cells, and HSG cells (Table I and Fig. 2, A–C). AG73, a cell-adhesive peptide derived from the laminin α1 chain G domain, was used as a positive control (8). EF-2 (DFMTLFLAHGRLVFMFNVG, chain 2765) promoted fibroblast and HT-1080 attachment, whereas no activity was observed on the HSG cells. EF-5 chains (58) showed strong cell attachment activity with all of the cell types. EF-1 (DYATLQLQEGRLHFMFDLG, α5 chain 2747–2765) promoted fibroblast and HT-1080 attachment, whereas no activity was observed with the HSG cells. EF-5 (SPSLVFLNLGHIFVQAQTEGP, α5 chain 3304–3323) weakly promoted fibroblast attachment but was inactive with the other cell types. EF-3 (RDSFVALYSEGHVIFALG, α3 chain 2266–2284) showed no cell attachment activity with any of the cells tested.

The morphological appearance of fibroblasts on the peptides differed (Fig. 2D). On the AG73 peptide, fibroblasts were spread with ruffles (Fig. 2, D–V), as previously described (40). EF-1 promoted extensive fibroblast spreading (Fig. 2, D–I), whereas the cells on EF-2 and EF-4 showed ruffling similar to that on AG73 (Fig. 2, D–II and D–III). Fibroblasts on EF-5 weakly attached and showed a morphology similar to both EF-1 and AG73 (Fig. 2, D–IV).

Next, we tested the neurite outgrowth activity of five peptides with PC12 rat pheochromocytoma cells (Fig. 3). EF-1 and EF-3 promoted neurite outgrowth with PC12 cells (Fig. 3, C and D), whereas EF-1, EF-2, and EF-5 did not show activity (Fig. 3, A, B, and E). These results suggest that the homologous EF peptides have cell type-specific cell attachment and neurite outgrowth activities.

Organization of Actin Filaments and Localization of Vinculin—Next, we focused on the EF-1, EF-2, EF-4, and EF-5 peptides, which promoted fibroblast attachment, and examined the organization of actin filaments and localization of vinculin on the peptide-coated plates (Fig. 4). On the AG73 peptide, actin filament spikes were associated with membrane ruffles, as previously described (40), and no focal contacts with vinculin were observed (Fig. 4E). The EF-1 peptide induced well organized actin stress fibers and focal contacts containing vinculin (Fig. 4A). In contrast, EF-2 and EF-4 induced a morphology similar to AG73 with filopodia and ruffles, and no focal contact was observed (Fig. 4, B and C). EF-5 did not induce actin stress fibers or focal contacts (Fig. 4D). These results demonstrate that the cellular responses to the peptides are different.

Effects of Heparin and EDTA on Fibroblast Attachment—Next, we evaluated the effects of heparin and EDTA on fibroblast attachment to the peptides (Fig. 5). We used AG73 and laminin-1 as controls for heparin-dependent and cation-dependent cell attachment, respectively. Heparin strongly inhibited attachment on EF-2 and EF-4, whereas cell attachment on EF-1 and EF-5 was not inhibited by heparin. In contrast, EDTA significantly inhibited cell attachment on EF-1, but no effect on EF-2 and on EF-4-mediated attachment was observed. Further, fibroblast attachment to EF-5 was enhanced by EDTA. Fibroblasts attached to the EF-1 peptide in a cation-dependent manner, suggesting that EF-1 promoted integrin-mediated cell adhesion. However, cell attachment on EF-2 and EF-4 was inhibited by heparin, suggesting that these peptides have the potential to interact with membrane-associated heparin/heparan sulfate proteoglycans.

Attachment of 293T Cells Transfected with Either Syndecan-2 or Glypican-1 to Peptides—Syndecans and glypicans are cell surface heparan sulfate proteoglycans. Human neonatal dermal fibroblasts express syndecan-2 based on reverse transcriptase-PCR and Western blotting analysis (29). To examine whether syndecan-2 was involved in cell adhesion to EF-2 and EF-4, we used 293T cells overexpressing syndecan-2 and glypican-1 (29) and analyzed cell attachment (Fig. 6). 293T cell adhesion to EF-2 and EF-4 was significantly enhanced (more than 2-fold) by overexpression of syndecan-2 and slightly enhanced by overexpression of glypican-1. Overexpression of either proteoglycan had no effect on cell attachment to EF-1. These results indicate that cell attachment to EF-2 and EF-4 mainly involved syndecan-2.

Effect of Anti-integrin Antibodies on Cell Attachment to Peptides—Since EF-1 promoted cation-dependent fibroblast at-
Comparison with those on AG73; anti-integrin antibodies, including anti-attachment to EF-2 (data not shown). Other function-blocking contacts was evaluated on the following subjective scale:

- Stress fiber
- Filopodia/lamellipodia
- ND
- Filopodia/lamellipodia
- –
- Filopodia/lamellipodia
- –
- Heparin
- Heparin
- Heparin
- Heparin

For cell attachment assays, various amounts of peptides were coated on 96-well plates as described under “Materials and Methods.” Human neonatal dermal fibroblasts, HT1080 human fibrosarcoma cells, and HSG cells were used. In all cases, the biological activities of the peptides were quantitated and evaluated relative to those observed with AG73. Cell attachment was evaluated on the following subjective scale: +++; adhesion comparable with that on AG73; +, weak adhesion compared with that on AG73, –, no adhesion.

For neurite outgrowth assays with PC12 rat pheochromocytoma cells, various amounts of peptides were coated on the wells as described under “Materials and Methods.” Neurite outgrowth was evaluated on the following subjective scale: ++, promote neurite outgrowth; –, inactive.

Inhibitory effect

Receptor analysis

| Peptide | Cell attachment<sup>a</sup> | Neurite outgrowth<sup>b</sup> (PC12) | Actin organization<sup>c</sup> (fibroblast) | Focal contact<sup>d</sup> (fibroblast) | Inhibitory effect<sup>e</sup> (fibroblast) | Receptor analysis<sup>f</sup> (fibroblast) |
|---------|-----------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|-------------------------------|
| EF-1    | ++                          | +                              | Stress fiber                    | +                               | EDTA                            | α<sub>1</sub>β<sub>1</sub> integrin |
| EF-2    | ++                          | +                              | –                               | –                               | Heparin                         | Syndecan-2                    |
| EF-3    | –                           | –                              | –                               | –                               | ND                              | –                             |
| EF-4    | ++                          | ++                             | Filopodia/lamellipodia          | ND                              | ND                              | ND                            |
| EF-5    | +                           | –                              | –                               | –                               | Heparin                         | ND                            |
| AG73<sup>g</sup> | ++                          | +                              | Filopodia/lamellipodia          | –                               | Heparin                         | Syndecan-2                    |

<sup>a</sup> For cell attachment assays, various amounts of peptides were coated on 96-well plates as described under “Materials and Methods.” Human neonatal dermal fibroblasts, HT1080 human fibrosarcoma cells, and HSG cells were used. In all cases, the biological activities of the peptides were quantitated and evaluated relative to those observed with AG73. Cell attachment was evaluated on the following subjective scale: +++, adhesion comparable with that on AG73; +, weak adhesion compared with that on AG73, –, no adhesion.

<sup>b</sup> For neurite outgrowth assays with PC12 rat pheochromocytoma cells, various amounts of peptides were coated on the wells as described under “Materials and Methods.” Neurite outgrowth was evaluated on the following subjective scale: ++, promote neurite outgrowth; –, inactive.

<sup>c</sup> Actin and vinculin were detected in the fibroblasts attached on peptides as described under Materials and Methods.

<sup>d</sup> For cell attachment inhibition assays, human fibroblasts were incubated in the presence of 10 μg/ml heparin or 5 mM EDTA as described under Materials and Methods.

<sup>e</sup> Receptors for cell attachment to EF-1, EF-2, and EF-4, which were inhibited by heparin or EDTA, were analyzed. Using 293T cells, which overexpressed syndecan-2, enhanced cell attachment to EF-2 and EF-4. Anti-α<sub>1</sub> and anti-β<sub>1</sub> integrin antibodies inhibited cell attachment to EF-1.

<sup>f</sup> ND, not done.

<sup>g</sup> AG73 (RKLQVQLSIRT, α<sub>1</sub> chain 2719–2730) (8) was used as a positive control.

Fig. 2. Cell attachment activity of the EF peptides. A–C, cell attachment of human fibroblast (A), HT1080 cells (B), and HSG cells (C). 96-Well plates were coated with various amounts of peptides, and cells were added to the wells for 1 h. Following staining with crystal violet, the attached cells were dissolved in 10% SDS, and the OD (570 nm) was measured. Triplicate separate experiments gave similar results. D, morphological appearance of fibroblasts on the peptides. Wells of a chamber slide were coated with 5 μg/well of EF-1 (D-I), EF-2 (D-II), EF-4 (D-III), and AG73 (D-IV) and 20 μg/well of EF-5 (D-V). Fibroblasts were added to the wells. After a 2-h incubation, attached cells were stained with crystal violet. Photographs were taken with a 100× objective on a microscope. Triplicate experiments gave similar results.

Cell Attachment Activity of a Recombinant Laminin α<sub>1</sub> Chain LG4-5 Module and Effect of the EF-1 Peptide on the Attachment—The interaction of the laminin α<sub>1</sub> chain LG4-5 module containing the EF-1 sequence with ααββ integrin was not previously reported. Therefore, we prepared a recombinant laminin α<sub>1</sub> chain LG4-5 module (rec-αLG4-5) and examined integrin-mediated cell attachment to the rec-αLG4-5 protein. Fibroblasts attached to rec-αLG4-5 in a dose-dependent manner (Fig. 8A). Heparin inhibited 70% of the attachment (Fig. 8B), suggesting that cell surface heparin/heparan sulfate chains are involved in the fibroblast attachment to rec-αLG4-5. EDTA also inhibited about 40% of the cell attachment, suggesting that a part of the cell attachment was medi-
ated by cation-dependent receptor(s), including integrins. An anti-β1 integrin subunit antibody slightly inhibited fibroblast attachment to rec-α1LG4-5, whereas an anti-α2 integrin subunit antibody did not (Fig. 8B). The same concentration of anti-β1 integrin antibody inhibited 90% of fibroblast attachment to laminin-1 (data not shown). Fibroblast attachment to rec-α1LG4-5 was inhibited in the presence of both anti-α2 and anti-β1 integrin antibodies (Fig. 8B). The EF-1 peptide was also able to inhibit attachment to the recombinant protein (Fig. 8B).

Taken together, interactions with α2β1 integrin and with cell surface proteoglycan(s) are involved in fibroblast attachment to the laminin α1 chain LG4-5 module, and the EF-1 site is important for the interaction with α2β1 integrin.

Active Core Sequences of EF-1—To determine the active core sequence of EF-1, we prepared systematically truncated N- and C-terminal peptides and tested their cell attachment activity using fibroblasts (Table II). EF-1d (LQLQEGRLHFMFDLG), an N-terminal truncated peptide of EF-1, with 4 amino acids removed, still retained full activity, whereas the activity of EF-1e, with a deletion of the N-terminal leucine and glutamine residues from EF-1d, was weaker than that of EF-1. When the amino-terminal LQ (EF-1e), LQLQE (EF-1f), or LQLQEG (EF-1g) were removed from EF-1d, a significant reduction in cell attachment activity was observed. Further, the removal of LQLQEG (EF-1h) from EF-1d resulted in total loss of activity. EF-1j (DYATLQLQEGRLHFMFD), a C-terminal truncated peptide of EF-1, still retained full activity, whereas the activity of EF-1k, with a deletion of the C-terminal aspartate residue from EF-1j, showed no activity. EF-1m (LQLQEGRLHFMFD), an N-terminal and C-terminal truncated peptide of EF-1, showed weak cell attachment activity. These results suggest that the 13-amino acid sequence LQLQEGRLHFMFD is crit-
cal for the cell attachment activity of EF-1. The cause of the reduced activity of EF-1m may be due to conformational changes, which are stabilized by the additional N-terminal amino acids.

EF-1 and EF-1m sequences contained methionine residues. Since the methionine residues were easily oxidized during the synthesis, they were replaced with norleucine residues in the EF-1 and EF-1m sequences. EF-1X (DYATLQLQEGRLHF3FDLGY3) and EF-1Xm (LQLQEGRLHFX3FD) promoted fibroblast attachment as well as EF-1 and EF-1m, respectively (Table II).

Cell Attachment Activity of Cyclic EF-1Xm Peptide—Next, we prepared a cyclic EF-1Xm peptide to evaluate the role of conformation in the biological response (Table II). The cys-EF-1Xm peptide significantly enhanced fibroblast attachment activity relative to the linear form (Fig. 9A). Fibroblast attached on cys-EF-1Xm showed well organized actin stress fibers and focal contacts (Fig. 9B). Further, fibroblast attachment to cys-EF-1Xm was significantly inhibited by EDTA and by anti-β1 and anti-β3 integrin antibodies (Fig. 9C), whereas other integrin antibodies and heparin did not affect cyc-EF-1Xm-mediated fibroblast attachment. These results indicate that the cyc-EF-1Xm peptide interacts with α2β1 integrin similar to that of a linear peptide EF-1.

Effect of Peptides on Cell Attachment to the EF-1 Peptide—Next, we examined the effect of various peptides on EF-1-mediated fibroblast attachment (Fig. 10). The EF-1 peptide showed inhibitory activity, as expected (Fig. 10). EF-1m slightly inhibited cell attachment. The cyc-EF-1Xm peptide strongly inhibited fibroblast attachment to EF-1 relative to the linear form. FIB-1, an RGDF (Arg-Gly-Asp) sequence-containing peptide derived from fibronectin that interacts with αvβ3 integrins (8, 41), did not affect EF-1-mediated cell attachment. The data suggest that peptide conformation is important for the interaction between the EF-1 sequence and α2β1 integrin and that the interaction is specific.

DISCUSSION

Previously, we identified the A3G75 and A4G82 sequences as cell attachment and heparin binding sites in the G domains of the human laminin α3 and the mouse laminin α4 chains, respectively (29–32). Based on the alignment of the LG4 sequences, the A3G75 and A4G82 sequences are homologous sites located in the connecting loop between the E and F strands (14, 35). Mutagenesis using recombinant LG4 proteins with serine or alanine substitution mutations of basic residues (Lys1421 and Arg1423, human laminin α3 chain; His1519 and Arg1523, mouse laminin α4 chain) in the E-F loop regions has suggested that the loop regions are critical for biological activity of the recombinant proteins (29, 31). Here, we have described five homologous peptides (EF-1 to EF-5) from the E-F domain of mouse laminin α3 and human laminin α4 chain that interact with αvβ3 integrin.

The EF-1 and EF-2 peptides were significantly inhibited by EDTA and by anti-β1 integrin antibodies (Fig. 9C), whereas other integrin antibodies and heparin did not affect cyc-EF-1Xm-mediated fibroblast attachment. These results indicate that the cyc-EF-1Xm peptide interacts with α2β1 integrin similar to that of a linear peptide EF-1.

Effect of Peptides on Cell Attachment to the EF-1 Peptide—Next, we examined the effect of various peptides on EF-1-mediated fibroblast attachment (Fig. 10). The EF-1 peptide showed inhibitory activity, as expected (Fig. 10). EF-1m slightly inhibited cell attachment. The cyc-EF-1Xm peptide strongly inhibited fibroblast attachment to EF-1 relative to the linear form. FIB-1, an RGDF (Arg-Gly-Asp) sequence-containing peptide derived from fibronectin that interacts with αvβ3 integrins (8, 41), did not affect EF-1-mediated cell attachment. The data suggest that peptide conformation is important for the interaction between the EF-1 sequence and α2β1 integrin and that the interaction is specific.

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Fig. 8. A, fibroblast attachment activity of the rec-α1LG4-5 protein. 96-Well plates were coated with various amounts of rec-α1LG4-5. Fibroblast attachment was studied as described in the legend to Fig. 2. Triplicate experiments gave similar results. B, effect of heparin, EDTA, anti-integrin subunit antibodies, and the EF-1 peptide on fibroblast attachment to the rec-α1LG4-5-coated plate. 96-Well plates were coated with rec-α1LG4-5 (0.5 μg/well). The inhibition assay using 10 μg/ml heparin, 5 mM EDTA, and 10 μg/ml ant-integrin antibodies was performed as described in the legends to Figs. 5 and 7. The inhibition assay was carried out in the presence of 100 μg/ml EF-1. Each value represents the mean of three separate determinations ± S.D. Duplicate experiments gave similar results. *p < 0.0005; **p < 0.005; ***p < 0.01.

TABLE II

| Peptide   | Sequence* | Cell attachment activity* |
|-----------|-----------|---------------------------|
| EF-1      | DYATLQLQEGRLHFMFDLG | ++                       |
| EF-1X     | DYATLQLQEGRLHFMFDLG | ++                       |
| EF-1a     | YATLQLQEGRLHFMFDLG  | ++                       |
| EF-1b     | AYTQSLQEGRLHFMFDLG  | ++                       |
| EF-1c     | LQATLQLQEGRLHFMFDLG| ++                       |
| EF-1d     | LQLQEGRLHFMFDLG     | ++                       |
| EF-1e     | LQRLQEGRLHFMFDLG    | ++                       |
| EF-1f     | GLRLQEGRLHFMFDLG    | ++                       |
| EF-1g     | RLRLQEGRLHFMFDLG    | ++                       |
| EF-1h     | LHRLQEGRLHFMFDLG    | ++                       |
| EF-1i     | DYTQSLQEGRLHFMFD    | ++                       |
| EF-1j     | DYTQSLQEGRLHFMFD    | ++                       |
| EF-1k     | DYTQSLQEGRLHFMFD    | ++                       |
| EF-1l     | DYTQSLQEGRLHFMFD    | ++                       |
| EF-1m     | LQLQEGRLHFMFD       | ++                       |
| EF-1n     | LQLQEGRLHFMFD       | ++                       |
| cyc-EF-1Xm| LQLQEGRLHFMFD       | ++                       |

* Sequences of the synthetic peptides are given in the single-letter code. All peptides have C-terminal amides. The single letter X denotes the norleucine residue.
* Activity was scored on the following subjective scale: ++, cell attachment comparable with that on EF-1; +, weak adhesion comparable with that on EF-1; −, no adhesion. Active core sequences are shown in boldface type.

indicate that the E-F loop sites in the α2 and α4 chains may play a critical role in diverse tissues where they are expressed. EF-1 showed cell attachment activity with fibroblasts and HT-1080 cells, whereas EF-5 promoted only fibroblast attachment. The α1 and α2 chains are expressed in the early embryo and in diverse adult tissues, respectively (1). They may interact with mesenchymal cells, but not with HSG cells, via the E-F loop sites in each. Further, PC12 cells promoted neurite outgrowth on EF-3 and EF-4. Laminin α chains are expressed in various neural tissues (1, 42). It is suggested that the E-F loop regions in the α3 and α4 chains are important for the interaction with neural cells, whereas the other sites may function in the α1, α2, and α5 chains.

Syndecans, a family of heparan sulfate proteoglycans, interact with extracellular matrices and various growth factors (43, 44). Previously, we defined the A3G75 site as a syndecan-2 and -4 binding site in fibroblasts (29) and the AG73 sequence as a syndecan-1 binding site in HSG cells (13, 25). In syndecan-mediated cell attachment to the heparin binding domain of fibronectin and to the AG73 peptide, actin filament spikes were associated with membrane ruffles, and no focal contacts with vinculin accumulation were observed (40, 45). In this study, actin organization and vinculin localization of fibroblasts on EF-2 and EF-4 were similar to that on AG73. Furthermore, cell attachment to EF-2 and EF-4 was inhibited by heparin and was significantly enhanced by overexpression of syndecan-2. These results suggest that cell attachment to EF-2 and to EF-4 is mainly mediated by syndecan-2.

Integrins are important cell surface receptors for laminins. Several integrin binding sites in laminin-1 have been identified. The LG1 to -3 modules are expressed in various tissues where they are expressed. Syndecan-1, which interacts with α5β1 integrins (1), is a receptor for collagen and laminin (46–48). Domain VI, an N-terminal domain of the laminin α chain, interacts with α5β1 and α6β1 integrins (48, 49). We observed well-developed actin stress fiber and focal contacts in fibroblasts on EF-1. Further, fibroblast attachment to the EF-1 peptide was inhibited by function-blocking anti-α5 and anti-β1 integrin antibodies. The EF-1 site is located in the α1 chain LG4 module. The E3 fragment, which is an enzymatically digested laminin-1 fragment that contains the α1 chain LG4-5 module, has heparin- and α-dystroglycan-binding activities (1, 44). The interaction between E3 and α5β1 integrin has not been previously reported. The β1 integrin subunit mediates HBL-100 human normal mammary and A375 human melanoma cell attachment to E3 (15). We prepared a recombinant α1 chain LG4-5 module and observed α5β1 integrin-mediated fibroblast attachment to the rec-α1LG4-5 protein. Additionally, the EF-1 peptide competi-
tively inhibited fibroblast attachment to this protein. Taken together, EF-1 is a new peptide specifically interacting with α2β1 integrin, and this sequence in the laminin α1 chain is a novel α2β1 integrin-binding site in laminin-1. The AG73 peptide (RKRLQVQLSIRT, mouse laminin α1 chain 2719–2730), which is derived from the LG4 module and binds to syndecans, significantly inhibited cell attachment to the rec-α1LG4-5 pro-
tein (data not shown) and E3 (25). These results suggest that cell surface syndecans and integrins interact with the laminin α1 chain LG4-5 module via the AG73 and EF-1 sites, respectively.

We found that EF-5 showed only weak attachment activity with fibroblasts which was enhanced by EDTA. Actin organization and vinculin localization within the fibroblasts on EF-5 differed from that of cells adhering via syndecans or integrins. Recently, we found that A5G77 (LVLFLNHGHFVA, mouse laminin α5 chain 3307–3318) peptide, contained within the EF-5 sequence, inhibited branching morphogenesis (50). However, the homologous 12-mer peptides of A5G77 from the α5 chain were not active. Further, the inhibition of branching morphogenesis by A5G77 resulted in a different morphology from that observed with AG73, a syndecan-binding peptide from the laminin α1 chain G domain (27). These results suggest that the EF-5 region may interact with different receptor(s), resulting in α5 chain-specific biological activity.

EF-3 promoted neurite outgrowth with PC12 cells. Previously, we described that a recombinant human laminin α3 chain LG4 module and a peptide, A3G75, located on the E-F loop region of the module also promote neurite outgrowth with PC12 cells (30). The A3G75 peptide inhibited PC12 cell attachment to the recombinant human laminin α3 chain LG4 module (30). These results indicate that this region of the α3 chain is important for neurite outgrowth.

Previously, several cell-adhesive peptides were cyclized and showed increased biological activity over their noncyclized form. A cyclic YIGSR (Tyr-Ile-Gly-Ser-Arg) peptide enhanced cell attachment activity and showed increased inhibition of tumor metastasis over that observed with linear peptide (51). Cyclic RGD peptides also showed increased inhibition in their blocking activity (52). Moreover, cyclic RGD peptides interact with different integrins, including α1β1, α5β1, and α2β1 integrins (53). In this study, cyclization of the linear EF-1Xm peptide significantly enhanced cell attachment activity and the
inhibitory effect on cell attachment to EF-1. The cyc-EF-1Xm peptide induced actin stress fiber and focal contact formation and interacted with αβ2 integrin similar to EF-1. These results indicate that integrin specificity of the EF-1 sequence is enhanced by the cyclization. Additionally, the cyclic A4G82 peptide involved in the EF-4 sequence, significantly increased heparin binding and cell attachment activity when compared with the linear peptide.2 Taken together, the loop conformation of the E-F regions is critical for specific binding to cell receptors.

Here, we demonstrated the biological activities of five homologous peptides derived from the E-F loop regions of the laminin α chain LG4 modules. These homologous peptides showed chain-specific cell attachment and neurite outgrowth activities and interacted with different cell surface receptors, such as αβ2 integrin and syndecan-2. These results suggest that the E-F loop regions are important in various biological activities of the laminin isoforms in tissue-specific locations.

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Biological Activities of Homologous Loop Regions in the Laminin α Chain G Domains
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