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Identification of Cell Adhesive Sequences in the N-terminal Region of the Laminin α2 Chain*[S]

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Background: The N-terminal region of the laminin α2 chain promotes laminin assembly and cell adhesion.
Results: Screening of 218 peptides revealed that 11 sequences derived from N-terminal laminin α2 chain promote cell adhesion.
Conclusion: Three amino acids in the N terminus LN domain are critical sites for integrin α2β1 binding.
Significance: Eleven active peptides may be useful as tools for the study of laminin-receptor interactions.

The laminin α2 chain is specifically expressed in the basement membrane surrounding muscle and nerve. We screened biologically active sequences in the mouse laminin N-terminal region of α2 chain using 216 soluble peptides and three recombinant proteins (rec-a2LN, rec-a2LN+, and rec-a2N) by both the peptide- or protein-coated plate and the peptide-conjugated Sepharose bead assays. Ten peptides showed cell attachment activity in the plate assay, and 8 peptides were active in the bead assay. Seven peptides were active in the both assays. Five peptides promoted neurite outgrowth with PC12 cells. To clarify the cellular receptors, we examined the effects of heparin and EDTA on cell attachment to 11 active peptides. Heparin inhibited cell attachment to 10 peptides, and EDTA significantly affected only A2-8 peptide (YHYVTITLDLQQ, mouse laminin α2 chain, 117–128)-mediated cell attachment. Cell attachment to A2-8 was also specifically inhibited by anti-integrin β1 and anti-integrin α2β1 antibodies. These results suggest that A2-8 promotes an integrin α2β1-mediated cell attachment. The rec-a2LN protein, containing the A2-8 sequence, bound to integrin α2β1 and cell attachment to rec-a2LN was inhibited by A2-8 peptide. Further, alanine substitution analysis of both the A2-8 peptide and the rec-a2LN+ protein revealed that the amino acids Ile-122, Leu-124, and Asp-125 were involved in integrin α2β1-mediated cell attachment, suggesting that the A2-8 site plays a functional role as an integrin α2β1 binding site in the LN module. These active peptides may provide new insights on the molecular mechanism of laminin-receptor interactions.

The heterotrimeric basement membrane glycoproteins laminins consist of α, β, and γ chains and form cross-shaped or Y-shaped structures (1, 2). Five α (α1–α5), three β (β1–β3), and three γ chains (γ1–γ3) have been identified, and 19 isoforms are formed by various combinations of each chain depending on the tissue and developmental stage (3, 4). Cross-shaped laminin heterotrimers contain either an α1, α2, α3B, or α5 chain (1). These four α chains consist of three major regions: an N-terminal short arm, a coiled-coil domain, and C-terminal five tandem modules of LG domains, whereas the α3A and α4 chain lack the N-terminal short arm region. The N-terminal short arm is important for laminin self-assembly and cell adhesion, the coiled-coil domain maintains the laminin heterotrimer formation, and the LG modules mediate cell attachment to laminin through various cell surface receptors (5). Each laminin α chain is tissue- and/or developmental stage-specifically expressed, and the cell surface receptors for the laminin isoforms are mainly determined by the type of laminin α chains.

The laminin α2 chain, which is a component of laminin-211 (α2β1γ1), laminin-221 (α2β2γ1), laminin-213 (α2β1γ3), laminin-212 (α2β1γ2), and laminin-222 (α2β2γ2), is expressed in the basement membranes surrounding skeletal muscle, peripheral nerve, brain, and capillaries (1, 2). Mutations in the human laminin α2 chain gene (*LAMA2*) cause congenital muscular dystrophy (6). Moreover, a disruption of the *LAMA2* gene in mice results in postnatal death within 5 weeks with congenital muscular dystrophy and patchy peripheral nerve demyelination, indicating that the expression of the laminin α2 chain is completely crucial for muscle regeneration and neuronal myelination (7). The laminin α2 chain LG1–3 module harbors cell binding activity through integrins α3β1, α6β1, and α7β1, and the interaction of integrins with the laminin α2 chain LG module is required for clustering of the acetylcholine receptor (8). α-Dystroglycan (α-DG)[2] interacts with both the LG1–3 and LG4–5 modules of the laminin α2 chain (8, 9). Major heparin-binding sites are mapped on the LG5 module and on the individual LG1, LG3, and LG4 and N-terminal short arm region (10–12). Mutagenesis analysis using recombinant LG4–5 module proteins showed that α-DG binding to the LG4–5 module required Arg-2803 in LG4 module, and heparin binding required several basic residues, including Arg-2803, Lys-2953, Lys-3030, Lys-3088, and Lys-3095 (9, 12). The short arm region interacts with integrins α1β1 and α2β1 (13). The recom-

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[2] The abbreviations used are: α-DG, α-dystroglycan; HDF, human neonatal dermal fibroblast; HSPG, heparan sulfate proteoglycan.

binant short arm region protein promoted PC12 cell attachment and neurite outgrowth, whereas C-terminal LG domains did not (13, 14). These results suggested that the short arm region may play an important role in the interaction between the α2 chain and neuronal cells.

Previously, we have screened biologically active sites using synthetic peptides to the laminin α chains and identified several active peptides (15–17). Many active peptides were derived from the LG modules, and some of them were suggested to play a critical role in the specific binding to cell surface receptors. AG73 (RKRLQVQLSIRT, mouse laminin α1 chain, residues 2719–2730) (15, 18), A3G756 (KNSFMALYLSKGRLVFALG, mouse laminin α3 chain, residues 1411–1429) (19), and A4G82 (TLFLAHGRLVFM, mouse laminin α4 chain, residues 1514–1525) (20) bind to syndecans and promote various biological activities, including cell adhesion (20, 21), migration (20, 21), neurite outgrowth (22, 23), branching morphogenesis (24), and tumor growth and metastasis (25, 26). EF-1 (DYATLQLQEGRLHFMFDLG, mouse laminin α1 chain, residues 2747–2765) specifically interacts with integrin α2β1 and induces cell spreading, focal adhesions, and production of actin stress fibers (17). Recently, we analyzed the mouse laminin α2 chain LN and LG domains, respectively. A2-7, A2-8, A2-20, and A2-21 from the LN domain promoted HT-1080 cell attachment activity and A2-7, A2-8, and A2-20 also promoted B16-BL6 cell attachment (27). From the LG domains, A2G78 (GLLFYMARINHA, mouse laminin α2 chain, residues 2796–2807) is a critical active sequence for both heparin/heparan sulfate proteoglycans (HSPGs) and α-DG binding, whereas A2G80 (VQLRNGFPYFSY, mouse laminin α2 chain, and 2812–2823) specifically binds to α-DG (12). However, the biologically active sites in the laminin α2 chain short arm region and coiled-coil domain have not yet been systematically investigated.

In the present work, we synthesized 218 overlapping peptides covering the entire laminin α2 chain short arm region and coiled-coil domain sequence and evaluated their cell attachment activity on peptide-coated plates and peptide-conjugated Sepharose beads using two different cell types. Neurite outgrowth activity of the peptides was also examined. The identified active sequences in terms of cellular receptors were further analyzed.

EXPERIMENTAL PROCEDURES

Peptide Synthesis—All of the peptides were manually synthesized by the *N*-(9-fluorenyl) methoxycarbonyl (Fmoc) solid phase method with a C-terminal amide as described previously (12). The 218 synthetic peptides were purified by reverse phase HPLC on a Mightysil RP-18 GP 250–10 column (Kanto Chemical Co. Inc., Tokyo, Japan) using a gradient elution with water/acetonitrile containing 0.1% trifluoroacetic acid. Purity and identity of the peptides were confirmed by analytical HPLC and electrospray ionization mass spectrometry at the Central Analysis Center, Tokyo University of Pharmacy and Life Sciences. The 216 peptides were dissolved in Milli-Q water and used for experiments with the exception of A2-53 and A2-55, which were insoluble and not used for experiments.

Antibodies—Rat monoclonal antibody against human integrin α6 (GoH3) was purchased from AMAC (Westbrook, ME). Mouse monoclonal antibodies against human integrin α1 (FB12), α2 (P1E6), α3 (P1B5), αv (P3G8), β1 (6S6), and α2β1 (BHA2.1) were purchased from Millipore Co. Ltd. (Billerica, MA).

Cells and Culture—Human neonatal dermal fibroblasts (HDFs) (Cell Applications Inc., San Diego, CA), C2C12 cells, a mouse myoblast cell line (CRL-1772; ATCC, Manassas, VA), and 293T cells (Invitrogen) were cultured in DMEM containing 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). PC12 cells, a rat pheochromocytoma cell line (28), were cultured in DMEM, containing 7.5% horse serum (Invitrogen), 7.5% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. The cells were maintained at 37 °C in an humidified 5% CO_2, 95% air atmosphere.

Construction of Expression Vectors—cDNA was synthesized using mRNA from mouse kidney by the Superscript III (Invitrogen) and amplified by the KOD plus (Toyobo Life Science, Osaka, Japan) as follows: 216–1723 nucleotides of the mouse laminin α2 chain, 5′-CCCAAGCTTCGGCGACAGTCCCAAGCG-3′ (forward 1) and 5′-CCCTCGAGAACCATCAAGCAGGGATTTC-3′ (reverse 1). A DNA sequence of the mouse laminin α2 chain LN-LEa domain (a2LN+) was inserted into the XhoI and HindIII sites of the pCEP4dPur vector, which expresses the His and FLAG tag on its N terminus under BM-40 signal peptide (pCEPa2LN+) (29). Laminin α2 chain LN-LEa domain with N-terminal tags and signal peptide were inserted into pEF4/V5-His (Invitrogen) with KpnI and NotI sites, and a mouse laminin α2 chain LN-LEa domain-expressing plasmid (pEFa2LN+; 216–1723 nucleotides) was obtained. PCR product using pCEPa2LN+ amplified by 5′-GGAATTCCACCATGAGGGCCTGGATCTTCTTTC-3′ (forward 2) and 5′-CCTCGAGACATCCCGCCAACTGAAATATC-3′ (reverse 2) was inserted to pEF4/V5-His with SpeI and EcoRV to obtain a mouse laminin α2 chain LN domain-expressing plasmid (pEFa2LN; 216–993 nucleotides). The PCR product using the previous cDNA was amplified by a forward 1 primer and 5′-CGTGAGGTTGATGTTCATGG-3′ (reverse 3) and inserted into pEF2aLN+ with EcoRI and XbaI to construct the mouse laminin α2 chain short arm region-expressing plasmid (pEFa2N; 216–4931 nucleotides).

For preparation of mutant recombinant laminin α2 chain LN-LEa (rec-a2LN+), we performed site-directed mutagenesis using a QuikChange site-directed mutagenesis kit (Agilent Technologies, Inc., Santa Clara, CA). The following amino acid residues of the laminin α2 chain LN domain were substituted with alanine (m1: Ile-122; m2: Leu-124 and Asp-125; and m3: Thr-123).

Expression and Purification of Recombinant Proteins—The recombinant protein expression vectors were transfected into 293T cells using Lipofectamine 2000 (Invitrogen). The transfected cells were maintained for 2 days, then split at a 1:6 ratio in DMEM containing 10% FBS, and incubated with 200 μg/ml zeocin (Invitrogen) for 3 days for selection. The selected cells were maintained with 100 μg/ml zeocin. To prepare recombinant proteins, nearly confluent cells were cultured without serum for 3 days, and the condition medium was collected. The medium was cleared of debris by centrifugation, and Complete EDTA-free (Roche Applied Science) was added to decrease

proteolysis. Nickel-charged agarose resins (Probond; Invitrogen) were added to the conditioned medium. After incubation overnight at 4 °C, the resins were transferred to a column and washed with 0.05% Tween 20 in PBS (T-PBS), containing 10 mM imidazole (Wako Pure Chemical Industries, Osaka, Japan). His-tagged recombinant proteins were eluted with T-PBS, containing 250 mM imidazole and 500 mM NaCl. Purified proteins were dialyzed against PBS and quantified using the BCA protein assay kit with bovine serum albumin as a standard (Thermo Fisher Scientific, Rochester, NY). Purity was determined by reducing SDS-PAGE followed by Coomassie Brilliant Blue R-250 (Wako) staining and Western blotting. The CD (J-720; JASCO, Tokyo, Japan) spectrum analysis did not show significant differences in rec-a2LN+ mutant proteins.

Cell Attachment Assay Using Synthetic Peptides and Recombinant Proteins—The cell attachment assay using synthetic peptides and recombinant proteins was performed in 96-well plates (Thermo Fisher Scientific). 96-well plates were coated with various amounts of proteins in 50 μl of PBS overnight at 4 °C. Peptides were also coated on the plates by drying overnight. Then the substrate-coated wells were blocked with 1% heat-denatured BSA (Sigma-Aldrich) in DMEM at 37 °C for 1 h and then washed with 0.1% BSA in DMEM. The cells were detached with 0.05% trypsin-EDTA (Invitrogen) and were allowed to recover for 20 min at 37 °C. After they were washed with 0.1% BSA in DMEM, the cells were plated in 0.1% BSA in DMEM at 2.0×10^4 cells/100 μl/well and incubated at 37 °C for 1 h in 5% CO_2. The medium was removed by aspiration, and the attached cells were fixed and stained with 0.2% crystal violet aqueous solution in 20% methanol for 15 min. After the wells were washed, 1% SDS solution (150 μl) was added to lyse the cells. The optical density at 570 nm was measured in a plate reader Safire (Tecan Austria GmbH Grödig, Austria) or observed under a BZ-8000 microscope before lysing by SDS (Keyence, Osaka, Japan). All of the cell attachment assays were carried out at least three times with triplicate wells for each test concentration.

Cell Attachment Assay Using Peptide-conjugated Sepharose Beads—The synthetic peptides were coupled to CNBr-activated Sepharose 4B (GE Healthcare) as described previously (21). The peptides (200 μg) were incubated with the CNBr-activated Sepharose (30 mg) in 1 ml of 0.1 M $NaHCO_3$, containing 0.5 M NaCl (pH 8.3), for 1 h at room temperature. Then the beads were blocked with 1 M ethanolamine (pH 8.0) and washed with 0.1 M acetate buffer (pH 4.0), containing 0.5 M NaCl, and 0.1 M Tris-HCl (pH 8.0), containing 0.5 M NaCl. The cells were resuspended in DMEM containing 0.1% BSA (1×10^5 cells/100 μl) and incubated with 3 mg/50 μl peptide bead solution for 1 h. The attached cells were stained with 0.2% crystal violet aqueous solution in 20% methanol. After removal of unattached cells, the attached cells were observed under a BZ-8000. Each peptide was assayed using HDFs and C2C12 cells. Each assay was repeated at least twice.

Cell Attachment Assay Using Peptide-Chitosan Membranes—Cell attachment assays using peptide-chitosan membranes were performed on 96-well plates as described previously (30, 31). After 50 μl/well of maleimidobenzoyloxy-chitosan solution was added and dried, the plates were washed with 1% $NaHCO_3$ and then washed with PBS. For conjugation of peptides to chitosan membranes, a Cys-Gly-Gly sequence was added at the N terminus of the peptides as described previously (30). 5 mM of peptide solution was added to the wells and incubated for 2 h. Then the plates were used for the cell attachment assay as described above.

Inhibition Assay—For inhibition of cell attachment to peptide-coated plates and to peptide-chitosan membranes, 96-well plates were prepared as described above. The cells were preincubated for 15 min at 37 °C in the presence of either 10 μg/ml heparin (Sigma-Aldrich), 5 mM EDTA (Wako Pure Chemicals), 10 μg/ml various cell adhesive peptides, and 10 or 30 μg/ml of integrin antibodies. HDFs (2.0×10^4 cells/100 μl/well) were incubated at 37 °C in 5% CO_2 on the peptide-coated plates for 30 min and evaluated. Each assay was repeated at least three times.

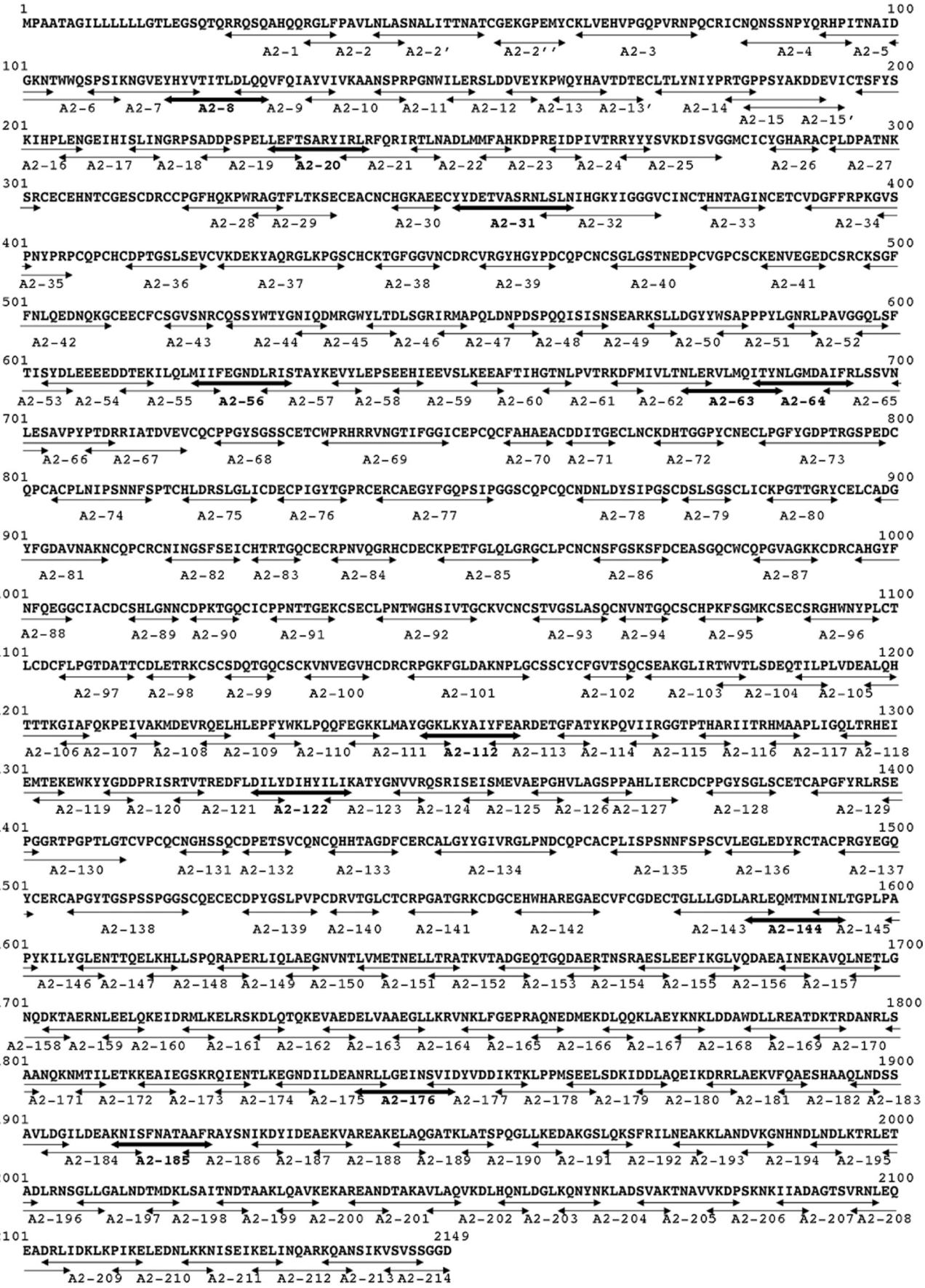
Neurite Outgrowth Assay—Neurite outgrowth was performed in 96-well plates and assessed as described previously (32). PC12 cells were primed with 100 ng/ml of NGF (Invitrogen) for 24 h prior to the assay. The cells were resuspended in DMEM/F-12 containing 30 nM Na_2SeO_3, 100 μg/ml transferrin, 20 nM progesterone (Sigma-Aldrich), 5 μg/ml insulin (Invitrogen), and 100 ng/ml NGF. The cells were added to 96-well plates at 3.0×10^3 cells/100 μl/well, incubated for 24 h, fixed with 20% formalin, and stained with 0.2% crystal violet. One hundred cells in each well were viewed under a BZ-8000, and the percentage of the active cells, which had neurites that extended twice the cell diameter in length and/or longer, was determined. Analyses of neurite outgrowth were carried out in triplicate.

RESULTS

Peptides from the N-terminal Region of Mouse Laminin α2 Chain—We prepared 218 peptides from the N-terminal region of mouse laminin α2 chain (positions 24–2149) to identify biologically active sequences (Fig. 1). For the screening, the peptides were generally synthesized as 12-amino acid residues in length and overlapped with neighboring peptides by four amino acids. If the N-terminal amino acid was either glutamate or glutamic acid, one amino acid was extended at the N terminus to avoid pyroglutamine formation. Cysteine residues were deleted from the peptide design to avoid the influence of disulfide bonds on cell adhesive activity. Cysteine-flanking sequences of less than 5 amino acids and N-terminal signal peptides were omitted. The activities of peptides A2-1 to A2-24 were determined in our previous work (27).

Cell Attachment Activity of Laminin α2 Chain Synthetic Peptides—The cell attachment activity of the soluble 216 peptides was evaluated using both the coated plate method and conjugated Sepharose beads, respectively. To eliminate false positive results, we used two different assays. The coated plate method determines morphological differences and dose dependence of cell attachment, whereas the Sepharose bead conjugation method regulates the amount of peptides. AG73, which was identified as the strongest peptide for cell attachment in the mouse α1 chain LG4 module, and its scrambled peptide AG73T were used as positive and negative controls, respectively (21).

In the coated plate method, nine peptides (A2-8, A2-31, A2-56, A2-63, A2-112, A2-122, A2-144, A2-176, and A2-185)


1 100
MPAATAGILLLLLLGTLEGSQTQRRQSQAHQQRGLFPAVLNLASNALITTNATCGEKGPEMYCKLVEHVPGQPVRNPQCRICNQNSSNPYQRHPITNAID
A2-1 A2-2 A2-2′ A2-2′′ A2-3 A2-4 A2-5
101 200
GKNTWWQSPSIKNGVEYHYVTITLDLQQVFQIAYVIVKAANSPRPGNWILERSLDDVEYKPWQYHAVTDTECLTLYNIYPRTGPPSYAKDDEVICTSFYS
A2-6 A2-7 A2-8 A2-9 A2-10 A2-11 A2-12 A2-13 A2-13′ A2-14 A2-15 A2-15′
201 300
KIHPLENGEIHISLINGRPSADDPSPELLEFTSARYIRLRFQRIRTLNADLMMFAHKDPREIDPIVTRRYYYSVKDISVGGMCICYGHARACPLDPATNK
A2-16 A2-17 A2-18 A2-19 A2-20 A2-21 A2-22 A2-23 A2-24 A2-25 A2-26 A2-27
301 400
SRCECEHNTCGESCDRCCPGFHQKPWRAGTFLTKSECEACNCHGKAEECYYDETVASRNLSLNIHGKYIGGGVCINCTHNTAGINCETCVDGFFRPKGVS
A2-28 A2-29 A2-30 A2-31 A2-32 A2-33 A2-34
401 500
PNYPRPCQPCHCDPTGSLSEVCVKDEKYAQRGLKPGSCHCKTGFGGVNCDRCVRGYHGYPDCQPCNCSGLGSTNEDPCVGPCSCKENVEGEDCSRCKSGF
A2-35 A2-36 A2-37 A2-38 A2-39 A2-40 A2-41
501 600
FNLQEDNQKGCEECFCSGVSNRCQSSYWTYGNIQDMRGWYLTDLSGRIRMAPQLDNPDSPQQISISNSEARKSLLDGYYWSAPPPYLGNRLPAVGGQLSF
A2-42 A2-43 A2-44 A2-45 A2-46 A2-47 A2-48 A2-49 A2-50 A2-51 A2-52
601 700
TISYDLEEEEDDTEKILQLMIIFEGNDLRISTAYKEVYLEPSEEHIEEVSLKEEAFTIHGTNLPVTRKDFMIVLTNLERVLMQITYNLGMDAIFRLSSVN
A2-53 A2-54 A2-55 A2-56 A2-57 A2-58 A2-59 A2-60 A2-61 A2-62 A2-63 A2-64 A2-65
701 800
LESAVPYPTDRRIATDVEVCQCPPGYSGSSCETCWPRHRRVNGTIFGGICEPCQCFAHAEACDDITGECLNCKDHTGGPYCNECLPGFYGDPTRGSPEDC
A2-66 A2-67 A2-68 A2-69 A2-70 A2-71 A2-72 A2-73
801 900
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A2-74 A2-75 A2-76 A2-77 A2-78 A2-79 A2-80
901 1000
YFGDAVNAKNCQPCRCNINGSFSEICHTRTGQCECRPNVQGRHCDECKPETFGLQLGRGCLPCNCNSFGSKSFDCEASGQCWCQPGVAGKKCDRCAHGYF
A2-81 A2-82 A2-83 A2-84 A2-85 A2-86 A2-87
1001 1100
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A2-88 A2-89 A2-90 A2-91 A2-92 A2-93 A2-94 A2-95 A2-96
1101 1200
LCDCFLPGTDATTCDLETRKCSCSDQTGQCSCKVNVEGVHCDRCRPGKFGLDAKNPLGCSSCYCFGVTSQCSEAKGLIRTWVTLSDEQTILPLVDEALQH
A2-97 A2-98 A2-99 A2-100 A2-101 A2-102 A2-103 A2-104 A2-105
1201 1300
TTTKGIAFQKPEIVAKMDEVRQELHLEPFYWKLPQQFEGKKLMAYGGKLKYAIYFEARDETGFATYKPQVIIRGGTPTHARIITRHMAAPLIGQLTRHEI
A2-106 A2-107 A2-108 A2-109 A2-110 A2-111 A2-112 A2-113 A2-114 A2-115 A2-116 A2-117 A2-118
1301 1400
EMTEKEWKYYGDDPRISRTVTREDFLDILYDIHYILIKATYGNVVRQSRISEISMEVAEPGHVLAGSPPAHLIERCDCPPGYSGLSCETCAPGFYRLRSE
A2-119 A2-120 A2-121 A2-122 A2-123 A2-124 A2-125 A2-126 A2-127 A2-128 A2-129
1401 1500
PGGRTPGPTLGTCVPCQCNGHSSQCDPETSVCQNCQHHTAGDFCERCALGYYGIVRGLPNDCQPCACPLISPSNNFSPSCVLEGLEDYRCTACPRGYEGQ
A2-130 A2-131 A2-132 A2-133 A2-134 A2-135 A2-136 A2-137
1501 1600
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A2-138 A2-139 A2-140 A2-141 A2-142 A2-143 A2-144 A2-145
1601 1700
PYKILYGLENTTQELKHLLSPQRAPERLIQLAEGNVNTLVMETNELLTRATKVTADGEQTGQDAERTNSRAESLEEFIKGLVQDAEAINEKAVQLNETLG
A2-146 A2-147 A2-148 A2-149 A2-150 A2-151 A2-152 A2-153 A2-154 A2-155 A2-156 A2-157
1701 1800
NQDKTAERNLEELQKEIDRMLKELRSKDLQTQKEVAEDELVAAEGLLKRVNKLFGEPRAQNEDMEKDLQQKLAEYKNKLDDAWDLLREATDKTRDANRLS
A2-158 A2-159 A2-160 A2-161 A2-162 A2-163 A2-164 A2-165 A2-166 A2-167 A2-168 A2-169 A2-170
1801 1900
AANQKNMTILETKKEAIEGSKRQIENTLKEGNDILDEANRLLGEINSVIDYVDDIKTKLPPMSEELSDKIDDLAQEIKDRRLAEKVFQAESHAAQLNDSS
A2-171 A2-172 A2-173 A2-174 A2-175 A2-176 A2-177 A2-178 A2-179 A2-180 A2-181 A2-182 A2-183
1901 2000
AVLDGILDEAKNISFNATAAFRAYSNIKDYIDEAEKVAREAKELAQGATKLATSPQGLLKEDAKGSLQKSFRILNEAKKLANDVKGNHNDLNDLKTRLET
A2-184 A2-185 A2-186 A2-187 A2-188 A2-189 A2-190 A2-191 A2-192 A2-193 A2-194 A2-195
2001 2100
ADLRNSGLLGALNDTMDKLSAITNDTAAKLQAVKEKAREANDTAKAVLAQVKDLHQNLDGLKQNYNKLADSVAKTNAVVKDPSKNKIIADAGTSVRNLEQ
A2-196 A2-197 A2-198 A2-199 A2-200 A2-201 A2-202 A2-203 A2-204 A2-205 A2-206 A2-207 A2-208
2101 2149
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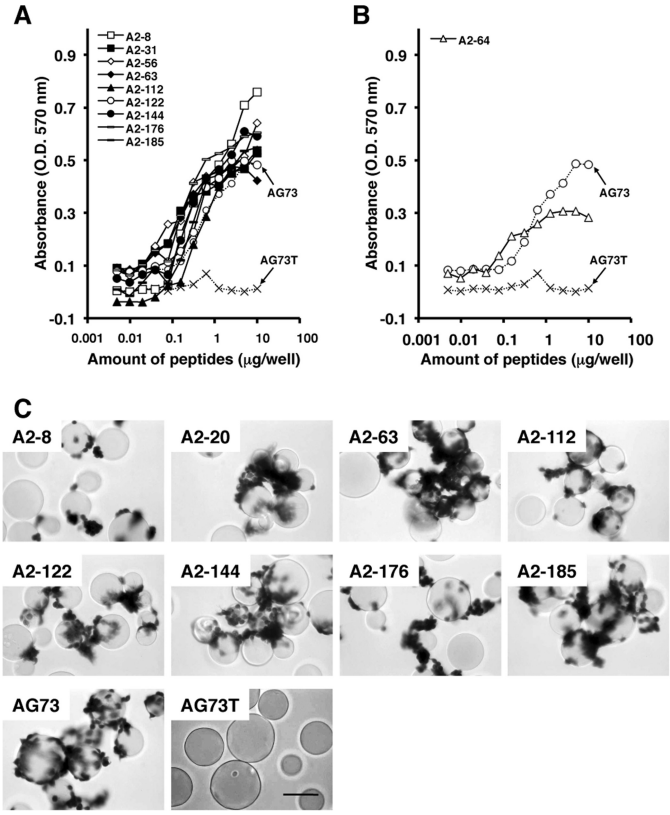

FIGURE 2. **Cell attachment to the peptide-coated plates and peptide-Sepharose beads.** *A* and *B*, 96-well plates were coated with various amounts of synthetic peptides and examined for cell attachment activity using HDFs. The cells were added to the wells for 1 h. After the cells were stained with 0.2% crystal violet in 20% methanol, the attached cells were dissolved in 1% SDS, and the A_{570} (*O.D.*) was measured. *C*, HDFs were allowed to attach to peptide-Sepharose beads for 1 h and were then stained with 0.2% crystal violet in 20% methanol. The AG73 peptide (RKRLQVQLSIRT), which has the strongest cell attachment activity in the α1 chain G domain, and its scrambled peptide AG73T (LQQRRSVLRTKI) were used as positive and negative controls, respectively. The data are expressed as the means of triplicate results. Triplicate experiments gave similar results. *Bar*, 100 μm.

showed strong cell attachment activity with human neonatal dermal fibroblasts (HDFs) in a dose-dependent manner (Fig. 2*A*), and the A2-8 peptide significantly promoted cell spreading (data not shown). These cell attachment activities were comparable with that of AG73. The A2-64 peptide promoted cell attachment, but the activity was weaker than that of AG73 (Fig. 2*B*). The rest of the peptides did not promote HDF attachment. We next evaluated cell attachment activity using C2C12 cells. Eight peptides (A2-8, A2-56, A2-63, A2-112, A2-122, A2-144, A2-176, and A2-185) had strong C2C12 cell attachment activity comparable with that of AG73 (supplemental Fig. S1*A*). Two peptides (A2-31 and A2-64) showed weak C2C12 cell attachment activity (supplemental Fig. S1*B*). None of the other peptides promoted C2C12 attachment.

Next, we conjugated the 216 soluble peptides to CNBr-activated Sepharose beads and tested their cell attachment activity using HDFs and C2C12 cells. HDFs attached to eight peptide-conjugated beads (A2-8, A2-20, A2-63, A2-112, A2-122, A2-144, A2-176, and A2-185) (Fig. 2*C*). None of the remaining peptide-conjugated beads promoted HDF attachment. We also evaluated the cell attachment activity of peptide-conjugated beads using C2C12 cells (supplemental Fig. S1*C*). Both cell types attached to the same eight peptide-conjugated beads.

Ten peptides promoted cell attachment to the two different cells in the peptide-coated plate assay, and eight peptides showed cell attachment activity to the two different cell types in the peptide-conjugated bead assay. Seven peptides showed cell attachment activity in both assays. Three peptides were active only in the peptide-coated plate assay (Table 1). A2-20 showed cell attachment activity only in the peptide-conjugated bead assay using HDFs and C2C12 cells. On the other hand, we previously screened HT-1080 and B16-BL6 cell attachment activity and found that A2-7, A2-8, and A2-20 promoted HT-1080 and B16-BL6 cell attachment in the coated plate assay, and A2-21 promoted HT-1080 Sepharose bead cell attachment activity (27). These results indicate that cell attachment to A2-20 is cell type-specific.

Effect of Heparin and EDTA on Cell Attachment to Peptide-coated Plates—To determine the cellular receptors, we next evaluated the effect of heparin and EDTA on HDF attachment to the 11 active peptides (Fig. 3). AG73, which interacts with syndecans and promotes heparin-dependent cell attachment, and EF-1, which interacts with integrin α2β1 and promotes a divalent cation-dependent cell adhesion (17), were used as controls. To evaluate the inhibitory effect of EDTA and heparin on cell adhesive peptides on the 96-well plates, A2-20 was conjugated with a chitosan membrane in the well. HDF attachment to seven peptides (A2-20, A2-56, A2-112, A2-122, A2-144, A2-176, and A2-185) was significantly inhibited by heparin, but not by EDTA. These seven peptides promoted heparin-dependent cell attachment, suggesting that they interact with HSPGs on the cell surface. HDF attachment to three peptides (A2-31, A2-63, and A2-64) was inhibited by both heparin and EDTA. These data suggest that A2-31, A2-63, and A2-64 interact with both HSPGs and integrin(s) for cell attachment. HDF attachment to A2-8 was inhibited by only EDTA. HDFs attached to A2-8 in a divalent cation-dependent fashion, suggesting that the A2-8-mediated cell attachment involves integrin(s).

Biological Activity of Laminin α2 Chain Short Arm Recombinant Proteins—Cell adhesive peptides were mainly localized in the short arm region (Fig. 4). In addition, A2-8 promoted cation-dependent cell attachment activity and is located in the LN domain. Next, we prepared recombinant proteins composed of the short arm region (rec-a2N; 191 kDa), the LN-LEa domain (rec-a2LN+; 71 kDa), and LN domain (rec-a2LN; 37 kDa) (Fig. 4, *A* and *B*), and examined them in the cell attachment assay using HDFs. rec-a2N, rec-a2LN+, and rec-a2LN promoted cell attachment in a dose-dependent manner (Fig. 4*C*). The molecular weights of these three recombinant proteins are different, suggesting that rec-a2N promoted the strongest cell attachment activity on a molar basis. Cell attachment to three recombinant proteins was significantly inhibited by heparin, EDTA,

FIGURE 1. **Sequences and peptides from the N-terminal region of laminin α2 chain.** The sequences were derived from the mouse N-terminal region of laminin α2 chain (46). *Arrows* denote the locations of peptides. *Thick lines* represent active peptides. A2-53 and A2-55 were insoluble and were not used for the experiments.

TABLE 1
Biological activities of the synthetic peptides derived from the N-terminal region of laminin α2 chain

Peptide	Sequence	Cell attachment				HDF attachment inhibition[c]	Neurite outgrowth (PC12)[d]
		HDF		C2C12			
		Plate[a]	Bead[b]	Plate	Bead		
A2-8	YHYVTITLDLQQ	++	+	++	+	EDTA	−
A2-20	LEFTSARYIRLR	−	+	−	+	Heparin	−
A2-31	YYDETVASRNLSLN	++	−	+	−	Heparin/EDTA	+
A2-56	MIIFEGNDLRIS	++	−	++	−	Heparin	+
A2-63	TNLERVLMQITYN	++	+	++	+	Heparin/EDTA	+
A2-64	ITYNLGMDAIFR	+	−	+	−	Heparin/EDTA	−
A2-112	GGKLKYAIYFEA	++	+	++	+	Heparin	+
A2-122	DILYDIHYILIK	++	+	++	+	Heparin	+
A2-144	ARLEQMTMNINL	++	+	++	+	Heparin	+
A2-176	ANRLLGEINSVI	++	+	++	+	Heparin	+
A2-185	AKNISFNATAAF	++	+	++	+	Heparin	+
AG73[e]	RKRLQVQLSIRT	++	+	++	+	Heparin	+
AG73T[e]	LQQRRSVLRTKI	−	−	−	−	−	−

[a] For the cell attachment assays on plates, various amounts of peptides were coated on 96-well plates as described under "Experimental Procedures." HDFs and C2C12 cells were used. In all cases, cell attachment to the peptide-coated plates was quantitated and assessed relative to those observed with AG73 and evaluated on the following subjective scale: ++, adhesion comparable with that on AG73; +, weak adhesion compared with that on AG73; −, no adhesion.
[b] For the cell attachment assay on beads, the peptides were coupled to CNBr-activated Sepharose 4B as described under "Experimental Procedures." HDFs and C2C12 mouse myoblast cells were used. +, active; −, inactive.
[c] EDTA and heparin inhibited attachment of HDFs to the peptide-coated plates. Inhibited compounds (EDTA or heparin) are shown.
[d] For the neurite outgrowth assay with PC12 rat pheochromocytoma cells, various amounts of peptides were coated on 96-well plates as described under "Experimental Procedures." Neurite outgrowth was evaluated on the following subjective scale: +, active; −, inactive.
[e] AG73 (RKRLQVQLSIRT, mouse laminin α1 chain 2719–2730) and its scrambled peptide AG73T (LQQRRSVLRTKI, a scrambled AG73) were used as positive and negative controls, respectively.

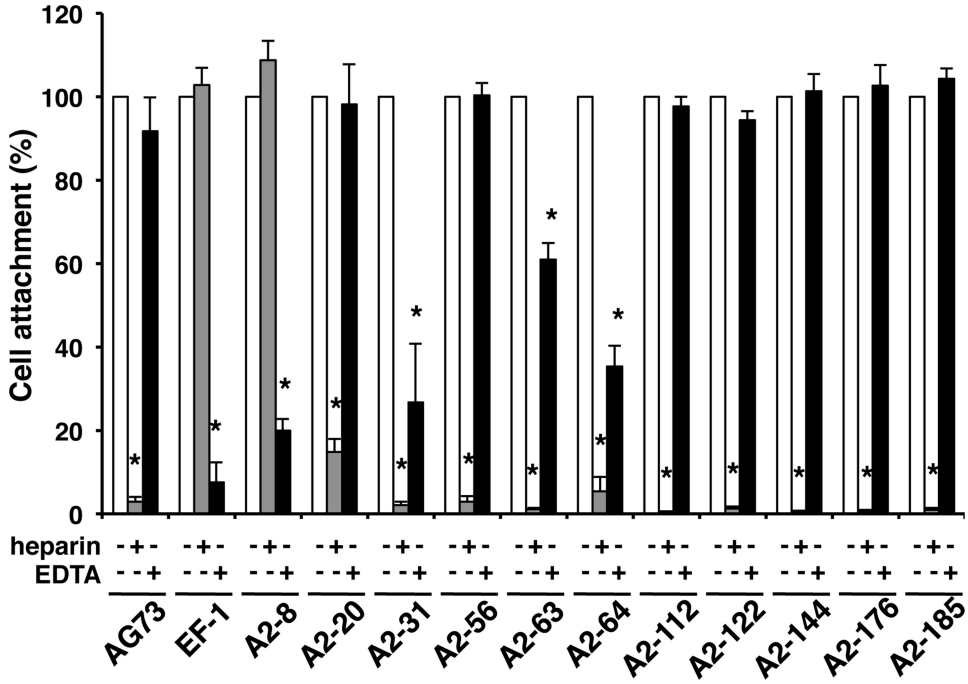

FIGURE 3. **Effect of heparin and EDTA on the attachment of HDFs to the 11 active peptides.** HDFs were allowed to attach to the peptide-coated plates in the absence (*white bars*) or presence of either 10 μg/ml of heparin (*gray bars*) or 5 mM EDTA (*black bars*). 96-well plates were coated with 0.5 μg/well of the cell adhesive peptides. Because A2-20 did not have cell attachment activity, an A2-20-chitosan matrix was used as described under "Experimental Procedures." Either 10 μg/ml heparin or 5 mM EDTA was added to the cell suspensions, and then the cells were added onto the peptide-coated plates. After a 30-min incubation, the attached cells were stained with 0.2% crystal violet in 20% methanol, and the adherent cells were counted under a microscope in three different fields that were selected at random (0.67 mm^2/field). AG73, a heparin binding peptide, and EF-1, an integrin α2β1 binding peptide, were used as control. Each value represents the mean of three separate determinations ± S.D. (*versus* each control). Triplicate experiments gave similar results. *, $p < 0.01$.

and anti-β1 and -α2β1 integrin antibodies (Fig. 4*D* and supplemental Fig. S2). These results suggest that cell attachment to these three proteins is mainly mediated by heparin and integrin α2β1 and that the N-terminal LN domain is a dominant region for integrin α2β1 as previously described (13).

Effect of Peptides on Cell Attachment to rec-a2N—Next, we examined the effect of the eight cell adhesive peptides derived from short arm region (A2-8, A2-20, A2-31, A2-56, A2-63, A2-64, A2-112, and A2-122) on HDF attachment to rec-a2N (Fig. 5*A*) and the non-cell adhesive peptide, A2-1, was used as a control. Cell attachment to rec-a2N was inhibited by all cell adhesive peptides (Fig. 5*A*). A2-8, A2-20, A2-31, A2-56, and A2-63 decreased cell attachment to rec-a2N more than 50% at 25 μg/ml. In addition, we examined the effect of four integrin-binding peptides derived from laminin and from collagen on the cell attachment to rec-a2N (Fig. 5*B*). Cell attachment to

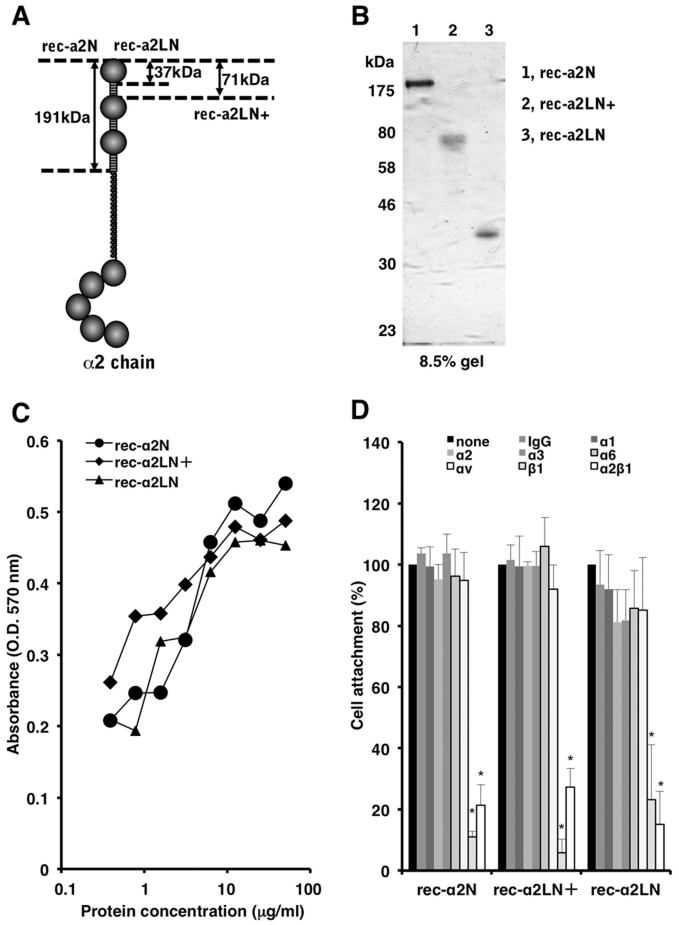

FIGURE 4. **Biological activities of three different recombinant proteins.** *A*, three different laminin α2 chain short arm recombinant proteins, rec-a2N, rec-a2LN+, and rec-a2LN, were prepared, and their cell attachment activities and the inhibitory effects of integrin functional blocking antibodies were tested. *B*, Coomassie Brilliant Blue R-250 staining represents the three different recombinant proteins. *Lane 1*, rec-a2N; *lane 2*, rec-a2LN+; *lane 3*, rec-a2LN. *C*, 96-well plates were coated with various amounts of recombinant proteins and examined for cell attachment activity using HDFs. The cells were added to the wells for 1 h. After the cells were stained with 0.2% crystal violet in 20% methanol, the attached cells were dissolved in 1% SDS, and the A_{570} (*O.D.*) was measured. *D*, 96-well plates were coated with 10 μg/well of each recombinant protein. The cell suspensions in the presence of anti-integrin antibodies were preincubated at room temperature for 15 min. Then the cells were added to the wells and incubated for 30 min. After staining with crystal violet, the attached cells were counted under a microscope in three different fields, which were selected at random (each field is 0.67 mm^2). Each value represents the mean of three separate determinations ± S.D. (*versus* none). Duplicate experiments gave similar results. *, $p < 0.05$.

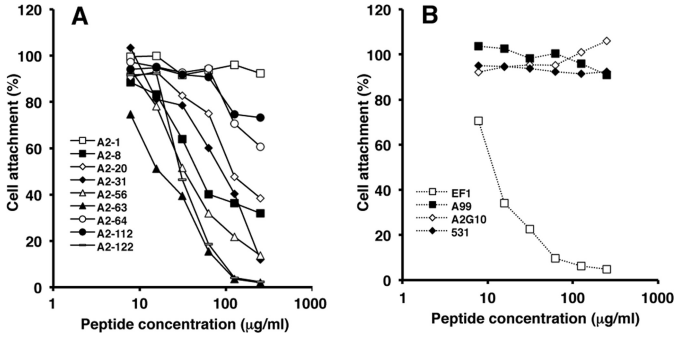

FIGURE 5. **Effect of 9 active peptides from short arm region and various integrin-binding peptides on the attachment of HDFs to rec-a2N.** 96-well plates were coated with 10 μg/well of rec-a2N proteins. *A*, various amount of cell adhesive peptides from the laminin α2 chain (A2-8, A2-20, A2-31, A2-56, A2-63, A2-64, A2-112, and A2-122), control peptide A2-1, and *B*, extracellular matrix-derived integrin-binding peptides (EF-1, integrin α2β1 binding (17); A99, integrin αvβ3 binding, AGTFALRGDNPQG, mouse laminin α1 chain, residues 1141–1153 (30); A2G10, integrin α6β1 binding, SYWYRIEASRTG, mouse laminin α2 chain, residues 2173–2184 (33); and 531, integrin α3β1 binding, GEFYFDLRLKGDKY, human collagen α1 (IV), residues 531–544 (34)) were 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 staining with crystal violet, the attached cells were counted under a microscope in three different fields, which were selected at random (each field is 0.67 mm^2). Each value represents the mean of three separate determinations (*versus* none). Duplicate experiments gave similar results.

rec-a2N was strongly inhibited by the EF-1 peptide, which binds to integrin α2β1 (Fig. 5*B*). In contrast, the other integrin-binding peptides (A99 from the mouse laminin α1 chain, which interacts with integrin αvβ3 (30); A2G10 from the mouse laminin α2 chain LG module, which interacts with integrin α6β1 (33); and 531 from human collagen type IV, which interacts with integrin α3β1 (34)) did not affect cell attachment to rec-a2N (Fig. 5*B*). These results suggest that the A2-8, A2-20, A2-31, A2-56, and A2-63 sequences play a critical role in cell attachment to rec-a2N and that the rec-a2N promotes integrin α2β1-mediated cell attachment.

Neurite Outgrowth Activity of the Synthetic Peptides and Recombinant Proteins—The 216 soluble peptides, rec-a2N, and rec-a2LN were evaluated for neurite outgrowth activity using PC12 cells (Fig. 6). Eight peptides (A2-20, A2-56, A2-63, A2-112, A2-122, A2-144, A2-176, and A2-185) and rec-a2N promoted neurite outgrowth (Fig. 6). In contrast, the remaining peptides, including A2-8 and rec-a2LN, did not promote neurite outgrowth. These results suggest that eight active sequences in the N-terminal region of laminin α2 chain have potential to interact with neuronal cells, and the active sites are not in the N-terminal LN module. Based on the cell attachment assays using HDFs and C2C12 cells and neurite outgrowth assay, we have identified 11 biologically active peptides (Table 1).

Effect of Anti-integrin Antibodies on Cell Attachment to A2-8 and Effect of Integrin-binding Peptides on Cell Attachment to rec-a2LN—Because HDF attachment to A2-8 was cation-dependent and A2-8 promoted strong cell spreading activities, we examined the inhibitory effect of anti-integrin antibodies on the HDF attachment to A2-8. Cell attachment to A2-8 was ~50% blocked by 10 μg/ml of anti-β1 integrin antibodies, and the other antibodies, anti-α1, -α2, -α3, -α6, and -αv, had no affect on HDF attachment to A2-8 (Fig. 7*A*). Further, 30 μg/ml of anti-α2β1 antibody blocked by 50%, anti-α2 antibody slightly blocked, and anti-β1 completely blocked HDF attachment to A2-8, respectively. Next, we examined the effect of two cell adhesive peptides (A2-8 and A2-20) derived from LN domain and four integrin-binding peptides on HDF attachment to rec-a2LN (Fig. 7, *B* and *C*). A2-8 significantly inhibited cell attachment to rec-a2LN, and the activity was higher than that of A2-20, and the negative control A2-1 did not affect the cell attachment activity of rec-a2LN (Fig. 7*B*). Further, cell attachment to A2-8 was inhibited by EF-1, which interacts with integrin α2β1 (Fig. 7*C*). In contrast, the other integrin-binding peptides (A99, A2G10, and 531) did not reduce cell attachment to rec-a2LN (Fig. 7*C*). This was the same finding as the inhibition assay with rec-a2N (Fig. 5). These results suggest that the A2-8 sequence

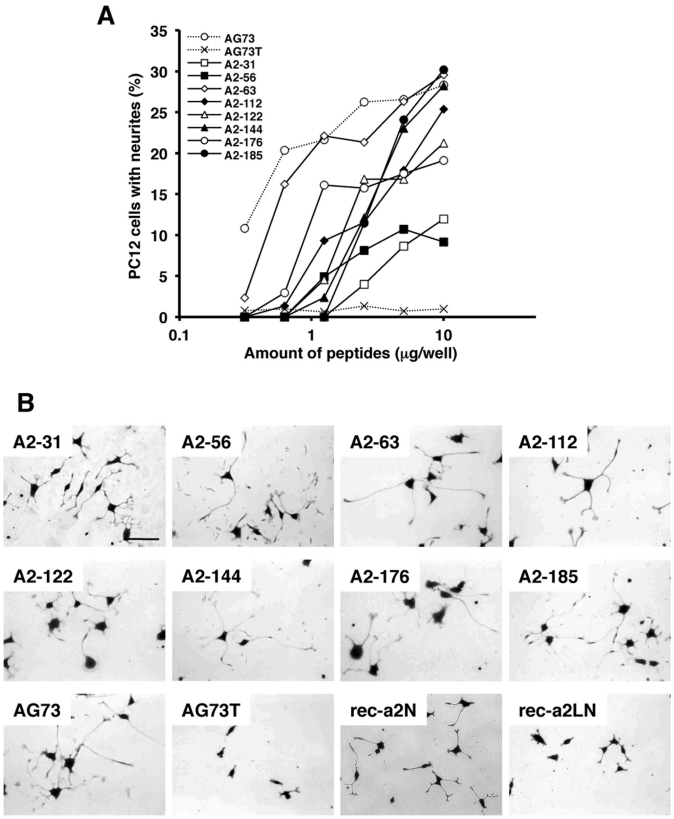

FIGURE 6. **Neurite outgrowth of PC12 cells on the peptide-coated plates.** *A*, 10 μg/well of peptides were coated on 96-well plates. PC12 cells (3.0×10^3 cells/well) were seeded in the wells and incubated for 24 h. After the cells were fixed and stained, the percentage of cells with neurites was determined as described under "Experimental Procedures." Triplicate experiments gave similar results. *B*, the PC12 cells were incubated on 10 μg/well of peptides and proteins for 24 h. After fixation and staining, the cells were photographed using BZ-8000 microscope. *Bar*, 100 μm.

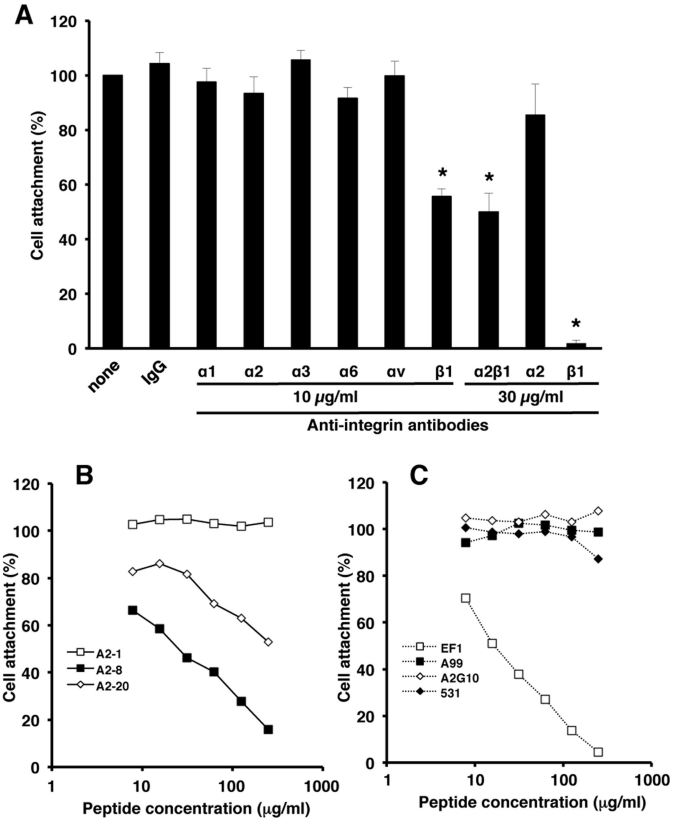

FIGURE 7. **Effect of anti-integrin antibodies on cell attachment to A2-8 and effect of various peptides on cell attachment to rec-a2LN.** *A*, 96-well plates were coated with 10 μg/well of A2-8. 10 and/or 30 μg/ml of anti-integrin antibodies were added to the cell suspensions and preincubated at room temperature for 15 min. Then the cells were added to the wells and incubated for 30 min. After staining with crystal violet, the attached cells were counted under a microscope in three different fields, which were selected at random (each field is 0.67 mm^2). Each value represents the mean of three separate determinations ± S.D. (*versus* none). *B* and *C*, 96-well plates were coated with 10 μg/well of rec-a2LN proteins. Various concentrations of A2-8, A2-20, and integrin-binding peptides were added to the cell suspension and preincubated at room temperature for 15 min. Then the cells were added to the wells and incubated for 30 min. After staining with crystal violet, the attached cells were counted as described in above. Each value represents the mean of three separate determinations (*versus* none). Duplicate experiments gave similar results. *, $p < 0.01$.

plays a critical role in cell attachment to rec-a2LN via binding to integrin α2β1.

Effect of Alanine-substituted A2-8 Peptides on Cell Attachment and Spreading—To determine the cell adhesive core sequences in A2-8, we evaluated the cell attachment activity of the alanine-substituted A2-8 peptides using HDFs (Table 2). Two alanine-substituted peptides (A2-8-ii, A2-8-xii) exhibited cell attachment activity comparable with that on A2-8 (Fig. 8*A*). In contrast, cell attachment activities were partially decreased in the peptides with alanine substituted at the first Tyr (A2-8-i), third Tyr (A2-8-iii), fourth Val (A2-8-iv), fifth Thr (A2-8-v), seventh Thr (A2-8-vii), tenth Leu (A2-8-x), and eleventh Gln (A2-8-xi) (Fig. 8*B*). Cell attachment activities were significantly decreased in the peptide with alanine substituted at the sixth Ile (A2-8-vi), eighth Leu (A2-8-viii), and ninth Asp (A2-8-ix) (Fig. 8*C*). In addition, the cells attached to A2-8-vi, -viii, and -ix but exhibited no spreading, whereas the cells attached to A2-8 and exhibited extensive spreading (Fig. 8*D*). These results suggest that the sixth Ile residue (Ile-122), eighth Leu residue (Leu-124), and ninth Asp residue (Asp-125) are important for the cell attachment and spreading activity of A2-8.

Preparation and Biological Activity of Mutant rec-a2LN+—We have found that A2-8 significantly decreased cell attachment to rec-a2N and rec-a2LN. We next prepared mutant recombinant laminin α2 LN proteins (rec-a2LN) with alanine substitutions within the A2-8 sequence to confirm the importance of the A2-8 sequence on cell attachment to LN domain. Unfortunately mutant rec-a2LN was not secreted from the cell.

TABLE 2
HDF attachment activity of alanine-substituted peptides of A2-8

Peptide	Sequence	Substituted residue	HDF attachment[a]
A2-8	YHYVTITLDLQQ		+++
A2-8-i	AHYVTITLDLQQ	Tyr	++
A2-8-ii	YAYVTITLDLQQ	His	+++
A2-8-iii	YHAVTITLDLQQ	Tyr	++
A2-8-iv	YHYATITLDLQQ	Val	++
A2-8-v	YHYVAITLDLQQ	Thr	++
A2-8-vi	YHYVTATLDLQQ	Ile	+
A2-8-vii	YHYVTIALDLQQ	Thr	++
A2-8-viii	YHYVTITADLQQ	Leu	+
A2-8-ix	YHYVTITLALQQ	Asp	+
A2-8-x	YHYVTITLDAQQ	Leu	++
A2-8-xi	YHYVTITLDLAQ	Gln	++
A2-8-xii	YHYVTITLDLQA	Gln	+++

[a] Various amounts of peptides were coated on 96-well plates as described under "Experimental Procedures." HDF attachment was evaluated on the following subjective scale: +++, adhesion compared with that on A2-8; ++, weak adhesion compared with that on A2-8; +, very weak adhesion compared with that on A2-8.

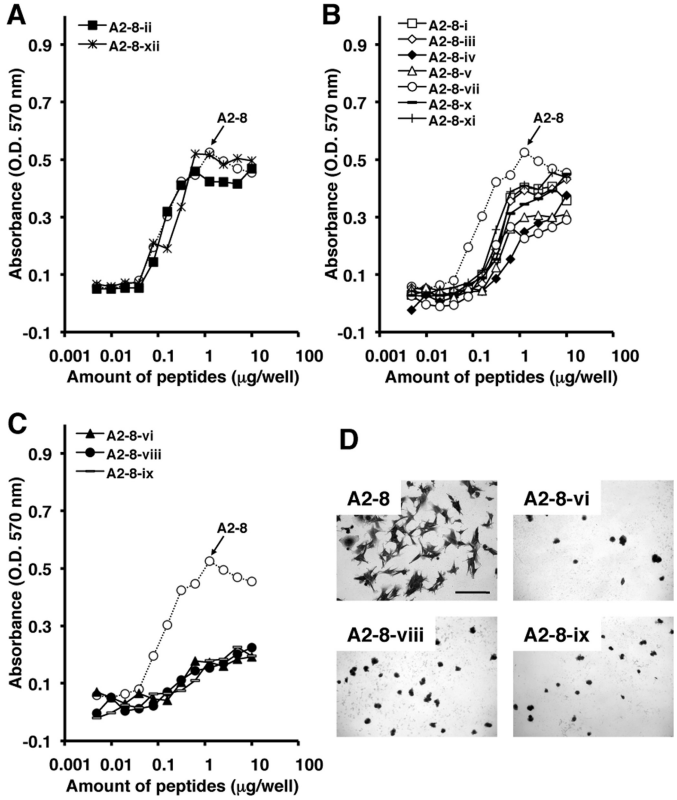

FIGURE 8. **Effect of alanine substitutions on A2-8 cell attachment activity.** 96-well plates were coated with various amounts of 12 Ala-substituted A2-8 peptides (Table 2) and examined for cell attachment activity using HDFs. *A–C*, HDFs were added to the wells for 1 h. After the cells were stained with 0.2% crystal violet in 20% methanol, the attached cells were dissolved in 1% SDS, and the A_{570} (*O.D.*) was measured. *D*, HDFs were allowed to attach to peptide-coated plates for 90 min and were then stained with 0.2% crystal violet in 20% methanol. The data are expressed as mean of triplicate results. Triplicate experiments gave similar results. *Bar*, 100 μm.

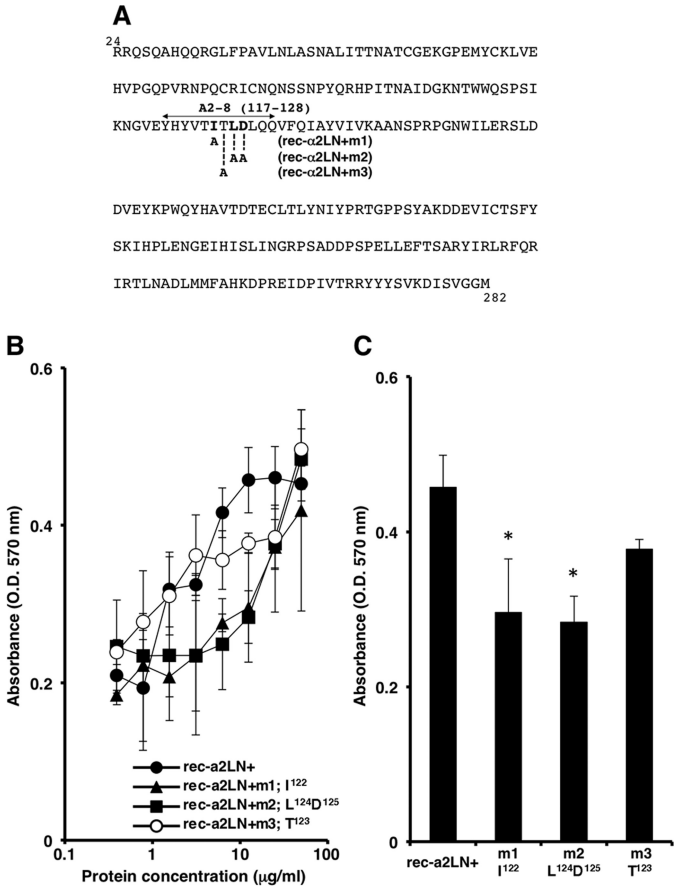

FIGURE 9. **Effect of alanine substitutions on cell attachment activity of rec-a2LN+.** *A*, mutant rec-a2LN+ proteins that have alanine substitutions in the A2-8 site (rec-a2LN+m1: Ile-122; rec-a2LN+m2: Leu-124 and Asp-125; and rec-a2LN+m3: Thr-123) were prepared. *B*, 96-well plates were coated with various concentrations of the protein solutions and examined for cell attachment activity using HDFs. The cells were added to the wells for 1 h. After the cells were stained with 0.2% crystal violet in 20% methanol, the attached cells were dissolved in 1% SDS, and the A_{570} (*O.D.*) was measured. *C*, the *p* value was analyzed at maximal cell attachment activity (25 μg/ml). Each value represents the mean of three separate determinations ± S.D. Triplicate experiments gave similar results. *, $p < 0.01$.

Therefore, we inserted an Ala-substituted mutation into recombinant laminin α2 LN-LEa domain (rec-a2LN+). Three different mutant recombinant proteins, rec-a2LN+m1, rec-a2LN+m2, and rec-a2LN+m3, were secreted and purified. The CD spectra of four recombinant proteins exhibited similar results, suggesting that LEa domain provides structural stability to the LN domain as previously reported (35). Two mutant proteins contained alanine substitutions at the critical amino acid residues for cell attachment to A2-8 (rec-a2LN+m1: Ile-122; rec-a2LN+m2: Leu-124; and Asp-125), and rec-a2LN+m3 (Thr-123) was prepared as a control with an Ala substitution between Ile-122 and Leu-124 (Fig. 9*A*). The cell attachment activity of rec-a2LN+m1 and rec-a2LN+m2 was partially decreased relative to the activity of rec-a2LN+ WT and rec-a2LN+m3 (Fig. 9, *B* and *C*). These results suggested that Ile-122, Leu-124, and Asp-125 are involved in the integrin α2β1-mediated cell attachment to the LN domain of the laminin α2 chain.

DISCUSSION

The laminin α chain interacts with a variety of cellular receptors, including integrins, HSPGs, and α-DG, and these interactions are required for the function of basement membranes to signal cellular responses (1, 5). Five tandem globular domains of the LG module that are located on the laminin α chain C terminus are considered to be the major cell adhesive region in the laminin α chain. In addition, many studies had identified numerous cell adhesive sites in the laminin α chain LG module. Previously, we have screened biologically active sequences in the LG module of the five murine laminin α chains using recombinant proteins and synthetic peptides (15, 20, 32, 36–38). We have reported that AG10, AG32 (TWYKIAFQRNRK, mouse laminin α1 chain, residues 2370–2381) (15, 39), A2G10, and MA3G27 (NAPFPKLSWTIQ, mouse laminin α3 chain, residues 1851–1862) (37) interact with integrin α6β1, and EF-1 binds to integrin α2β1 (17). Additionally, we have also identified syndecan-binding sequences on AG73 (18, 21), MA3G70 (KPRLQFSLDIQT, mouse laminin α3 chain, residues 2243–2254) (19), A4G82 (20), and A5G81 (AGQWHRVSVRWG, mouse laminin α5 chain, residues 3337–3348) (40), CD44 binding sequences on A5G27 (41), and α-DG binding sequences on A2G78 and A2G80 (12). On the other hand, a major functional role of the N-terminal region of the laminin α chain has been thought to be laminin-laminin assembly to form

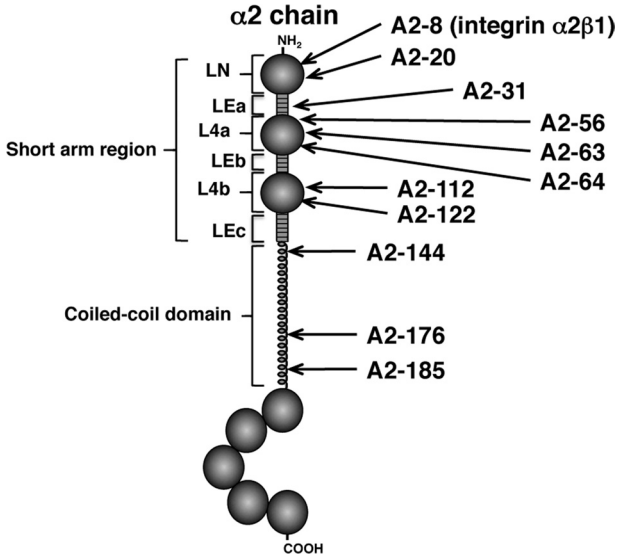

FIGURE 10. **Localization of cell adhesive peptides in N-terminal region of laminin α2 chain.** The localization of 11 HDF and C2C12 cell adhesive peptides is indicated. A2-8 binds integrin α2β1.

a mesh-like structure (5). Although a number of reports demonstrated the cell adhesive activity of the laminin α chain LG module, fewer studies have looked at the N-terminal region of laminin α chain. Analysis of laminin α1 and α2 chain N-terminal recombinant proteins revealed that the N-terminal globular module of the LN domain binds integrins α1β1 and α2β1 and heparin, and synthetic peptide analysis identified 25 cell adhesive sequences from the laminin α1 chain (14, 16). However, cell adhesive sites in N-terminal region of laminin α2 chain have not yet been identified. Here, we identified eight cell adhesive peptides from the short arm region and three from coiled-coil domain of laminin α2 chain and estimated the integrin α2β1-binding sequence in the LN domain using alanine-substituted A2-8 peptides and mutant recombinant proteins. The active peptide, A2-8, demonstrates the biological significance in the N-terminal region of laminin α2 chain. The systematic peptide screening approach is a useful strategy for identification of biologically active sequences in multifunctional large proteins such as laminin.

In this study, we have identified 11 biologically active sequences in the N-terminal region of laminin α2 chain by a systematic peptide screening (Table 1 and Fig. 10). Seven peptides (A2-8, A2-63, A2-112, A2-122, A2-144, A2-176, and A2-185) were identified as active sequences in both the peptide-coated plate and the peptide-conjugated bead assays. The other four sequences (A2-20, A2-31, A2-56, and A2-64) promoted cell attachment activity in either the plate or bead assay. A2-20 was active in the bead assay but was not active when coated on the plates. In contrast, A2-31, A2-56, and A2-64 were active in the plate assay but were not active when conjugated to the beads. These results indicated that both assays should be employed when screening for active peptides. It is likely that the differential activities are due to conformational changes and/or poor coating efficiencies on the plastic plates. We previously reported that A99 is active in the peptide bead but not active in the peptide plate assays (16). The activity similarity of A2-20 and A99 suggests that these peptides require a flexible conformation or higher coating efficiency to the plates. A2-31, A2-56, and A2-64 showed cell attachment activity in the plate assays, whereas these peptides were inactive in the bead assays. We have previously demonstrated that A208, an IKVAV-containing sequence in the laminin α1 chain, is active in the plate assays but is not active in the bead assays (16, 42). In recent our analyses, we found that A208 forms amyloid-like fibrils with β sheet structures in aqueous solution and promotes cell adhesion and neurite outgrowth (43, 44). These three peptides may have the potential to behave similarly to the A208 peptide and require a typical β sheet conformation for their biological activities. Further, seven of the eleven peptides are located in the three globular modules: LN, L4a, and L4b domain, suggesting that globular modules play an important role in the cell adhesive activity of the laminin α2 chain N terminus. Taken together, these results indicate that it is important to examine two different methods for identifying biologically active sites when performing a systematic peptide screening.

Ala-substituted A2-8 peptides analysis indicated that Ile-122, Leu-124, and Asp-125 may mediate cell attachment to A2-8 through integrin α2β1. To confirm whether these residues are engaged in integrin α2β1-mediated cell attachment to the laminin α2 chain LN-LEa domain, we created mutant recombinant proteins. The cell attachment activity of rec-a2LN+m1 (Ile-122 to Ala-122) and rec-a2LN+m2 (Leu-124 and Asp-125 to Ala-124 and Ala-125, respectively) was partially decreased. In this study, we identified three cell adhesive peptides, A2-8, A2-20, and A2-31, from the LN-LEa domain. Cell attachment activity of rec-a2LN was mainly inhibited by A2-8 and partially inhibited by A2-20. These results suggest that three residues are critical for the cell attachment activity of rec-a2LN+ through integrin α2β1 binding and that A2-20 and A2-31 may participate in cell attachment via heparin binding of rec-a2LN+ as previously reported (13). We further tested the inhibitory effect of anti-integrin α2β1 functional blocking antibody on cell attachment to mutant recombinant proteins (data not shown). The cell attachment to rec-a2LN+m1 and rec-a2LN+m2 was not inhibited by antibody, whereas it was inhibited to rec-a2LN+m3 (Thr-123 to Ala-123). These results suggested that Ile-122, Leu-124, and Asp-125 might be involved in integrin α2β1-mediated cell attachment to the LN domain of laminin α2 chain.

Recently, x-ray crystallographic analysis of the laminin α5 chain LN domain revealed the presence of five α helices, eight β strands, and three *N*-linked glycans in the LN domain of the laminin α5 chain (45). The alignment among the laminin α1, α2, and α5 chains indicated that A2-8 is located on the β2 strand and loop connection region between the β2-β3 strands. The active site of A2-8, including Ile-122, Leu-124, and Asp-125, is localized on the surface of the LN domain. The laminin α5 chain Asn-148, located on the LN domain β2 strand, is the second *N*-linked glycosylated site and is aligned to Thr-121 of the laminin α2 chain in A2-8 and Thr-115 of the laminin α1 chain. This demonstrates that the second *N*-glycosylated site of α5 chain LN domain is absent in both the α1 and α2 chain. The Thr-123, Leu-124, Asp-125, and Leu-126 on the α2 chain β2 strand in A2-8 are completely conserved sequences among α1,

α2, and α5 chains, and the A-12 (mouse laminin α1; WVTVTLDLRQVFQ, residues 113–125), which includes this conserved sequence, has strong cell attachment activity (16). These results suggested that β2 strand and loop connection region between the β2-β3 strands in the α1 and α2 chain are the critical sites for cell adhesive activity of the LN domain, whereas that of the α5 chain may be inactive because glycans on Asn-148 block access to this region. Further, the A2-8 sequence has a curved formation in LN domain. We previously identified an integrin α2β1-binding peptide EF-1 from the laminin α1 chain LG4 module. EF-1 is located on the E and F strand and the connecting loop region. We synthesized cyclic-EF-1 and found that cyclic-EF-1 had stronger cell adhesive activity than linear EF-1 (17). It is possible that cyclic-A2-8 may promote strong cell attachment activity and enhance the specificity of integrin α2β1 binding more potently than the linear A2-8 peptide.

We previously screened the laminin α1 chain short arm region and coiled-coil domain using 208 synthetic peptides and found 25 cell adhesive peptides using the fibrosarcoma cell line HT-1080 (16). In this study, we identified 11 cell adhesive peptides. The number of cell adhesive peptides in the laminin α1 chain and laminin α2 chains may indicate the cell type specificity and functional differences between the two laminin chains. The cell attachment activity of 10 cell adhesive peptides from laminin α2 chain (except A2-8) and 20 cell adhesive peptides from laminin α1 chain were significantly inhibited by heparin and showed that twice the number of heparin-binding peptides are located in the laminin α1 chain. The recombinant laminin α1 chain short arm region has stronger heparin binding activity than that of the laminin α2 chain (13), and we previously reported that the heparin binding activity of five laminin α chains LG4–5 module are related to the number of heparin-binding peptides (40), suggesting that these active peptides sequences are involved in the heparin binding of laminin. The sequence identity of these two chains is 70% in the short arm region and 52% in the coiled-coil domain. Eight peptides of the α2 chain and 17 peptides of the α1 chain are mapped to the short arm region, and sequence alignment analysis demonstrated that five peptides (A2-8 and A-12: WVTVTLDLRQVFQ; A2-20 and A-24: LLEFTSARYIRL; A2-63 and A-65: ANVTHLLIRANY; A2-112 and A-112: VLIKGGRARKHV; and A2-122 and A-119: LSNIDYILIKAS) are located at almost the same position, whereas there are no homologous peptides in the coiled-coil domain between the α1 and α2 chain-derived peptides. This suggests that five homologous peptides are critical for the cell adhesive activity of the N-terminal region of the laminin α1 and α2 chains, and other peptides sequence and the coiled-coil domain may contribute to the diversity of the two chains.

In conclusion, we have identified 11 biologically active sequences from the N-terminal region of the mouse laminin α2 chain. Because cell attachment activity of 10 active sequences except A2-8 was inhibited by heparin and these 10 peptides had basic amino acid residues (Arg or Lys), it is likely that basic amino acids are essential for cell attachment activity through HSPGs, such as syndecans, whereas the integrin α2β1-binding peptides, A2-8, did not include basic amino acids. We have identified A2-8 as an integrin α2β1-binding peptide, and Ile-122, Leu-124, and Asp-125 in the A2-8 sequence are involved in the cell adhesive activity of the LN domain via integrin α2β1 binding. The receptor-specific interactions need to be investigated further, because extracellular matrix proteins recognize multiple cell surface molecules, and sorting out the multiple biological effects of extracellular matrix proteins on cell behavior and signaling pathways is complex. Short peptides are simple tools for defining individual cell responses, receptor interactions, and signaling pathways. Previously, we identified an integrin α2β1 binding peptide (EF-1) and a syndecan binding peptide (AG73) from the laminin α1 LG4 domain, respectively (29). The EF-1 peptide promoted cell spreading and AG73 promoted strong cell attachment activity. When we created mutant rec-a1LG4, EF-1 mutated rec-a1LG4 lost cell spreading activity, and AG73 mutated rec-a1LG4 decreased cell attachment activity. This suggested that the EF-1 site in rec-a1LG4 promotes cell spreading, and the AG73 site in rec-a1LG4 promotes cell attachment, respectively. Our identified 11 active peptides could be involved in the biological activities related to the laminin α2 chain and would be useful for studying the molecular mechanisms of laminin-receptor interactions.

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