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Identification of Active Sequences in the L4a Domain of Laminin α5 Promoting Neurite Elongation

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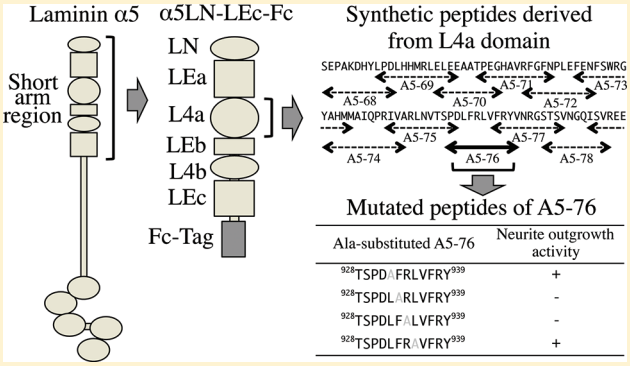
ABSTRACT: Laminin α5 is an extracellular matrix protein containing multiple domains implicated in various biological processes, such as embryogenesis and renal function. In this study, we used recombinant proteins and synthetic peptides to identify amino acid residues within the short arm region of α5 that were critical for neurite outgrowth activity. The short arm of α5 contains three globular domains (LN, L4a, and L4b) and three rodlike elements (LEa, LEb, and LEc). Recombinant proteins comprised of the α5 short arm fused with a Fc tag produced in 293 cells were assayed for PC12 (pheochromocytoma) cell adhesion and neurite outgrowth activities. Although it did not have cell attachment activity, neurite outgrowth was promoted by the recombinant protein. To narrow the region involved in neurite outgrowth activity, two truncated recombinant proteins were produced in 293 cells. A recombinant protein lacking L4a and LEb lost activity. Furthermore, we synthesized 78 partially overlapping peptides representing most of the amino acid sequences of L4a and LEb. Of the peptides, A5−76 [mouse laminin α5 928−939 (TSPDLFRLVFRY) in L4a] exhibited neurite outgrowth activity. Mutagenesis studies showed that Phe^{933} and Arg^{934} were involved in neurite outgrowth activity. Moreover, inhibition assays using anti-integrin monoclonal antibodies showed that neurite outgrowth on the α5 short arm was partially mediated by integrin α1β1. However, the antibodies to integrin α1 and β1 did not inhibit neurite elongation on the A5−76 peptide. These results suggest that in addition to cellular interactions with the active site in the L4a domain, the binding of integrin α1β1 seems to modulate neurite elongation on the short arm of α5.



Laminins make up a family of heterotrimeric glycoproteins that assemble into basement membranes. They are involved in not only cell−matrix but also matrix−matrix interactions in basal laminae. All laminins are composed of three subunits, designated α, β, and γ chains. Of the three subunits, α chains play pivotal roles in the biological activities of laminins. Until now, five α, three β, and three γ chains have been identified. To date, 18 different laminin heterotrimers have been found to be synthesized and secreted by various cells.[1] Of the five laminin α chains, laminin α5 is widely expressed in fetal and adult tissues.[2,3] The α5 chain associates with either β1 or β2 and γ1 or γ3 to form laminin-511 (α5, β1, γ1), laminin-521 (α5, β2, γ1), and laminin-523 (α5, β2, γ3).[2,4] Targeted disruption of laminin α5 in *Lama5*−/− mice results in embryonic lethality with defects in neural tube closure, digit separation, placentation, glomerulogenesis, lung lobe separation, intestinal smooth muscle development, tooth morphogenesis, and submandibular gland epithelial morphogenesis.[5−11] Additional experiments with grafts from *Lama5*−/− embryos have shown that laminin α5 is required for hair follicle development[12,13] and lung maturation.[14] These results suggest that laminin α5 plays multiple roles in the development and establishment of tissue architecture.

All five laminin α chains have a laminin type globular (LG) domain at their C-termini, which consists of five homologous domains (LG1−LG5). Adhesion of cells to laminins is mainly mediated by the binding of various receptors, including integrin and non-integrin receptors, to the LG domain.[1] Several groups have attempted to identify binding sites for integrins, syndecans, and α-dystroglycan within LG domains of laminin α chains.[15,16] The binding sites for Lu/B-CAM and α3β1/α6β1 integrins are localized on LG1−LG3,[17,18] whereas α-dystroglycan binds primarily to the LG4−LG5 tandem.[19] Expression of a mutant α chain with the entire α5 LG domain replaced with the α1 LG domain had minimal ameliorative effects on the defects observed in *Lama5*−/− embryos,[20] suggesting that the α5 LG domain harbors most of the functionality of the α5 chain with regard to mediating developmental processes. Furthermore, molecular dissection of the laminin α5 LG domain in vivo revealed that the LG1−LG2 tandem harbors most of the functionality of the α5 LG domain, whereas the α5 LG3−LG5 tandem is involved in glomerular filtration and postsynaptic maturation.[21,22]

α chains differ considerably in the length of their N-terminal short arms. These short arms consist of rodlike elements on the

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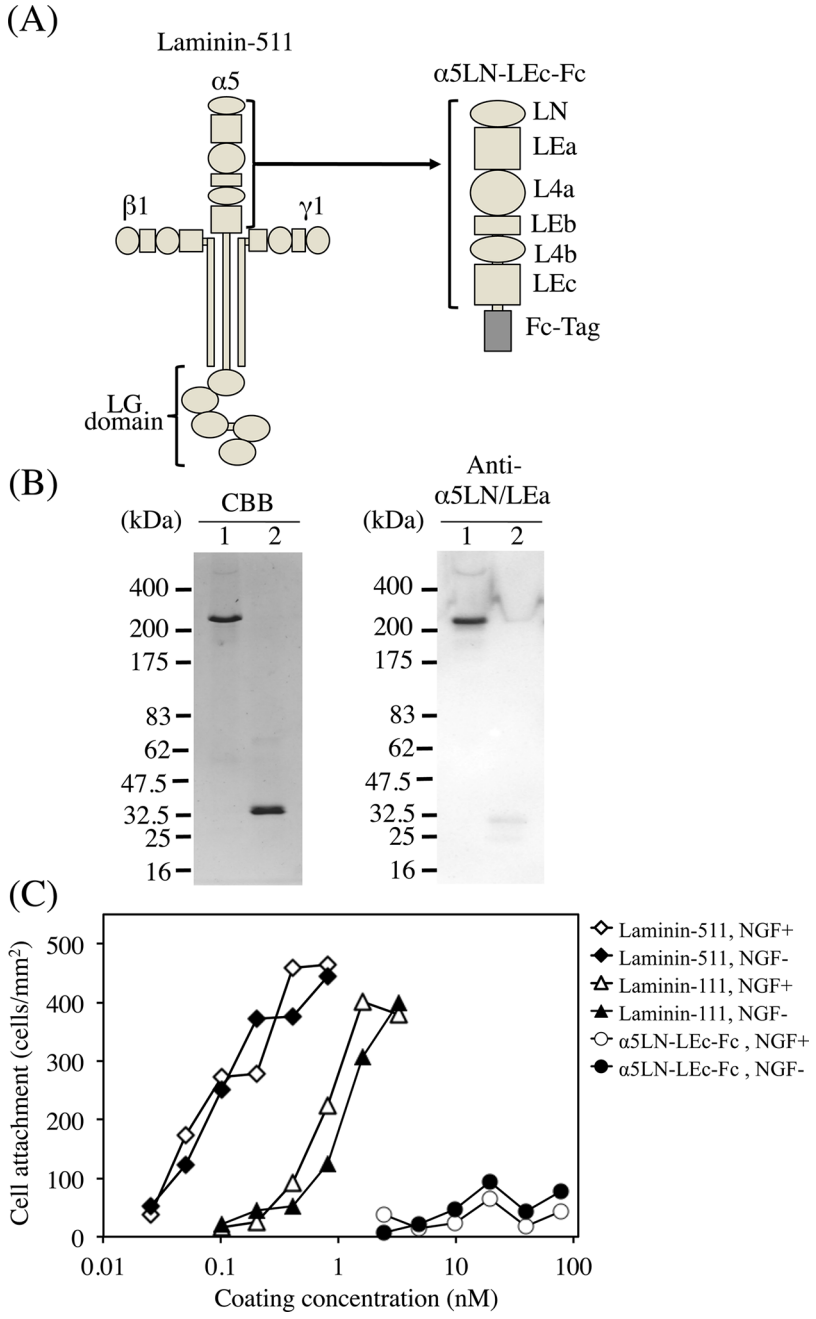

Figure 1. Attachment of rat PC12 cells to the short arm of laminin α5. (A) Diagram of laminin-511 and the α5 short arm. The recombinant protein consisted of the α5 short arm fused with a dimerizing human IgG_1 Fc tag. (B) α5LN-LEc-Fc and Fc purified from conditioned medium of the transfectants were subjected to SDS−PAGE on a 5 to 15% gel under reducing conditions. α5LN-LEc-Fc (lane 1) and Fc (lane 2) were stained with Coomassie Brilliant Blue (left). Recombinant proteins were immunoblotted with the anti-α5LN/LEa antibody (right). Molecular mass standards are indicated. (C) After incubation for 1 h, the attached cells were stained with a 0.2% crystal violet aqueous solution in 20% methanol for 10 min. PC12 cells readily attached to laminin-511 and -111 but did not adhere well to the α5LN-LEc-Fc protein. Similar results were obtained in three independent experiments.

basis of tandem arrays of laminin-type epidermal growth factor-like (LE) modules and globular domains.[23] The globular domains are also classified as laminin N-terminal (LN) domain and L4 modules. Of the laminin α chains, α5 has the longest short arm.[24] It consists of three globular and three LE modules [LN-LEa-L4a-LEb-L4b-LEc (see Figure 1A)]. Previous in vitro studies have shown that the short arm of the α5 chain harbors sites for cell adhesion and self-assembly. The LN domain binds to intgerin $\alpha 3\beta 1$ and heparin/heparan sulfate.[25] Our previous studies using a set of overlapping peptides identified two cell adhesive sequences on the α5 LN module.[26] Moreover, two RGD sequences present in the L4b domain bind β1 and $\alpha V\beta 3$ integrins,[27] and polymerization of laminins is mediated by their LN domains.[28,29] Recently, Hussain et al showed that Glu^{202} on the surface loop of the LN domain was required for the formation of a laminin network.[30] The same domain architecture is also found in netrin, an important class of axon guidance molecules.[31] Because laminin α5 is also distributed in neuronal tissues,[32] the short arm of α5 could be involved in neuronal elongation. However, the biological

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functions of the α5 short arm are still not completely understood.

Here we found that the short arm of laminin α5 exhibited neurite outgrowth activity. To narrow the active region within the laminin α5 short arm, we produced a series of deleted recombinant proteins and examined their neurite outgrowth activities. We also synthesized a set of partially overlapping peptides covering most of the amino acid sequence of the active region within the laminin α5 short arm. Amino acid residues involved in neurite outgrowth activity were identified using these synthetic peptides. We also characterized cellular interactions with the α5 short arm using function-blocking receptor antibodies.

EXPERIMENTAL PROCEDURES

Cell Culture. Human embryonic kidney (HEK293) and rat pheochromocytoma (PC12) cells were purchased from American Type Culture Collection (Manassas, VA). HEK293 cells were maintained in DMEM containing 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin. PC12 cells were cultured in DMEM containing 7.5% horse serum (Invitrogen, Carlsbad, CA), 7.5% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin.

Antibodies and Proteins. Polyclonal antibodies against laminin α5 domain LN/LEa and L4b were gifts from Dr. Sasaki (Oita University School of Medicine, Oita, Japan).[27,29] Hamster monoclonal antibodies against rat integrin α1 (Ha31/8), α2 (Ha1/29), and β1 (Ha2/5) were purchased from BD Biosciences (Franklin Lakes, NJ). Mouse monoclonal antibodies against rat integrin α3 were purchased from the Developmental Studies Hybridoma Bank (Iowa City, IA). Mouse laminin-111 was purchased from BD Biosciences. Recombinant laminin-511 was produced in HEK293 cells triply transfected with mouse laminin α5, β1, and γ1 chains and purified as previously described.[17] Recombinant human IgG_1 Fc (Fc) was produced and characterized as described previously [17]

Construction of Expression Vectors. An expression vector containing deleted short arm segments of laminin α5 fused with a Fc tag was prepared as follows. A cDNA clone encoding full-length mouse laminin α5 was provided by J. Miner (Washington University School of Medicine, St. Louis, MO). A human IgG_1 Fc expression vector was prepared as described in our previous study.[17] To generate α5LN-LEc-Fc containing the LN−LEc domains, we used the EcoRV site in the sequence encoding the L4b domain. The DNA segment encoding the LN−L4b domains and part of the LEb domain was isolated from the cDNA clone encoding full-length laminin α5 digested with EcoRI and EcoRV. The DNA fragment encoding the L4b−LEc domains was also amplified with sense (5′-GGAATTCACCCAGCGAGGTGATATCTTCATT-3′) and antisense (5′-GCTCTAGACCTTCACAGTGTATGCCATGGCC-3′) primers and digested with EcoRV and XbaI. Both DNA fragments were inserted into the EcoRI and XbaI sites of the human IgG_1 Fc expression vector mentioned above. To generate α5LN-LEa-Fc (LN−LEa domains) and α5LN-LEb-Fc (LN−LEb domains), we used two XbaI sites in the sequence encoding the LN domain and human IgG_1 Fc expression vector. The DNA fragment encoding the part of the LN and LEa or LEb domains was amplified with sense (5′-CGGAATTCTGGAGCGGTTTGGACCTCGGACTC-3′) and antisense (5′-CGTCTAGACCGCACCTGCAGGCACCTGTCTTTGG-3′ or 5′-CGTCTAGACCAGAGTTCCCACAGCGCTCTGTGGC-3′) primers. Each DNA fragment was digested with XbaI and inserted into the XbaI site of the α5LN-LEc-Fc expression vector. For all polymerase chain reactions, KOD plus DNA polymerase (TOYOBO, Osaka, Japan) was used according to the manufacturer's instructions.

Expression and Purification of Recombinant Proteins. HEK293 cells were transfected with laminin α5 N-terminal expression vectors using Lipofectamine 2000 (Invitrogen), and stable clones were selected using 100 μg/mL Zeocin (Invitrogen). The conditioned media were prepared as follows. The confluent cells were incubated in serum-free DMEM for 4 days. The conditioned media were harvested and clarified by sequential centrifugation at 500 rpm for 10 min and 10000 rpm for 20 min. The collected media were precipitated with ammonium sulfate at 80% saturation. The resulting precipitates were collected by centrifugation at 10000 rpm for 30 min and then dissolved in and dialyzed against PBS(−). The 30-fold concentrated media were used for purification. The recombinant proteins were purified from the conditioned media by Protein-A Sepharose (GE Healthcare, Piscataway, NJ). The eluted fractions were pooled and dialyzed against PBS(−).

Electrophoretic Analysis and Immunoblotting. SDS−PAGE was conducted on 5 to 15% gels under reducing conditions. Proteins were visualized by Coomassie Brilliant Blue staining. For immunoblotting, sample proteins were separated by SDS−PAGE and transferred onto PVDF membranes. Proteins on the membrane were reacted with primary antibodies followed by incubation with a secondary antibody conjugated with horseradish peroxidase (GE Healthcare). Bound antibodies were visualized with ECL detection regents (GE Healthcare).

Synthetic Peptides. All peptides were manually synthesized by the 9-fluorenylmethoxycarbonyl (Fmoc)-based solid-phase method with a C-terminal amide as described previously.[33] The respective amino acids were condensed manually in a stepwise manner using diisopropylcarbodiimide-*N*-hydroxybenzotriazole on a Rink amide resin (Merck KGaA, Darmstadt, Germany). The resulting protected peptide resins were deprotected and cleaved from the resin using a trifluoroacetic acid/thioanisole/*m*-cresol/ethanedithiol/Milli-Q water mixture (80:5:5:5:5, v/v) at 20 °C for 3 h. Crude peptides were precipitated and washed with ethyl ether and then purified by reverse-phase high-performance liquid chromatography (HPLC) (using a C18 column with a gradient of Milli-Q water with acetonitrile containing 0.1% trifluoroacetic acid). The purity and identity of the synthetic peptides were confirmed by electrospray ionization mass spectrometry, at the Central Analysis Center of the Tokyo University of Pharmacy and Life Sciences.

Cell Adhesion Assays. Cell adhesion assays using recombinant proteins were performed as described previously.[17] Briefly, 96-well plates (Nunc, Roskilde, Denmark) were incubated with recombinant proteins at 4 °C overnight and then blocked with PBS(−) containing 1% bovine serum albumin for 1 h at 37 °C. PC12 cells treated with or without NGF were suspended in serum-free DMEM at a density of 4×10^5 cells/mL; 50 μL of the cell suspension was added to wells coated with the proteins. After incubation at 37 °C for 1 h, the attached cells were fixed with 4% formaldehyde and stained with Diff-Quik (International Regents Corp., Kobe, Japan). The stained cells were counted under a microscope.

Neurite Outgrowth Assay Using Recombinant Proteins and Synthetic Peptides. Neurite outgrowth assays using recombinant proteins and synthetic peptides were

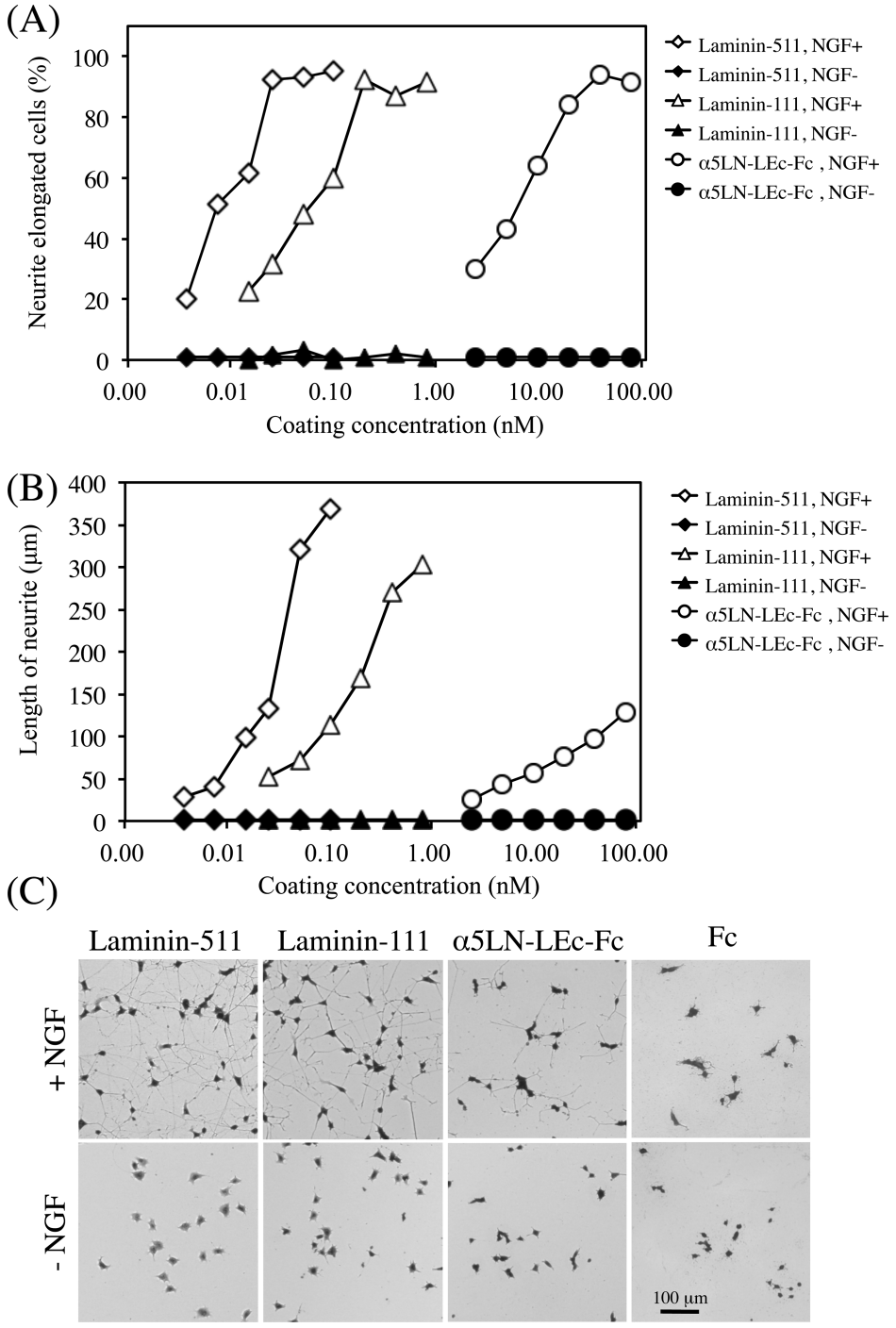

Figure 2. Neurite outgrowth from PC12 cells on the short arm of laminin α5. (A) Percentage of PC12 cells on the different proteins with neurites. Triplicate experiments gave similar results. (B) Length of neurites on the PC12 cells cultured on the recombinant proteins. (C) Morphology of PC12 cells on the various proteins, as indicated. Ninety-six-well plates were coated with laminin-511 (0.1 nM), laminin-111 (1.0 nM), α5LN-LEc-Fc (40.0 nM), and Fc (40.0 nM). PC12 cells treated with (top) or without (bottom) NGF were added to the wells. After incubation for 24 h, the cells were stained with Diff-Quik and photographed under a microscope. The bar is 100 μm.

performed in 96-well plates (Nunc). The 96-well plates were coated with the proteins as described above. Ten micrograms of peptides in Milli-Q water (50 μL) was added to each well, followed by drying overnight. After being coated with proteins or peptides, the plates were washed with DMEM/F12 (Invitrogen). PC12 cells were primed with 100 ng/mL nerve growth factor (NGF, Roche Diagnostics GmbH, Mannheim, Germany) for 24 h prior to the assay. The PC12 cells were then collected by agitation, allowed to recover in the cultured medium for 30 min at 37 °C in 5% CO_2, and then washed three times with DMEM/F12. After being washed, cells were resuspended in DMEM/F12 containing 100 μg/mL transferrin (Sigma, St. Louis, MO), 20 nM progesterone (Sigma), 30 nM Na_2SeO_3 (Wako, Osaka, Japan), 5 μg/mL insulin (Invitrogen), and 100 ng/mL NGF. The cells were added to 96-well plates at a density of 3.0×10^3 cells/well. After incubation at 37 °C for 24 h in 5% CO_2, the cells were fixed with 20% formalin and then stained with crystal violet or Diff-Quik. Images captured

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using Biozero (Keyence, Osaka, Japan) were imported into ImageJ for measuring the length of neurites and for layout. In each well (three fields), the cells with >20 μm long neurites were counted as neuronal cells. Inhibition assays using anti-integrin monoclonal antibodies were performed on the basis of the neurite outgrowth assays described above. PC12 cells were suspended in DMEM/F12 as described above and preincubated with 10 μg/mL monoclonal antibodies against different integrins at room temperature for 10 min. The preincubated cells were transferred to plates coated with the recombinant proteins and synthetic peptides. After incubation at 37 °C for 24 h, the cells were stained, and the length of neurites was measured as described above.

RESULTS

PC12 Cell Attachment Activities of Recombinant Proteins. A recombinant protein that contained the entire short arm of mouse laminin α5 was prepared using a mammalian cell expression system. The short arm of mouse laminin α5 was fused with the Fc tag, the human IgG_1 Fc portion (Figure 1A). α5LN-LEc-Fc exhibited a single band at 250 kDa via SDS−PAGE under reducing conditions (Figure 1B). The purified protein reacted with the anti-mouse laminin α5 domain LN/LEa polyclonal antibody. Although we tried to produce a monomeric short arm fused with a six-His tag, it was not expressed in the mammalian cell expression system. The α5 short arm, dimerized via the Fc tag, was efficiently produced and stable in the culture media. Recombinant mouse laminin-511 was prepared as described in our previous study.[17] Previous studies showed that the α5 short arm exhibits cell attachment activity for HT1080 fibrosarcoma cells.[25] We evaluated the cell attachment activity of the recombinant proteins using rat pheochromocytoma PC12 cells treated with or without NGF. PC12 cells readily attached to laminin-511 in a dose-dependent manner (Figure 1C). Treatment with NGF slightly increased the extent of attachment of cells to laminin-111. The cells treated with NGF often exhibited a spread shape with pseudopodia (data not shown). On the other hand, even though the cells were treated with NGF, PC12 cells did not attach well to α5LN-LEc-Fc.

Neurite Outgrowth Activities of Recombinant Proteins. Previous studies showed that laminin-111, -211/221, and -332 stimulate neurite outgrowth activity of PC12 and dorsal root ganglion cells.[34−36] In this study, we examined the neurite outgrowth activity of laminin-511 and -111 and α5LN-LEc-Fc. Laminin-511 promoted neurite elongation by NGF-treated PC12 cells more effectively than did laminin-111 (Figure 2A). Neurite outgrowth induced by laminin-511 was also observed at the lower protein concentrations active for cell attachment. On the other hand, although PC12 cells did not attach to α5LN-LEc-Fc, they elongated neurites on substrata coated with it. The higher concentrations of α5LN-LEc-Fc were required to promote neurite outgrowth activity, though the length of neurites on α5LN-LEc-Fc was ∼30% of that on laminin-511 (Figure 2B). Because the 96-well plates were not blocked with bovine serum albumin, the cells seemed to attach to the culture substrate. The morphology of NGF-treated PC12 cells on α5LN-LEc-Fc was similar to those on laminin-511 (Figure 2C). These results indicate that although the α5LN-LEc protein did not exhibit cell attachment activity, there were active sequences modulating neurite elongation.

Mapping of the Neurite Outgrowth-Promoting Sites in the Short Arm of Laminin α5. To narrow the region involved in neurite outgrowth activity, two deletion mutant proteins (Figure 3A) were produced in HEK293 cells. The

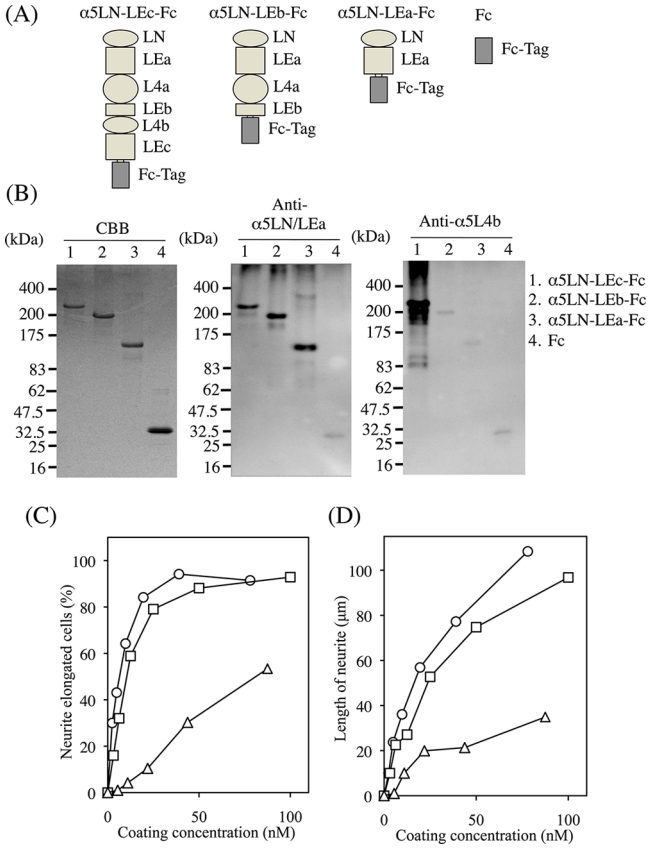

Figure 3. Neurite outgrowth of rat pheochromocytoma cells, PC12 cells, on the truncated recombinant proteins. (A) Diagram of the deletion mutant proteins designed to narrow the domains involved in neurite outgrowth activity. The deletion mutant proteins were fused with an IgG_1 Fc tag. (B) The mutant proteins purified from conditioned media of HEK293 transfectants were subjected to SDS−PAGE on a 7.5% gel under reducing conditions: lane 1, αLN-LEc-Fc; lane 2, α5LN-LEb-Fc; lane 3, α5LN-LEa-Fc; lane 4, Fc. Protein was stained with Coomassie Brilliant Blue. (C) Percentage of PC12 cells with neurites on the recombinant proteins. 96-well plates were coated with various amounts of α5LN-LEc-Fc (○), α5LN-LEb-Fc (□), and α5LN-LEa-Fc (△). PC12 cells treated with NGF were incubated in the wells. Triplicate experiments gave similar results. (D) Length of neurites on the PC12 cells cultured on the recombinant proteins. Images captured using a microscope were imported into ImageJ for the measurement of neurite length.

purified proteins were subjected to SDS−PAGE under reducing conditions (Figure 3B, left). The mutant proteins migrated at molecular weights predicted from their cDNA sequences. The purified proteins reacted with the anti-mouse laminin α5 domain LN/LEa polyclonal antibody (Figure 3B, middle). The recombinant full-length short arm fusion protein (α5LN-LEc-Fc) reacted with the anti-mouse laminin α5 domain L4b antibody, but as expected, α5LN-LEa-Fc and α5LN-LEb-Fc did not (Figure 3B, right). These results suggest that the recombinant proteins are properly produced.

These proteins were coated onto wells of 96-well plates. The coating efficiency was examined using an enzyme-linked immunosorbent assay with the anti-laminin α5 domain LN/LEa polyclonal antibody; the coating efficiency of the deletion mutant proteins was not different from that of the full-length

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protein (data not shown). The deletion of α5 domains L4b and LEc did not affect the neurite outgrowth activity of PC12 cells; as for α5LN-LEc-Fc, ∼80% of the cells exhibited neurite outgrowth at 20 nM α5LN-LEb-Fc (Figure 3C). Similarly, the lengths of neurites increased in a dose-dependent manner (Figure 3D). However, the additional deletion of α5L4a and LEb domains, leaving only LN and LEa domains, significantly decreased neurite outgrowth activity (Figure 3C,D). These results indicate that the major neurite outgrowth-promoting sites are located in α5L4a and/or LEb domains. As shown in Figures 2 and 3, the length of neurite readily responds to the alteration of the substratum at high coating concentrations better than the percentage of neurite-elongated cells. Hereafter, neurite length was measured to detect moderate effects of peptides and antibodies.

Neurite Outgrowth Activity of Synthetic Peptides Derived from Domains L4a and LEb of Laminin α5. To attempt to identify active sites in the α5L4a-LEb segment, we prepared 78 peptides from these domains (Figure 4) to screen

Figure 4. Sequences and peptides from the L4a and LEb domains of laminin α5. The sequence was derived from mouse laminin α5 (UniProtKB/Swiss-Prot entry Q61001). Locations of peptides are indicated by dotted arrows. The active peptide sequence in neurite outgrowth assays is denoted with a bold arrow.

for neurite outgrowth activity. These peptides were dissolved in Milli-Q water, coated on plastic culture wells, and tested in neurite outgrowth assays. Only peptide A5−76 (TSPDLFRLVFRY), derived from the L4a domain, promoted neurite outgrowth (Figure 5A). The 77 remaining peptides did not promote neurite outgrowth in PC12 cells (data not shown).

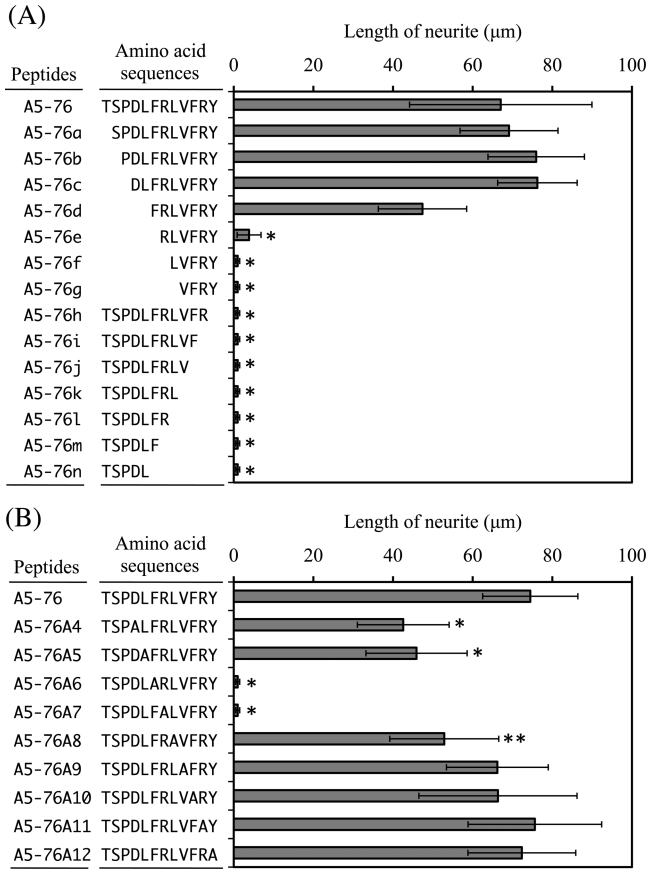

Figure 5. Neurite outgrowth of PC12 cells on A5−76 and its mutated peptides. Neurite outgrowth activity of the truncated N-terminal and C-terminal peptide derivatives of A5−76 (A) and the alanine-substituted A5−76 peptides (B). Ten micrograms of peptides was coated on 96-well plates. PC12 cells (3000 cells/well) were seeded in the plates. After incubation for 24 h, cells were fixed and stained. The lengths of neurites were determined as described in Experimental Procedures. Each column represents the mean ± the standard deviation. $^{*}p < 0.01$; $^{**}p < 0.05$.

Core Sequence of the A5−76 Peptide Imparting Neurite Outgrowth Activity. Because A5−76 exhibited neurite outgrowth activity, the structural requirements were examined using systematically N- and C-terminally truncated peptide variants (Figure 5A). A5−76e (RLVFRY), with a deletion of six N-terminal residues from A5−76, lacked neurite outgrowth activity. Likewise, deletion of the C-terminal tyrosine from A5−76 abolished the activity. These results indicate that the seven-amino acid sequence FRLVFRY is critical for neurite outgrowth activity of the A5−76 peptide. Furthermore, we synthesized Ala-substituted A5−76 peptides to identify crucial amino acid residues for neurite outgrowth activity (Figure 5B). When the Phe and Arg at positions 6 and 7, respectively, were substituted with Ala, the neurite outgrowth activity of A5−76 was decreased. Neurite outgrowth activity was also significantly decreased on three peptides that were substituted with Ala at positions 4, 5, and 8.

Inhibitory Effects of Function-Blocking Antibodies to Integrins on Neurite Outgrowth. Expression of integrin α1β1 in PC12 cells is regulated by NGF and involved in neurite outgrowth.[37] Integrin α1β1 binds to the α chain short arm of laminin-111.[38] We also showed that adhesion of hepatocellular carcinoma cells to laminin-511 was mediated via integrin

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$\alpha1\beta1$.[39] To examine whether neurite outgrowth on α5LN-LEc-Fc was mediated via integrin $\alpha1\beta1$, we applied function-blocking antibodies in the neurite outgrowth assay. As shown in Figure 6, neurite outgrowth on α5LN-LEc-Fc was partially

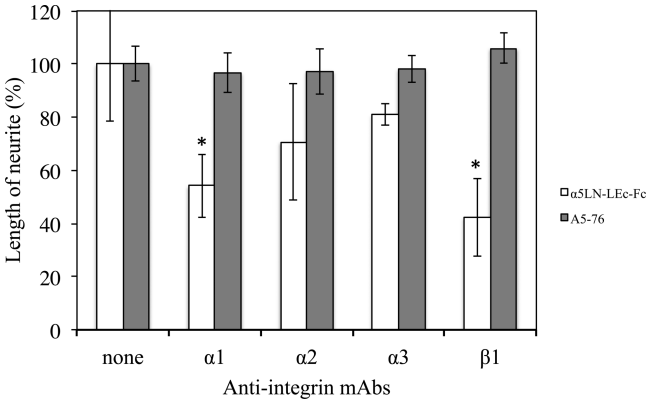

Figure 6. Inhibitory effects of integrin-blocking antibodies on neurite outgrowth on α5LN-LEc-Fc and the A5−76 peptide. NGF-treated PC12 cells preincubated with the function-blocking antibody to integrins were added to recombinant protein- or peptide-coated wells. After incubation for 24 h, the cells were stained, and the lengths of neurites were measured as described in Experimental Procedures. Each column represents the mean ± the standard deviation. *$p < 0.01$.

inhibited by anti-integrin $\alpha1$ and $\beta1$ monoclonal antibodies. The results indicated that although the binding of integrin $\alpha1\beta1$ to α5LN-LEc-Fc was not enough for cell attachment activity, the interaction with integrin $\alpha1\beta1$ was required for neurite elongation. On the other hand, neurite outgrowth on the A5−76 peptide was not inhibited by the anti-integrin antibodies, suggesting that non-integrin receptors mediate the residual neurite outgrowth activity of α5LN-LEc-Fc after blocking the integrins.

DISCUSSION

The successful production of recombinant protein containing the entire short arm of laminin $\alpha5$ dimerized with the Fc tag allowed us to examine the function of this region. Previous studies showed that adhesive sites were located in the short arm of laminin $\alpha5$.[25−27] Although HT1080 (human fibrosarcoma) and HuH-7 (human hepatocellular carcinoma) cells could attach to α5LN-LEc-Fc (unpublished data), PC12 cells treated with and without NGF did not attach to α5LN-LEc-Fc. Adhesion of cells to α5LN-LEc-Fc seems to depend on the expression and activation level of cell surface receptors, such as integrins and proteoglycans.

Laminin-511 exhibited cell attachment activity at lower concentrations than did laminin-111. Similarly, the neurite outgrowth activity of laminin-511 was stronger than that of laminin-111 and α5LN-LEc-Fc. These results suggest that efficient cell attachment is a requirement for neurite elongation. In this study, we also showed that the A5−76 peptide, derived from domain L4a of $\alpha5$, exhibited only neurite outgrowth activity. Our previous report showed that seven peptides derived from the $\alpha5$ C-terminal LG domain exhibited not only cell attachment activity but also neurite outgrowth activity.[40] Compared with the $\alpha5$ short arm, the LG domain of $\alpha5$ harbors many neurite outgrowth-promoting sites. Therefore, the short arm of the $\alpha5$ chain might be expected to be less active for neurite outgrowth than the fully assembled laminin-511, as we found. The lengths of neurites on α5LN-LEc-Fc were also shorter than that on laminin-511. Laminin-511 promotes not only cell attachment but also cell migration;[41] the velocity of neurite elongation on α5LN-LEc-Fc may be slower than that on laminin-511.

A neurite outgrowth-promoting site on the L4a domain of laminin $\alpha5$ was identified using a combination of recombinant proteins and synthetic peptides. We showed that ^{933}FRLVFRY^{939} is an active core sequence in the α5L4a domain involved in neurite outgrowth activity. Our results also show that A5−76h (TSPDLFRLVFR), with a deletion of Tyr from A5−76, almost completely lacked neurite outgrowth activity. In contrast, A5−76A12 (Tyr → Ala mutant of A5−76) showed neurite outgrowth activity comparable to that of A5−76. The results showed that the side chain of the Tyr residue did not directly contribute to the activity. They also suggested that the carboxyl-terminal amide Ala of A5−76A12 contributed to neurite elongation. However, Ala substitution does not usually lead to additional activity on peptides, and the carboxyl-terminal amide is occupied as a peptide bond in the native molecule. Therefore, the carboxyl-terminal amide Ala is unlikely to contribute neurite outgrowth activity. To clarify the difference between both peptides, we analyzed circular dichroism spectra of A5−76, A5−76h, and A5−76A12 (data not shown). Although A5−76A12 maintained an α helical structure similar to that of A5−76, A5−76h exhibited an unstable α helical structure. It may therefore be necessary to maintain an α helical structure in the C-terminus of A5−76 for neurite outgrowth activity. We also found that Phe^{933} and Arg^{934} in this sequence are critical for neurite elongation. In human laminin $\alpha5$, Arg^{934} is replaced with Trp. Therefore, Phe^{933} is likely more critical for neurite outgrowth. In the future, we will need to produce the laminin $\alpha5$ short arm mutated at Phe^{933} and Arg^{934} and examine neurite outgrowth on the mutant proteins. In our previous studies, we showed that several other peptides derived from laminin chains exhibit neurite outgrowth activity.[40,42−46] We aligned 36 peptide sequences that exhibited neurite outgrowth activity. Of the peptides, AG26 [SPNGLLFYLASNG (from laminin $\alpha1$)] and A5G73 (RNRLHLSMLVRP) contained the LXXL motif in their sequences. In this study, A5−76A5 and A5−76A8 exhibited partially decreased neurite outgrowth activity. These results suggest that the LXXL motif promotes neurite outgrowth. The alignments did not reveal any longer significant homologies. Thus, different parts of the $\alpha5$ chain may have specific neurite outgrowth promoting roles in neuronal tissue.

Inhibition assays using anti-integrin antibodies showed that the neurite outgrowth on α5LN-LEc-Fc was partially mediated by integrin $\alpha1\beta1$. However, the anti-integrin $\alpha1$ and $\beta1$ antibodies incompletely inhibited neurite outgrowth on α5LN-LEc-Fc, suggesting that other cell surface receptors are involved in neurite elongation. At the nerve terminal surface, SV2, a synaptic vesicle protein, forms a complex with $\alpha5$-containing laminin.[47] SV2 is also expressed in hippocampal neurons and PC12 cells.[48] Although there is no evidence that SV2 can bind to the $\alpha5$ short arm, it is a reasonable candidate molecule for the binding to A5−76.

In some neurite outgrowth assays, EDTA or heparin was also added to the culture media. After being cultured for 24 h, PC12 cells detached and died in the presence of EDTA. On the other hand, we observed inhibitory effects of heparin on neurite elongation. Because heparin may affect the viability of PC12 cells (as with EDTA), we could not clarify whether heparin

directly inhibited neurite elongation on A5−76 peptides. Our previous study showed that several peptides derived from the LG domain of laminin α2 exhibit neurite outgrowth activity.[46] Furthermore, EDTA and/or heparin inhibited adhesion of cells to these peptides. Although we do not present the data here, we observed that EDTA and heparin inhibited the adhesion of human fibrosarcoma cells to A5−76 peptides. Therefore, it remains possible that neurite elongation of PC12 cells on A5−76 is mediated through a cell surface heparin sulfate proteoglycan.

In summary, we have shown that there is a neurite outgrowth-promoting site on the L4a domain of laminin α5. Although a peptide containing the site exhibited neurite outgrowth activity, the lengths of neurites on the peptide were shorter than on the fully assembled laminin-511 trimer. We have reported that chitosan membranes conjugated with peptides can regulate cellular responses.[49,50] Chitosan membranes conjugated with A99a (ALRGDN) or AG73 (RKRLQVQLSIRT), peptides derived from laminin α1, promote neurite outgrowth.[51] Recently, we showed that although the EF1zz peptide derived from laminin α1 exhibits cell attachment activity but not neurite outgrowth activity, the peptide is still able to enhance neurite elongation induced by AG73.[52] A5−76, together with the cell adhesive peptides linked to chitosan membranes, may mimic the neurite outgrowth activities of laminin-511.

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Notes

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ABBREVIATIONS

NGF, nerve growth factor; LE domain, laminin epidermal growth factor-like domain; LG domain, laminin-type globular domain; PBS(−), Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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