Identification of Neurite Outgrowth Active Sites on the Laminin α4 Chain G Domain†

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ABSTRACT: The laminin α4 chain is widely distributed in various mesodermal tissues, including the perineurium of peripheral nerves, dorsal root ganglion (DRG), skeletal muscle, and capillaries, and plays important roles in synaptic specialization at the neuromuscular junction and in microvascular formation. The C-terminal globular domain (G domain) of the laminin α4 chain was previously found to be critical for heparin binding and cell attachment activity. Here, we focused on neurite outgrowth activity of the laminin α4 chain G domain. We found that the recombinant α4 chain G domain protein (rec-α4G) promoted neurite outgrowth of rat pheochromocytoma PC12 cells. When 114 overlapping synthetic peptides that covered the entire G domain were tested for neurite outgrowth activity, nine peptides were active, but the 105 remaining peptides did not exhibit activity. Three of the nine active peptides, A4G6 (LAIKNDNLVYVY), A4G20 (DVISLYNFKHIY), and A4G107 (VIRDSNVVQLDV), strongly promoted neurite outgrowth of PC12 cells. A4G107 was found to form amyloid-like fibrils in Congo red, X-ray, and electron microscopy analyses. We also synthesized cyclic peptides to evaluate their conformational requirements. Cyclic peptide A4G82X (cyc-A4G82X;TLFLAHGRLVFX, where X is norleucine) significantly enhanced neurite outgrowth activity, but the rest of the cyclic peptides eliminated the activity. The A4G82 sequence is located on the loop region, suggesting that the activity of A4G82 is required for a loop conformation. These peptides also exhibited neurite outgrowth activity with dorsal root ganglion (DRG) explants and with DRG cells from E14.5 mouse embryos, indicating that they are active in both neuronal cell lines and native neuronal cells. Taken together, the data suggest that the peptides from the laminin α4 chain G domain promote neurite outgrowth activity via a specific conformation.

Laminins are a family of heterotrimeric extracellular matrix glycoproteins and are a major component of basement membranes. Laminins consist of α, *β*, and *γ* chains, and at least 15 isoforms have been identified with various combinations of five α, three *β*, and three *γ* chains (*1*−*4*). The laminin isoforms appear to have tissue-specific distributions and have various biological functions, including cell adhesion, neurite outgrowth, tumor metastasis, and angiogenesis (*1*, *4*). The functions of individual chains and isoforms have been elucidated from studies on genetic diseases and by targeted gene disruption in mice (*5*−*11*).

Laminins promote neurite outgrowth of peripheral and central nervous system neurons and many neuronal cell lines (*12*). Laminins have potential as a directional cue for migrating axons (*13*, *14*) and an important guidance molecule for developing axons (*15*). Several peptides derived from laminins have been found to promote neurite outgrowth (*16*−*19*). Large globular domains (G domain), consisting of LG1−LG5 modules, at the carboxyl terminus of laminin α chains have been suggested to play an important role in cellular interactions and biological activities (*20*). Several active peptides from the G domains of laminin α1and α2 chains have been shown to stimulate neurite outgrowth (*21*). We also reported that the LG4 and LG5 modules of the laminin α3 chain G domain exhibited neurite outgrowth activity (*22*).

The laminin α4 chain is a component of three laminins: laminin 8 (α4:*β*1:*γ*1), laminin 9 (α4:*β*2:*γ*1), and the recently identified laminin 14 (α4:*β*2:*γ*3) (*1*, *23*). The α4 chain lacks the N-terminal short arm and is expressed in cells of a mesenchymal origin, such as endothelial cells and adipocytes (*24*, *25*). The laminin α4 chain is widely distributed in various mesodermal tissues, as well as the perineurium of peripheral nerves, dorsal root ganglion (DRG), heart, kidney, skeletal muscle, capillaries, and endothelium (*26*−*28*). Previous work using laminin α4-deficient mice has indicated that

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the laminin α4 chain has a significant role in newly formed capillary basement membranes and transsynaptically coordinates pre- and postsynaptic differentiation (*29*, *30*). In the absence of laminin α4, the active zone and transmitter release site at the growth cone and the junctional fold at the muscle site are formed but are not precisely apposed to each other (*30*), suggesting that laminin α4 at the growth cone regulates neuronal outgrowth and neuromuscular junction formation. Moreover, loss of laminin α2 leads to upregulation of laminin α4 throughout muscle basal laminae (*28*, *31*). It was demonstrated that the laminin α4 G domain was proteolytically processed in cultured endothelial and schwannoma cells, and the LG4−LG5 module of the α4 G domain was released (*32*). The α4 G domain binds to heparin, sulfatides, and fibulins (*32*). Using the recombinant protein and a large set of overlapping peptides, active sequences of the laminin α4 G domain for cell attachment and syndecan-2 or -4 binding were identified (*33*−*35*).

In this paper, we describe a systematic screening of neurite outgrowth active sequences in the mouse laminin α4 G domain (residues 852−1816) using a recombinant protein and 114 overlapping synthetic peptides covering the G domain. We identified several peptides active for promoting neurite outgrowth of rat phenochromocytoma PC12 cells and mouse E14.5 embryo DRG explants and cells. We also evaluated their conformational requirement for activity.

MATERIALS AND METHODS

Recombinant Protein (rec-α4G) and Synthetic Peptides. A recombinant protein (rec-α4G) involving the mouse laminin α4 chain G domain (residues 852−1816) with a c-myc sequence at the C-terminus was expressed using *dhfr*-deficient CHO DG44 cells and purified with a heparin affinity column (HiTrap, Amersham Pharmacia Biotech, Uppsala, Sweden) and a gel filtration column (Superdex 200, Amersham Pharmacia Biotech), as previously described (*33*, *34*). The purity and amount of rec-α4G protein were monitored by 8% SDS−PAGE under reducing conditions. Pure fractions were combined, and the protein concentration was determined with the BCA assay (Pierce, Rockford, IL).

All peptides were synthesized manually using the 9-fluorenylmethoxycarbonyl (Fmoc)-based solid phase strategy and prepared in the C-terminal amide form as previously described (*34*, *36*). The purity and identity of the synthetic peptides were confirmed by reverse phase high-performance liquid chromatography (HPLC) and by fast atom bombardment mass spectral analysis at the GC-MS & NMR Laboratory, Graduate School of Agriculture, Hokkaido University. Two peptides (A4G98 and A4G99) were not soluble in aqueous solution and could not be purified by reverse phase HPLC. The A4G82 sequence contains a methionine residue at the C-terminus. To prevent oxidization during synthesis, the methionine residue was replaced with norleucine (A4G82X). Cyclic peptides were synthesized by adding cysteine to the carboxyl- and amino-terminal ends of each peptide (Table 2) and cyclizing under oxidative conditions (45:10:45 $CH_3COOH/DMSO/H_2O$ mixture), a modified method described previously (*37*).

Culture of Cells. PC12 cells (*38*) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY) containing 7.5% horse serum (Gibco), 7.5% fetal bovine serum (FBS, Gibco), 100 units/mL penicillin, and 100 μg/mL streptomycin (Gibco).

Table 1: Neurite Outgrowth Activity of Peptides on the Laminin α4 Chain G Domain[a]

peptide	sequence	neurite outgrowth[b]
A4G6	^{892}LAIKNDNLVYVY^{903}	+++
A4G20	^{1009}DVISLYNFKHIY^{1020}	++
A4G51	^{1261}LOPNGLLFYYTSG^{1273}	+
A4G82X	^{1514}TLFLAHGRLVFX$^{1525\,c}$	+
A4G93	^{1598}NVQITSVYSFSG^{1609}	+
A4G94	^{1611}LGNLOLNGASIT^{1622}	+
A4G95	^{1619}ASITSASOTFSVT^{1631}	+
A4G107	^{1729}VIRDSNVVOLDV^{1740}	+++
A4G116	^{1800}KAALVSGAVSINS^{1812}	+
AG73[d]	RKRLQVQLSIRT	+++

[a] The sequences and residues of the peptides were derived from the mouse laminin α4 chain (GenBank accession number AAC24725). [b] Neurite outgrowth-promoting activities of the peptides with PC12 cells were evaluated by the following subjective scale: +++, activity comparable to that on AG73; ++, activity apparent but weaker than that on AG73; +, activity significantly low. [c] The C-terminal residue of A4G82 is methionine (residue 1525). Because the methionine residue is easily oxidized during the synthesis, we replaced it with norleucine (X). This replacement did not change the HT1080 cell attachment activity (*34*). [d] AG73 was used as a positive control for neurite outgrowth activity (*21*, *42*).

Table 2: Sequences of Cyclic Peptides and Neurite Outgrowth Activity

peptide	sequence	neurite outgrowth[a]
A4G6	LAIKNDNLVYVY	+++
cyc-A4G6	CLAIKNDNLVYVYC	−
A4G20	DVISLYNFKHIY	++
cyc-A4G20	CDVISLYNFKHIYC	−
A4G82X	TLFLAHGRLVFX[b]	+
cyc-A4G82X	CTLFLAHGRLVFX[b]C	+++
A4G107	VIRDSNVVOLDV	+++
cyc-A4G107	CVIRDSNVVOLDVC	−
AG73[c]	RKRLOVOLSIRT	+++

[a] Neurite outgrowth-promoting activities of the peptides with PC12 cells were evaluated by the following subjective scale: +++, activity comparable to that on AG73; ++, activity apparent but weaker than that on AG73; +, activity significantly low; −, no activity. [b] C-Terminal methionine residue A4G82 was replaced with norleucine (X). [c] AG73 was used as a positive control for neurite outgrowth activity (*21*, *42*).

Neurite Outgrowth Assay Using a Recombinant Protein and Synthetic Peptides. The neurite outgrowth assay using proteins and peptides was performed in 96- and 24-well plates (Nunc), as previously described (*22*). The recombinant protein in 50 μL of 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, and 0.5 mM *N*-ethylmaleimide (buffer A) and laminin in 50 μL of Milli-Q water were added to 96-well plates and the plates incubated overnight at 4 °C. Synthetic peptides dissolved in 500 μL of Milli-Q water were added to 24-well plates and the plates dried overnight. Plates coated with proteins or peptides were washed with DMEM/F12 (Gibco). PC12 cells were primed with 100 ng/mL nerve growth factor (NGF, Roche Applied Science, Indianapolis, IN) 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), 20 nM progesterone (Sigma), 30 nM Na_2SeO_3

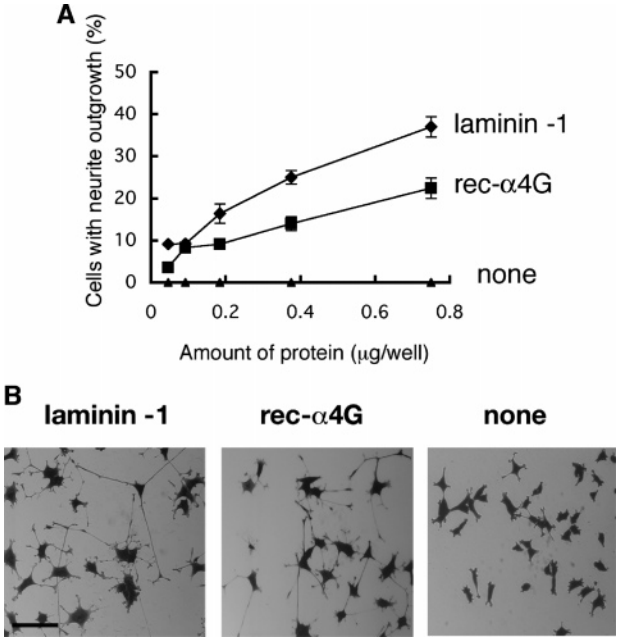

FIGURE 1: Neurite outgrowth activity of the recombinant rec-α4G protein in PC12 cells. (A) Various amounts of rec-α4G and laminin 1 were coated on 96-well plates, and PC12 cells (2×10^4 cells/well) were seeded in the plates. After being incubated for 24 h, cells were fixed and stained. The percentage of neurite outgrowth cells was determined as described in Materials and Methods. Data are expressed as mean ± SD of triplicate results. (B) The PC12 cells cultured on rec-α4G (1.2 μg/well) and laminin 1 (1.2 μg/well) for 24 h were stained with crystal violet and photographed on a microscope (200×). The scale bar is 100 μm.

(Wako, Osaka, Japan), 5 μg/mL insulin (Gibco), and 100 ng/mL NGF. The cells were added to 96- and 24-well plates at densities of 3.0×10^3 and 2.0×10^4 cells/well, respectively. The cells were incubated at 37 °C for 24 h and fixed with 20% formalin, and then the number of cells equal to or greater than twice the cell diameter was determined and averaged for each peptide tested.

A neurite outgrowth assay of DRG cells and explants was performed as described previously (*39*, *40*), with some modifications. DRG cells and explants were obtained from mouse E14.5 embryos, and other non-neuronal tissues were removed carefully. DRGs were collected in ice-cold PBS and incubated with 0.25% trypsin and DNase for 30 min at 37 °C. DRG cells collected by centrifugation (1000 rpm for 8 min). The cells and explants were incubated in 0.5% N2 supplement, 50 ng/mL NGF, and 1% PSM DMEM/F12 for 24 h on poly-L-lysine (10 μg/mL), and A4G6, A4G20, A4G82X, cyclic-A4G82X, and A4G107 (50 μg/mL) coated coverglasses. Neurite length was measured by confocal laser microscopy (Carl Zeiss LSM510 instrument).

Congo Red Binding Analysis. Peptides (1.0 mg/mL) in phosphate-buffered saline (PBS) and Congo red solution (100 μM in PBS) were mixed and incubated in disposable cuvettes for 24 h at room temperature. Absorption spectra were measured from 300 to 700 nm using a U-2000A UV−vis spectrophotometer (Hitachi Co. Ltd., Tokyo, Japan).

Congo Red Staining and Polarized Light Microscopy. A4G6, A4G20, and A4G107 were dissolved in PBS at a concentration of 5 mg/mL. The solutions were pipetted onto glass slides and dried overnight. The precipitates were stained with a 1% aqueous solution of Congo red for 1 h. After being

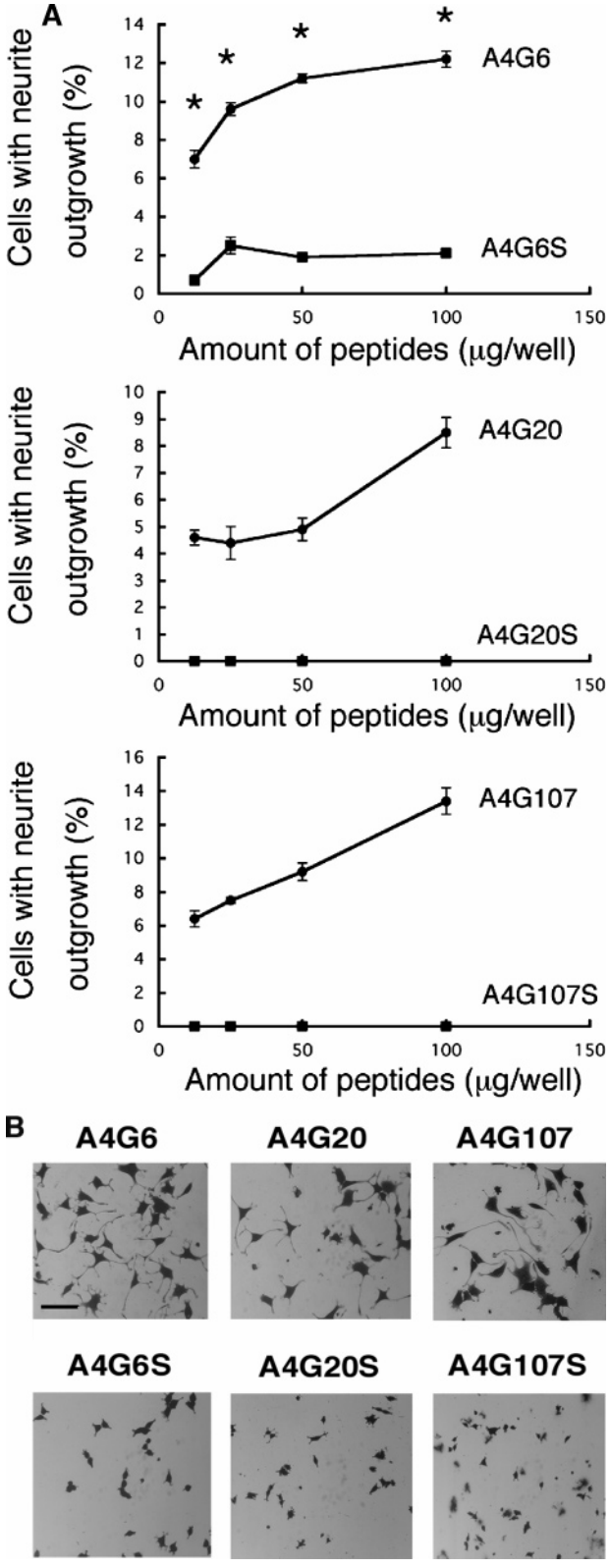

FIGURE 2: Neurite outgrowth of PC12 cells on synthetic peptides. (A) Various amounts of peptides were coated on 24-well plates. PC12 cells (2×10^4 cells/well) were seeded in the plates. After being incubated for 24 h, cells were fixed and stained. The percentage of neurite outgrowth cells was determined as described in Materials and Methods. Triplicate experiments gave similar results (asterisk indicates $p < 0.01$). (B) The PC12 cells were cultured on various peptides (50 μg/well) for 24 h, stained with crystal violet, and then photographed on a microscope (200×). S represents the scrambled peptide. The scale bar is 100 μm.

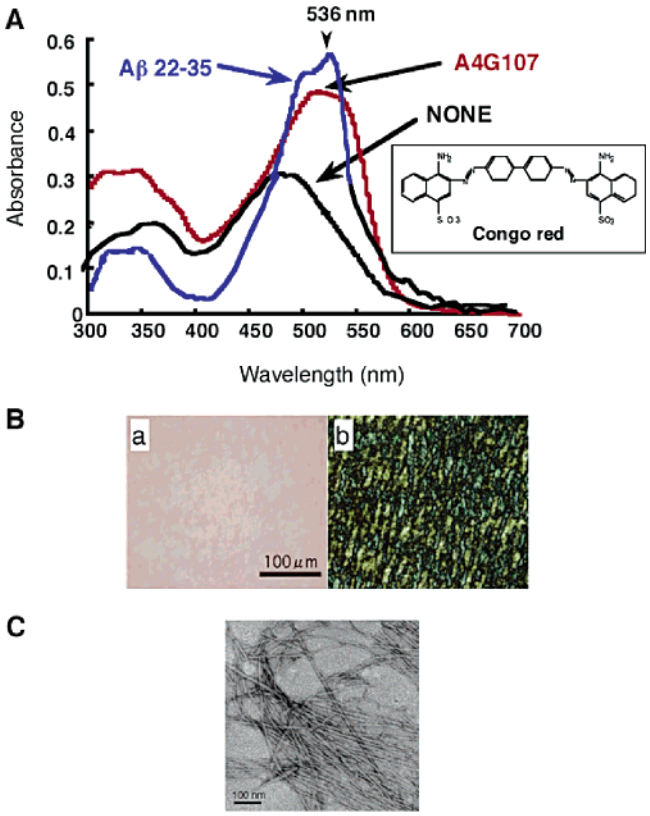

FIGURE 3: Congo red staining analysis and amyloid-like fibrils of A4G107. (A) A Congo red solution (100 μM) and a peptide solution (2 mg/mL) were mixed in PBS at room temperature for 24 h, and UV spectra were measured from 300 to 700 nm. (B) A4G107 in PBS (5 mg/mL) was pipetted onto a glass slide. After drying overnight, the precipitate was stained with a 1% aqueous solution of Congo red for 1 h. After being rinsed with pure acetone, samples were dehydrated as described in Materials and Methods. The specimens were mounted with a resin and observed in a microscope either under bright field illumination or between crossed polars. (C) Amyloid-like fibrils of A4G107. A4G107 (1 mg) was dissolved in PBS (200 μL), and the resulting gel was kept at 4 °C for 1 week. The amyloid-like fibrils were stained with a 5% aqueous solution of uranyl acetate and observed using an electron microscope.

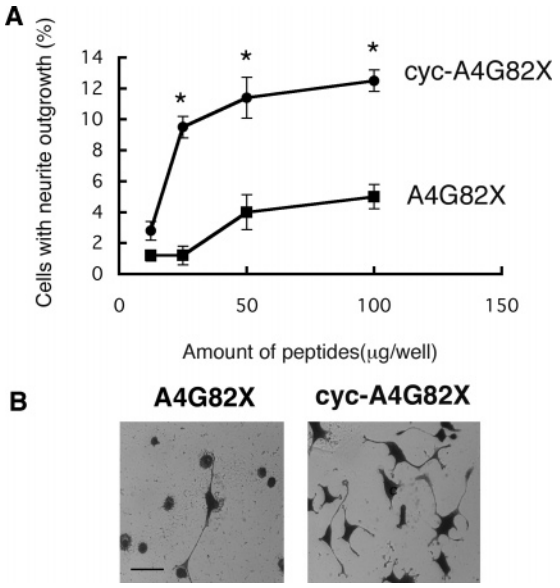

FIGURE 4: Neurite outgrowth activity of the cyclic peptide. (A) Various amounts of A4G82X and cyc-A4G82X peptides were coated on 24-well plates. PC12 cells (2×10^4 cells/well) were seeded in the plates. After being incubated for 24 h, cells were fixed and stained. The percentage of neurite outgrowth cells was determined as described in Materials and Methods. Assays was repeated three times (asterisk indicates $p < 0.01$). (B) The PC12 cells were cultured on A4G82X and cyc-A4G82X peptide-coated plates (50 μg/well) for 24 h, stained with crystal violet, and then photographed on a microscope (200×). The scale bar is 100 μm.

rinsed with pure acetone, the samples were dehydrated with 95 and 100% ethanol and then cleared with xylene. The specimens were mounted with a resin (malinol, Muto Pure Chemicals, Tokyo, Japan) and observed in a microscope (AX80, Olympus, Tokyo, Japan) either under bright field illumination or between crossed polar.

Electron Microscopy. Peptide gel in Milli-Q water (5 mg/mL) was applied onto a carbon-coated grid mesh with a thin Formvar film. The specimen was then negatively stained with a 5% aqueous solution of uranyl acetate and observed using a JEM-1200EX (JEOL, Tokyo, Japan) electron microscope at an acceleration voltage of 80 kV.

Immunocytochemistry. DRG explants were cultured for 24 h on peptide- or poly-L-lysine-coated coverglasses, fixed by 4% parafolmaldehyde in PBS for 10 min at room temperature, and blocked with 5% donkey serum (Chemicon International, Inc., Temecula, CA) and 1% BSA in PBS for 15 min. The primary antibody, rabbit anti-neurofilament (Sigma), was added and left at 4 °C overnight. The cells were then incubated with Alexa 488-conjugated donkey anti-rabbit IgG (Molecular Probes, Engene, OR) for 1 h at room temperature. After washing had been carried out, immunostained images were analyzed on a confocal laser microscope (Carl Zeiss LSM510 instrument).

RESULTS

Neurite Outgrowth Activity of the Rec-α4G Protein. A recombinant protein (rec-α4G) containing the mouse laminin α4 G domain and the c-myc sequence at the C-terminus was expressed in *dhfr*-deficient CHO DG44 cells and purified as previously described (*33*). Neurite outgrowth activity of the rec-α4G protein was tested using PC12 cells. Laminin 1 as a control promoted neurite outgrowth as described previously (*4*). The rec-α4G protein also promoted neurite outgrowth activity in a dose-dependent manner (Figure 1A,B). These results indicated that the laminin α4 G domain promotes neurite outgrowth and that active sequences exist in the domain.

Neurite Outgrowth Activity of Synthetic Peptides. We prepared 116 overlapping peptides covering the mouse laminin α4 G domain (*34*). One hundred fourteen peptides were soluble, but two peptides were not. The 114 soluble peptides were tested for their neurite outgrowth activity in PC12 cells. AG73 in the laminin α1 chain G domain was previously found to promote neurite outgrowth and was used as a control (*21*, *41*, *42*). Nine peptides promoted neurite outgrowth activity with PC12 cells (Table 1). A4G6, A4G20, and A4G107 showed especially strong neurite outgrowth activity in a dose-dependent manner, but scrambled peptides of these active peptide sequences were inactive (Figure 2A,B).

Congo Red Staining Analysis. The neurite outgrowth activity of IKVAV, derived from laminin α1, was recently reported to be closely related to amyloid-like fibril formation (*43*). Accordingly, the ability of the three active peptides to

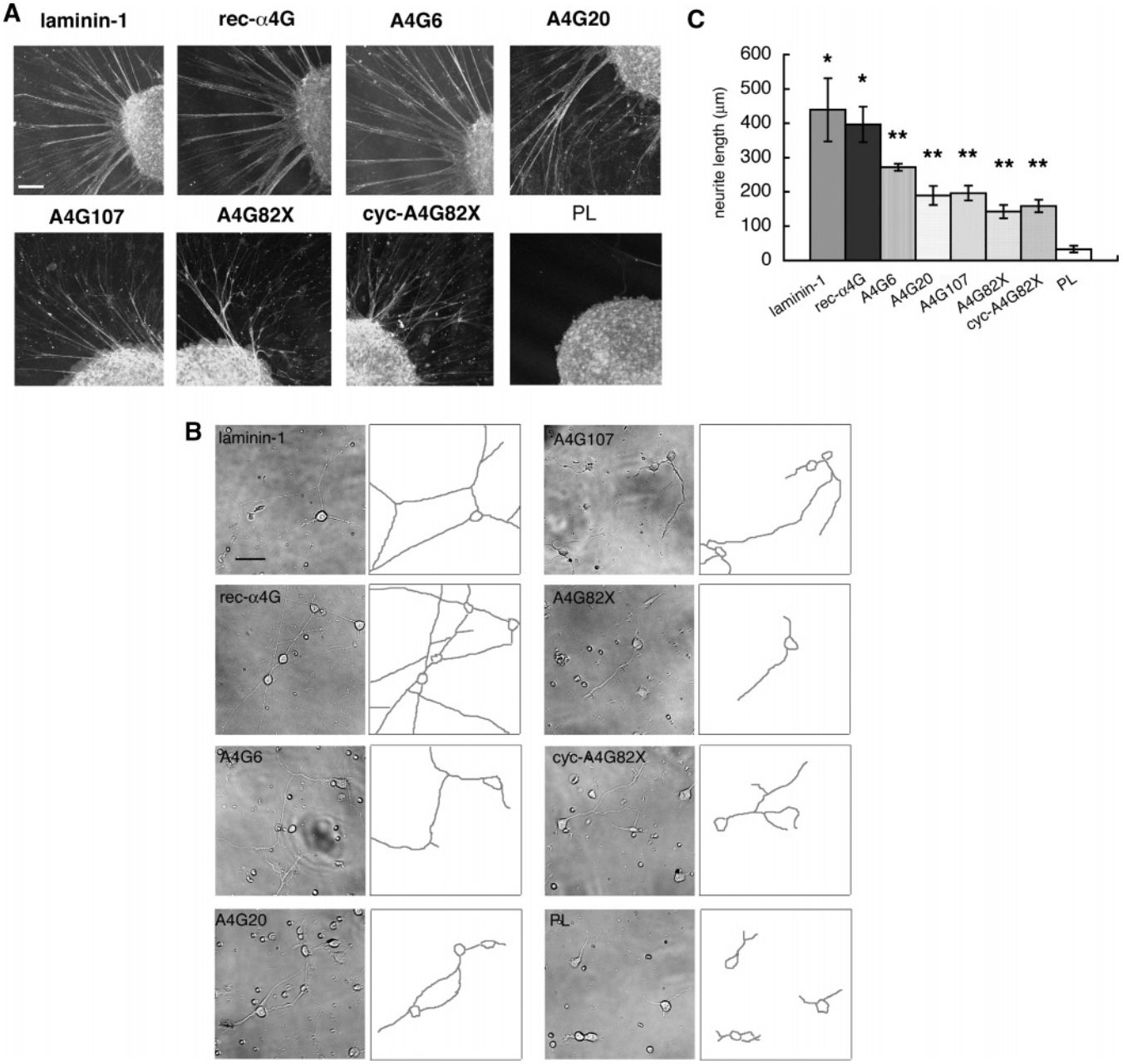

FIGURE 5: Neurite outgrowth of DRG explants (A) and cells (B) on peptide-, rec-α4G-, and laminin 1-coated coverglasses. DRG explants (A) and cells (B), prepared as described in Materials and Methods, were cultured for 24 h on poly-L-lysine (PL) (10 μg/mL)-, peptide (50 μg/well)-, rec-α4G (1.2 μg/mL)-, and laminin 1 (1.2 μg/mL)-coated coverglasses. (A) DRG explants were immunostained with the antibody against neurofilaments. (B) Primary cells from DRG were photographed on a confocal laser microscope (Carl Zeiss LSM510) and traced using NIH Image. (C) The neurite length of primary DRG cells ($n = 30$) was measured and analyzed using NIH Image (one asterisk indicates $p < 0.0001$; two asterisks indicate $p < 0.001$). The scale bar is (A) 50 or (B) 100 μm.

form amyloid-like fibrils was tested using Congo red (*44*). The absorption spectrum of the Congo red solution showed a peak at 486 nm as described previously (*43*, *45*). Two large peaks (at 512 and 536 nm) only appeared after the Congo red solution was incubated with A4G107 for 24 h (Figure 3A) but not when incubated with other peptides. These wavelengths were specific for the complex of Congo red and amyloid-like fibrils such as amyloid β-peptides (*45*). When the A4G107/Congo red solution was dried and examined under a polarizing microscope, the peptide precipitate exhibited birefringence, changing from red to green (Figure 3B). Negative staining electron microscopy showed that the A4G107 peptide formed a gel with the appearance of amyloid-like fibrils (Figure 3C). The scrambled peptide of A4G107 did not form amyloid-like fibrils (data not shown). These results suggest that the A4G107 peptide forms an amyloid-like structure and specifically binds to Congo red.

Neurite Outgrowth Activity of the Cyclic Peptide. We previously reported that the peptides, located in the loop region of the laminin α4 chain G domain, had biological activity (*22*, *34*). A4G82X, which is located in the loop region of the laminin α4 G domain, weakly promoted neurite outgrowth activity (Table 1). A4G82X and three other active peptides, A4G6, A4G20, and A4G107, were synthesized cyclically, and those peptides were tested for neurite outgrowth activity with PC12 cells (Table 2). The cyc-A4G82X peptide enhanced the activity (Figure 4), but the remaining cyclic peptides completely lost their activity (Table

2). These results suggest that the neurite outgrowth activity of the peptides depends on the conformation.

Neurite Outgrowth Activity of the Rec-α4G Protein and Synthetic Peptides on DRG Explants and Cells. The effects of rec-α4G and synthetic peptides (A4G6, A4G20, A4G82X, cyc-A4G82X, and A4G107) on mouse E14.5 embryo dorsal root ganglion (DRG) explants and cells were examined next. The rec-α4G protein showed a strong activity for neurite outgrowth of both DRG explants (Figure 5A) and cells (Figure 5B,C). The A4G6, A4G20, A4G82X, cyc-A4G82X, and A4G107 peptides also promoted neurite outgrowth of the DRG cells and explants, but poly-L-lysine did not exhibit activity at 24 h (Figure 5). Therefore, these peptides are active for promoting neurite outgrowth of native neuronal cells.

DISCUSSION

The laminin α4 chain is expressed in the perineurium of the peripheral nerves and a part of the brain and is implicated in the regulation of neuronal network formation (*1*, *23*, *26*, *32*). In this report, we showed that the recombinant laminin α4 G domain promoted neurite outgrowth, and we identified active sites for the activity within the domain by screening 114 synthetic peptides overlapping almost the entire laminin α4 G domain. We found that nine of the 114 peptides were active for neurite outgrowth of PC12 cells, and three of these peptides, A4G6, A4G20, and A4G107, exhibited stronger activity. We previously demonstrated that these three peptides were also strongly active for HT1080 cell attachment and heparin binding (*34*). A4G82X exhibiteded strong activity for HT1080 cell attachment and heparin binding (*34*), but we found that it was relatively weak for neurite outgrowth-promoting activity with PC12 cells. However, we found that cyclization of A4G82X significantly increased the activity, while all other cyclic peptides lost their neurite outgrowth-promoting activity, suggesting conformation-dependent activity.

Among the active peptides, A4G107 was found to form amyloid-like fibrils but A4G6 and A4G20 were not, suggesting that the amyloid-like structure is not a prerequisite for the neurite outgrowth-promoting activity of some peptides. However, the amyloid-like structure of A4G107 is likely necessary for neurite promoting activity, since the scrambled peptide of A4G107 lost amyloid formation and neurite outgrowth-promoting activity. As for A4G107, we recently reported that the IKVAV-containing peptide from the laminin α1 chain formed amyloid-like fibrils and was active for promoting neurite outgrowth and cell adhesion (*43*). We showed that these biological activities of the IKVAV-containing peptide were associated with amyloid-like fibril formation (*43*). Insoluble amyloid-like fibrils formed by assembly of β sheet domains are involved in several pathological conditions, including Alzheimer's and Huntington diseases (*46*, *47*). Laminin and its fragments interact with amyloid proteins and may be associated with amyloid formation (*48*, *49*). The IKVAV-containing peptide was shown to bind to the 110 kDa amyloid precursor protein (*48*). The A4G107 site of the laminin α4 chain may also interact with amyloid precursor proteins and be involved in amyloid plaque formation.

The G domain of laminin α chains has been implicated in many biological activities (*20*). Although it has a similar domain structure in all laminin α chains, its biological activities appear to be chain-specific (*20*). The laminin α4 chain G domain is proteolytically processed in vivo and in cultured endothelial and Schwannoma cells, resulting in the release of the LG4−LG5 part from the α4 chain (*32*). Both proteolytic fragments were able to bind heparin and cells (*32*, *35*); however, the biological significance of the processing is not clear. The LG4 subdomains of the laminin α chains consist of a 14-stranded β-sheet (A to N) sandwich structure, and several active sites in the connective region within the subdomain were identified. Recently, 19-mer peptides (EF peptides) were prepared from the E and F strands of the LG4 domains from five laminin α chains and compared for their activities (*37*). These studies showed that each EF peptide has unique cell type- and chain-specific biological activities. Peptide EF-4 from the laminin α4 chain, which contains the A4G82 sequence, attached to fibroblasts and promoted neurite outgrowth of PC12 cells (*37*). Using a crystal structure-based sequential alignment study of laminin α2 and α4 chains, A4G82 was shown to be located in the loop region between β strands in the LG4 module (*20*, *35*). The cyclic A4G82X peptide mimics the loop structure in this region. Therefore, neurite outgrowth-promoting activity of A4G82X is likely required for a loop conformation. Previously, we reported that the A3G75 peptide from the laminin α3 LG4 domain located on the loop region promotes cell adhesion and neurite outgrowth (*22*, *37*). A cyclic A3G75 may have stronger activity on neurite outgrowth than A3G75, similar to A4G82.

Recently, A4G6, A4G20, and cyclic A4G82X were found to interact with either syndecan-2 or -4 (*34*, *50*). Syndecans have been shown to work cooperatively with integrins (*51*) for cellular signaling, and the laminin α4 G domain binds both integrins and syndecans. It is possible that the neurite outgrowth-promoting activity of the peptides is regulated by both syndecans and integrins. This may explain why the peptides are less active for neurite outgrowth, compared to the recombinant rec-α4G protein, containing the whole G domain of the laminin α4 chain.

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