

Fig. 2. MS^n spectra of synthetic lactosylceramide of $C_{24:1}-d_{18:1}$ using ESI-ion trap mass spectrometer. (A) MS^n spectra ($n = 1-4$) of the lithiated molecular ion $[M + Li]^+$ with 1 mM LiCl in methanol at 400 °C. (B) MS^n spectra ($n = 1-3$) of the protonated molecular ion $[M + H]^+$ with 10 mM ammonium acetate in methanol at 200 °C. The collision energy was 100%. (C) Predicted cleavage scheme for lactosylceramide shows hexose (Hex), loss of hexoses (Y_0), long-chain base (w series), and fatty acyl chain (COR-H).

trum was associated with cleavage of the immediate N–CO bond on the MS^3 spectrum and identification of a long-chain base (w ion series) and a fatty acyl chain ($[Y'_0 - w' = COR - H]$) [7] (Fig. 2B). On the MS^3 spectrum, m/z 264 was found as a fragment ion of Y'_0 regarded as w'' ion, which was assigned to $d_{18:1}$, and the fatty acyl chain was identified as $C_{24:1}$ [7].

The use of sequential MS^n analysis allowed us to obtain the dissociation patterns of a single ion in multiple steps when that was compared with MS/MS analysis using FAB-MS or triple stage ESI/MS. The patterns were simple, thus allowing speculation on the structure of the molecular species.

Molecular species of lactosylceramides derived from porcine blood cells using infusion analysis

The molecular species of lactosylceramides derived from porcine blood cells were determined using infusion analysis. The MS spectrum for those ionized with 1 mM ammonium acetate is shown in Fig. 3.

The molecular species of lactosylceramides comprised more than 14 different structures from $C_{16:0}-d_{18:1}$ to $Ch_{26:1}-d_{18:1}$. The main species were $C_{22:0}-d_{18:1}$, $C_{24:1}-d_{18:1}$, $C_{24:0}-d_{18:1}$, $Ch_{24:1}-d_{18:1}$, and $Ch_{24:0}-d_{18:1}$, similar to the results of a previous study [11].

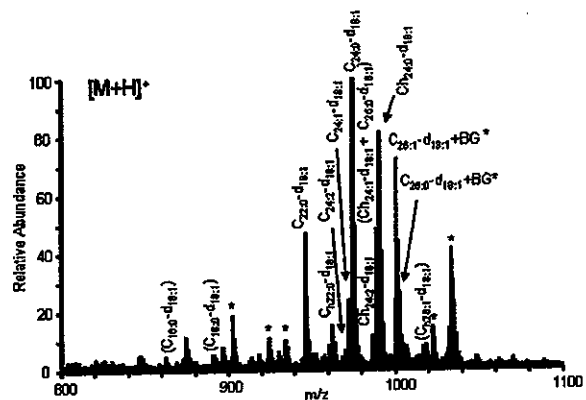


Fig. 3. ESI-MS spectrum of lactosylceramides derived from porcine blood cells ionized with ammonium salt using infusion analysis. Lactosylceramides were dissolved in 10 mM ammonium acetate in methanol at 10 ng/ μ l concentration, the ion transfer tube was set at 200 °C. The molecules were detected as protonated molecular ions, $[M + H]^+$. The parent formulae of lactosylceramides were characterized using other methods. *Represent those revealing background ions from the reagents. Some ions at m/z 1001 and 1003 were similar to the $[M + H]^+$ ions of $C_{26:1}-d_{18:1}$ and $C_{26:0}-d_{18:1}$ but they were distinguished from the background ions on MS^n analysis.

The use of infusion analysis was associated with one problem; it was impossible to distinguish fatty acyl chains between $C_n:x$ and $Ch(n-1):(x+1)$ by MS^n

analysis because of the same value of COR-H at m/z 364 for $C_{25:0}$ and $Ch_{24:1}$ fatty acyl chains.

LC-ESI-MS for classification of molecular species of lactosylceramides by single ion monitoring (SIM) mode

The molecular species of lactosylceramides were separated on a C_8 reverse-phase column and identified using the LCQ ion trap mass spectrometer by characterizing their fatty acyl chain and long-chain base moieties by MS^n analysis. Fig. 4A shows the three-dimensional (3D) HPLC mass chromatogram of 200 ng of total lactosylceramides derived from porcine blood cells. The abscissa, ordinate, and z axis indicate the retention time, relative abundance of ions, and m/z , respectively.

In the case of LC-MS, we used methanol containing 1 mM ammonium formate as a solvent because the formate produced stable and abundant $[M + H]^+$ ions and $[M + Na]^+$ ions were minimum as contaminants.

It was assumed that the long chain base of lactosylceramides has $d_{18:1}$ sphinganine, because this is the commonest type in animal tissues [17]. In Figs. 4B–E, $[M + H]^+$ ions of lactosylceramide molecular species were monitored in the SIM mode for classification according to fatty acyl carbon chain into (B) straight saturated ($n:0$), (C) straight unsaturated ($n:1$), (D) saturated and hydroxylated ($hn:0$), and (E) unsaturated and hydroxylated ($hn:1$) fatty acyl chains. We also monitored $d_{18:0}$ sphinganine, but no significant peak of lactosylceramides appeared in porcine blood cells (data not

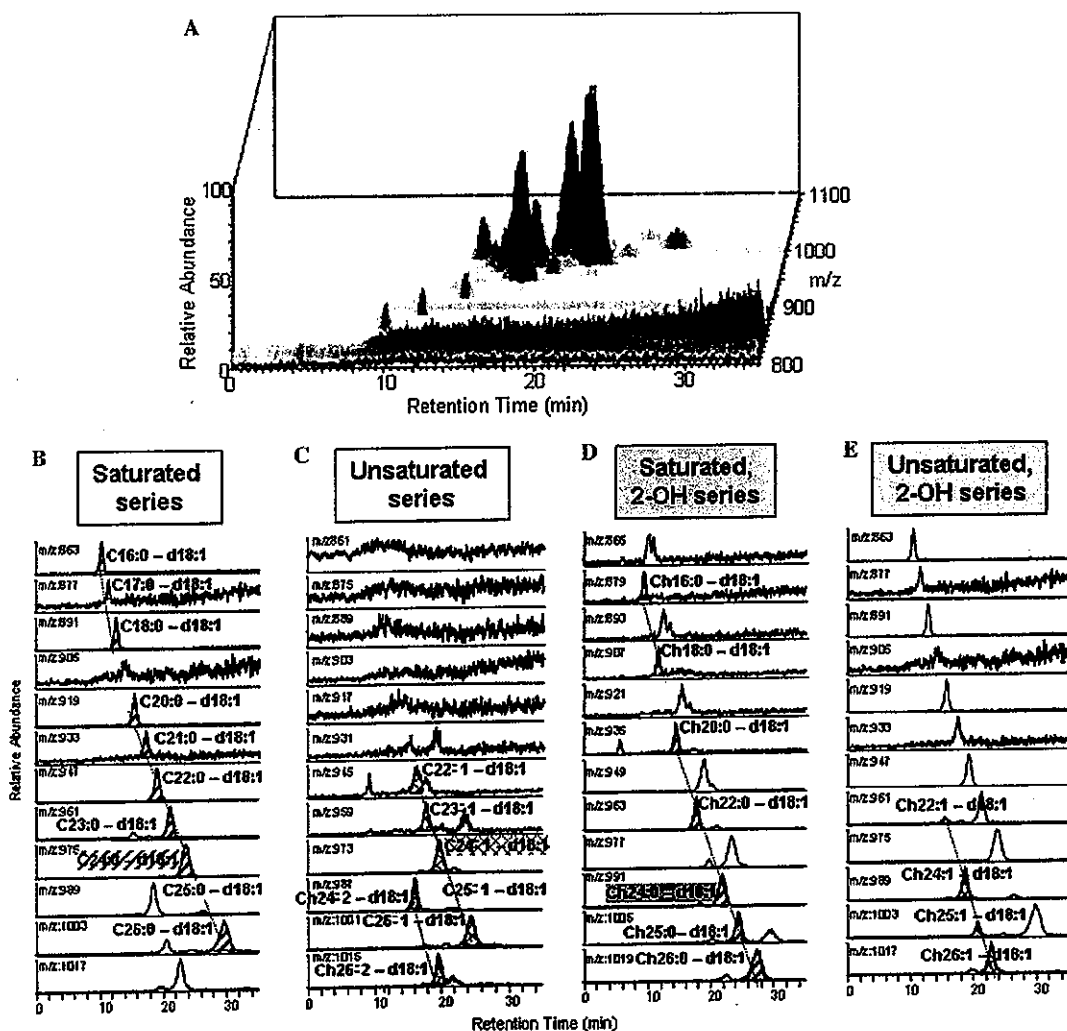


Fig. 4. Three-dimensional HPLC mass chromatogram of lactosylceramides derived from porcine blood cells and selected ion monitoring (SIM) chromatograms. (A) 3D-HPLC-ESI/MS chromatogram of 200 ng of lactosylceramide. SIM chromatograms monitored the $[M + H]^+$ ions of lactosylceramide molecular species classified according to fatty acyl carbon chain into (B) straight saturated ($n:0$), (C) straight unsaturated ($n:1$), (D) saturated and hydroxylated ($hn:0$), and (E) unsaturated and hydroxylated ($hn:1$).

shown). In the straight saturated molecular species (Fig. 4B), the retention time of lactosylceramides correlated with the length of their fatty acyl chain from 10 min for $C_{16:0}$ to 30 min for $C_{26:0}$. Although the $[M+H]^+$ ions of lactosylceramides at $C_{25:0}$ and $Ch_{24:1}$ had the same value at m/z 989 and could not be identified by infusion analysis, they were completely separated on the column. Their retention times were 17.3 min for $Ch_{24:1}$ and 24.6 min for $C_{25:0}$. The former had 2-hydroxylated fatty acyl chains and was more hydrophilic than the latter.

Lactosylceramides with the same carbon number of fatty acyl chain of saturated ($C_{24:0}$) at m/z 975, unsatu-

rated ($C_{24:1}$) at m/z 973, and 2-hydroxylated ($Ch_{24:0}$) at m/z 991 fatty acyl chain moiety appeared at 22.2, 18.6, and 20.8 min on the chromatogram, respectively (Figs. 4B–D). These results suggest that lactosylceramides classified as unsaturated, 2-hydroxylated, and saturated fatty acyl chain were eluted in that order on the column.

Determination of molecular species of porcine-blood cell-derived lactosylceramides

We also identified more than 33 different structures as molecular species from porcine-blood-cell-derived lactosylceramides (Fig. 5A). The profile was constructed

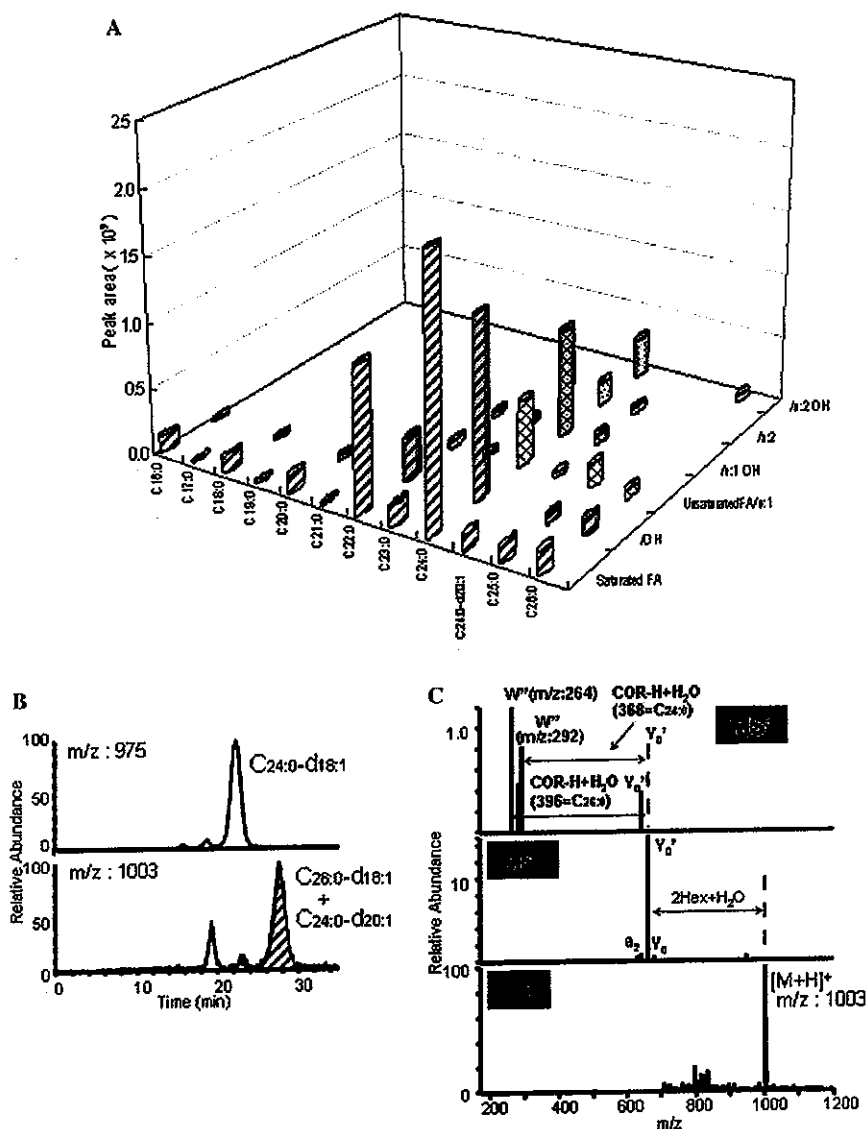


Fig. 5. Distribution of molecular species of lactosylceramides derived from porcine blood cells. (A) Abscissa represents the carbon unit of fatty acyl chain and long-chain base ($d_{18:1}$ is not shown). The intensity of lactosylceramides was estimated by the peak area on Figs. 3B–E. The ordinate represents the peak area. The z axis represents the class of fatty acyl chain such as saturated, unsaturated, and hydroxylated species. (B) The SIM chromatogram of the same $[M+H]^+$ ions at m/z 1003 for $C_{26:0}-d_{18:1}$ and $C_{24:0}-d_{20:1}$ and (C) MS^n analysis ($n = 1-3$) for m/z 1003.

based on the peak area of SIM chromatogram for lactosylceramides. Some lactosylceramides with long-chain base moiety at $d_{20:1}$ were confirmed by MS^3 analysis and are shown in Fig. 5C. The retention time of lactosylceramide at m/z 1003 for $C_{26:0}-d_{18:1}$ or $C_{24:0}-d_{20:1}$ was 28 min in Fig. 5B, but the m/z 264 and m/z 292 ions were produced on the MS^3 spectrum. This means that there were two other types of w'' ions associated with $d_{18:1}$ and $d_{20:1}$ sphingenines. Therefore, it was considered that separation of lactosylceramides was based on total carbon chain length and numbers of double bonds in the fatty acyl chain and long-chain base. Thus, lactosylceramides at C_n-d_x and $C_{n-2}-d_{x+2}$ were not separated on the column in the system, however, we could at least confirm by MS^3 analysis the presence or absence of the two types of lactosylceramides.

The molecular species of porcine-blood-cell-derived lactosylceramides contained more than 33 different structures, which were mainly $C_{24:0}-d_{18:1}$, $Ch_{24:0}-d_{18:1}$, $Ch_{24:1}-d_{18:1}$, $C_{24:1}-d_{18:1}$, and $C_{22:0}-d_{18:1}$, with 28 other minor species. Minor species of long-carbon-chain fatty

acids such as C_{22} and C_{26} were also identified as saturated, unsaturated, and hydroxylated species. The number of molecular species of lactosylceramides determined by LC-ESI/MS was twice that confirmed by infusion analysis. The amount of lactosylceramides used was 200 ng for determination of the $[M + H]^+$ ions and automatic MS^n analysis with LC-ESI/MS. In the case of infusion analysis, lactosylceramides at 10 ng/ μ l were infused into the ion source for 10–15 min depending on the number of MS^n analyses of $[M + H]^+$ ions manually. The sample amounts used for both analyses were not different and LC-ESI/MS was convenient and precise for determination of low-intensity $[M + H]^+$ ions.

Since the standard compound of the molecular species of lactosylceramides could not be reported and no product could be identified, we were unable to perform quantification analysis of these species. However, the SIM chromatograms of 10–200 ng of porcine-blood-cell-derived lactosylceramides showed almost linear relationships between the ratio of the peak area and the total amount of lactosylceramides (data not shown).

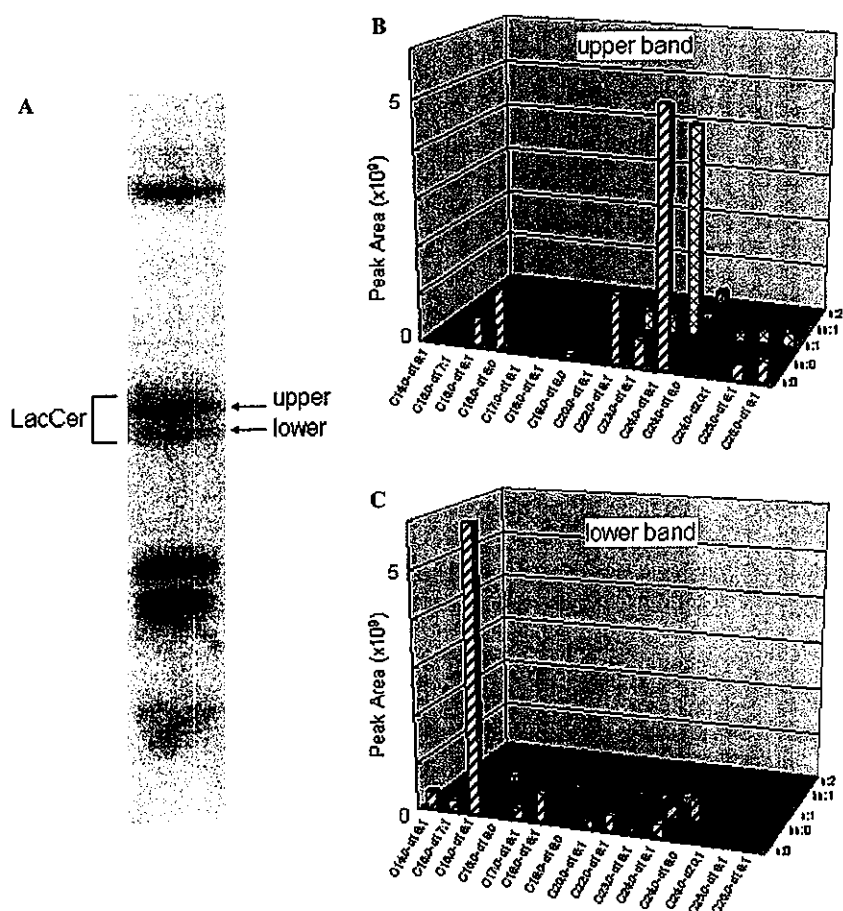


Fig. 6. Determination of molecular species of lactosylceramides derived from membrane microdomain of HL-60 cells. (A) The pattern of lactosylceramides for membrane microdomain fractionated from HL-60 cells separated on HPTLC. (B) Molecular species of lactosylceramides in the upper band and (C) those in the lower band derived from HL-60 cells (8×10^6 cells). The abscissa, ordinate, and z axis are similar to those used in Fig. 5.

Analysis of molecular species of lactosylceramides in the membrane microdomain fraction of HL-60 cells

Crude lipids were extracted from the membrane microdomain fraction of HL-60 cells using a mixture of chloroform and methanol (2:1 v/v). A fifth of the crude lipids was separated on HPTLC with a mixture solution of chloroform/methanol/water (65:25:4 v/v/v) and detected by primulin reagent. Lactosylceramides marked as upper and lower bands in Fig. 6A were removed and collected in each glass tube and crude lactosylceramides were extracted with a mixture of chloroform and methanol (2:1 v/v). A fifth of the crude lactosylceramides was loaded on a column and determined by LC-ESI-MSⁿ analysis, respectively. Figs. 6B and C show the distribution of molecular species of lactosylceramides in the upper and the lower bands, respectively. A total of 25 species of lactosylceramides was detected in the upper and lower bands on the HPTLC plate.

Lactosylceramides in the upper band consisted mainly of C_{24:0}-d_{18:1}, C_{24:1}-d_{18:1}, and C_{22:0}-d_{18:1} and those in the lower band were mainly C_{16:0}-d_{18:1} but higher carbon chain structures such as Ch_{24:0}-d_{18:1} fatty acyl chain moiety at *m/z* 991 were also detected. The high molecular species in the lower band seemed to be insufficiently separated on the HPTLC plate and rather coexisted with those of the upper band. It is possible, however, that the hydrophilic property of Ch_{24:0} could have affected the separation of lactosylceramides on the HPTLC plate. On the other hand, lactosylceramide for C_{16:0}-d_{18:0} also appeared in the upper band, suggesting that it might be more hydrophobic than lactosylceramide for C_{16:0}-d_{18:1} on the HPTLC plate. Numerous minor lactosylceramides that contained odd numbers of fatty acyl chains and saturated long-chain base moieties were identified also, although we could not clarify whether these structures have any biological relevance in HL-60 cells, of which about 70% differentiate into macrophage-lineage cells.

Further studies are necessary to identify key ions of various types of lactosylceramides such as branched, polyunsaturated fatty acyl moieties, and other types of sphingoids like phytosphingosine to characterize their structures and biological significance. There is also a need to determine the position of double bonds and hydroxylation in both fatty acyl chain and long-chain base moieties of lactosylceramides by LC-ESI ion trap mass spectrometry.

Conclusions

Using mass spectrometry, lactosylceramide was ionized with alkaline metals or ammonium salts to produce molecular ions of [M + Li/Na]⁺ at 400 °C or [M + H]⁺ at 200 °C. The [M + Li]⁺ ion was stable and not frag-

mented into fatty acyl chain and long-chain base moiety. However, MS³ analysis showed some dissociation of lactosylceramide [M + H]⁺ ion. The loss of two hexoses and water resulted in cleavage of the amide bond, which allowed information on the long chain base and fatty acyl moiety necessary to determine the molecular species of lactosylceramide to be obtained.

In the present study, we developed a useful and simple method to identify the molecular species of lactosylceramides using reverse-phase LC-ESI ion trap mass spectrometry. Using sequential MSⁿ analysis, we could obtain in multiple steps the dissociation patterns of a single ion. The patterns became simple and allowed us to speculate on the structure of the molecular species. The obtained molecular species were classified as [M + H]⁺ ions, which were produced with ammonium formate. In our analysis, 200 ng of lactosylceramides derived from porcine blood cells contained more than 33 molecular species. Lactosylceramides were eluted from a column depending on the length of their carbon chain and appeared in the order of unsaturated, 2-hydroxylated, and saturated fatty acyl chain with the same number of carbon atoms.

We also used our method to determine the molecular species of lactosylceramides present in the membrane microdomain fraction of HL-60 cells (about 70% were differentiated cells). Five species of major lactosylceramides and about 20 species of minor ones were detected in the upper and lower bands on the HPTLC plate. The biological relevance of the minor lactosylceramides, which consisted of about 20 species from C₁₆ to C₂₆, is not clear yet.

Acknowledgments

We thank Professor Sandro Sonnino and Dr. Alessandro Prinetti from the Department of Medical Chemistry, Biochemistry and Biotechnology, University of Milan, Italy for their generous gift of some synthetic lactosylceramides used in this study. This work was supported in part by Grants-in-aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan 16017293 for K.I. This work was supported, in part, by Thermo Electron Corp.

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Identification of Neurite Outgrowth Active Sites on the Laminin $\alpha 4$ Chain G Domain[†]

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Received November 9, 2004; Revised Manuscript Received January 16, 2005

ABSTRACT: The laminin $\alpha 4$ 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 $\alpha 4$ chain was previously found to be critical for heparin binding and cell attachment activity. Here, we focused on neurite outgrowth activity of the laminin $\alpha 4$ chain G domain. We found that the recombinant $\alpha 4$ chain G domain protein (rec- $\alpha 4$ G) 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 $\alpha 4$ 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 $\alpha 1$ and $\alpha 2$ chains have been shown to stimulate neurite outgrowth (21). We also reported that the LG4 and LG5 modules of the laminin $\alpha 3$ chain G domain exhibited neurite outgrowth activity (22).

The laminin $\alpha 4$ chain is a component of three laminins: laminin 8 ($\alpha 4:\beta 1:\gamma 1$), laminin 9 ($\alpha 4:\beta 2:\gamma 1$), and the recently identified laminin 14 ($\alpha 4:\beta 2:\gamma 3$) (1, 23). The $\alpha 4$ 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 $\alpha 4$ 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 $\alpha 4$ -deficient mice has indicated that

[†] Part of this work was supported by Grants-in-Aid from the Ministry of Health, Labor and Welfare and the High Technology Research Center Grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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the laminin $\alpha 4$ 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 $\alpha 4$, 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 $\alpha 4$ at the growth cone regulates neuronal outgrowth and neuromuscular junction formation. Moreover, loss of laminin $\alpha 2$ leads to upregulation of laminin $\alpha 4$ throughout muscle basal laminae (28, 31). It was demonstrated that the laminin $\alpha 4$ G domain was proteolytically processed in cultured endothelial and schwannoma cells, and the LG4–LG5 module of the $\alpha 4$ G domain was released (32). The $\alpha 4$ 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 $\alpha 4$ 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 $\alpha 4$ 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- $\alpha 4$ G) and Synthetic Peptides.

A recombinant protein (rec- $\alpha 4$ G) involving the mouse laminin $\alpha 4$ 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- $\alpha 4$ G 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 $\text{CH}_3\text{COOH}/\text{DMSO}/\text{H}_2\text{O}$ mixture), a modified method described previously (37).

Culture of Cells. PC12 cells (38) were cultured in

Table 1: Neurite Outgrowth Activity of Peptides on the Laminin $\alpha 4$ Chain G Domain^a

peptide	sequence	neurite outgrowth ^b
A4G6	⁸⁹² LAIKNDNLVYVY ⁹⁰³	+++
A4G20	¹⁰⁰⁹ DVISLYNFKHIY ¹⁰²⁰	++
A4G51	¹²⁶¹ LOPNGLLFYYS ¹²⁷³	+
A4G82X	¹⁵¹⁴ TLFLAHGRLVFX ¹⁵²⁵ ^c	+
A4G93	¹⁵⁹⁸ NVQITSVYSFSG ¹⁶⁰⁹	+
A4G94	¹⁶¹¹ LGNLONGASIT 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 $\alpha 4$ 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	(<i>tie</i> ;0) CLAIKNDNLVYVYC	–
A4G20	DVISLYNFKHIY	++
cyc-A4G20	(<i>tie</i> ;0) CDVISLYNFKHIYC	–
A4G82X	TLFLAHGRLVFX ^b	+
cyc-A4G82X	(<i>tie</i> ;0) CTLFLAHGRLVFX ^b C	+++
A4G107	VIRDSNVVOLDV	+++
cyc-A4G107	(<i>tie</i> ;0) CVIRDSNVVOLDVC	–
AG73 ^c	RKRLOVOLLSIRT	+++

^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).

7.5% fetal bovine serum (FBS, Gibco), 100 units/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin (Gibco).

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

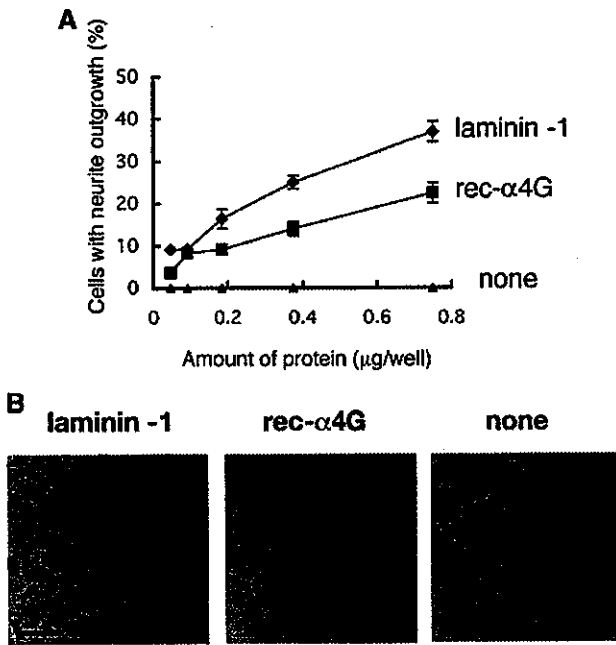


FIGURE 1: Neurite outgrowth activity of the recombinant rec- α 4G protein in PC12 cells. (A) Various amounts of rec- α 4G and laminin 1 were coated on 96-well plates, and PC12 cells (2×10^4 cells/well) were seeded in the plates. After being incubated for 24 h, cells were fixed and stained. The percentage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

(Wako, Osaka, Japan), 5μ g/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 (10μ g/mL), and A4G6, A4G20, A4G82X, cyclic-A4G82X, and A4G107 (50μ g/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 ($100 \mu\text{M}$ 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

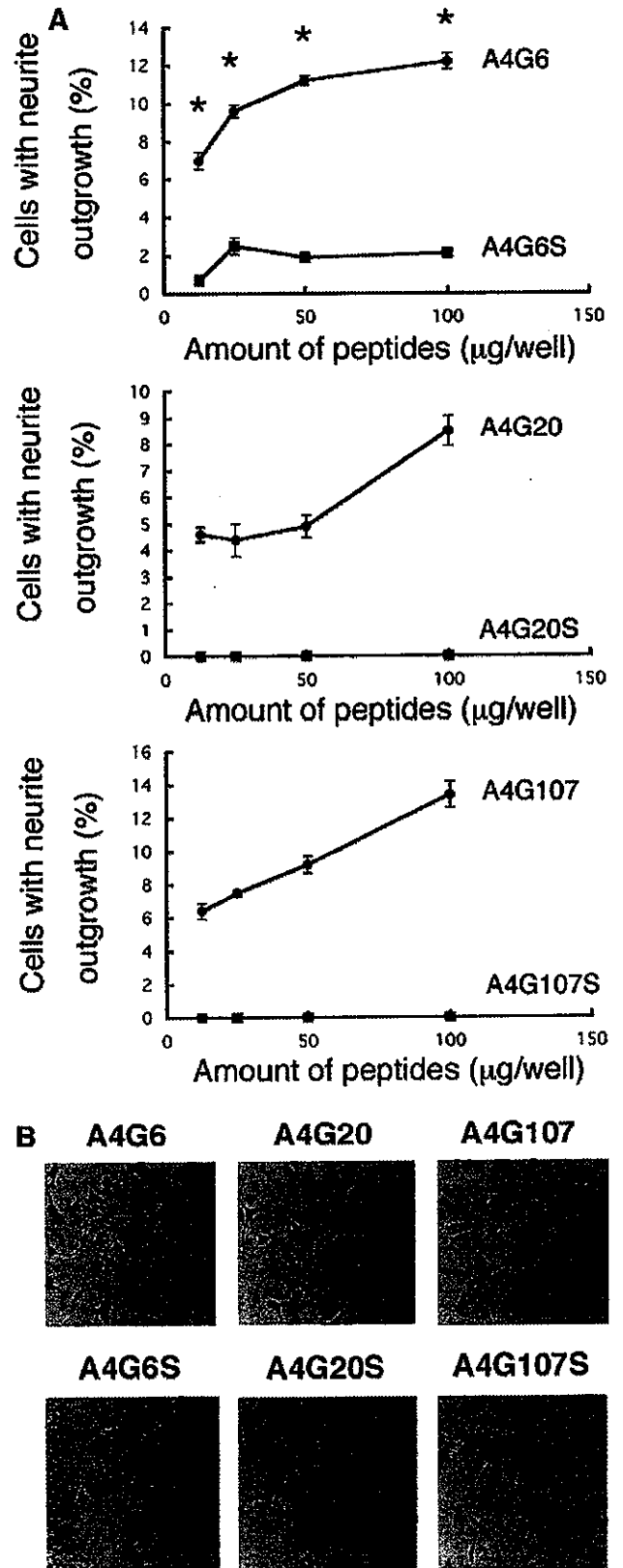


FIGURE 2: Neurite outgrowth of PC12 cells on synthetic peptides. (A) Various amounts of peptides were coated on 24-well plates. PC12 cells (2×10^4 cells/well) were seeded in the plates. After being incubated for 24 h, cells were fixed and stained. The percentage of neurite outgrowth cells was determined 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 (50μ g/well) for 24 h, stained with

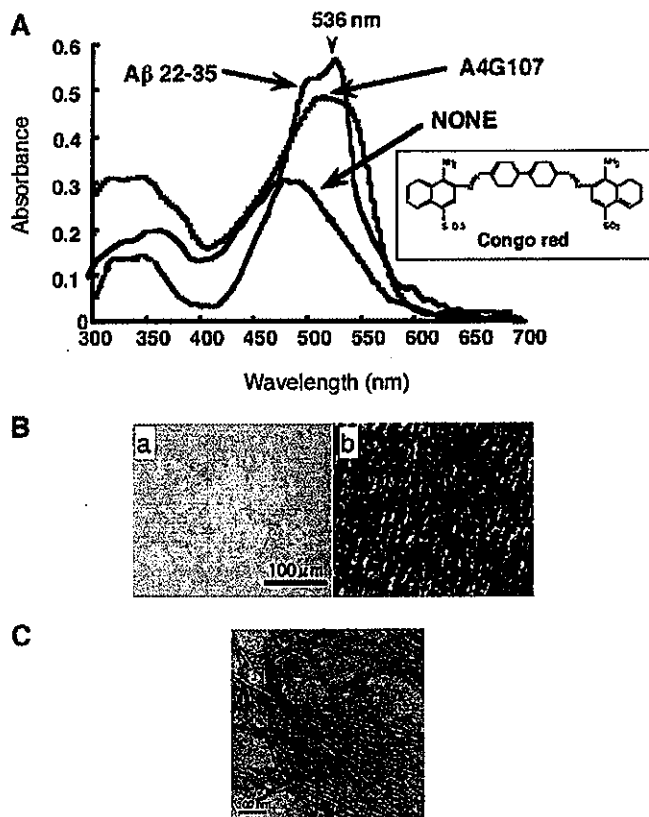


FIGURE 3: Congo red staining analysis and amyloid-like fibrils of A4G107. (A) A Congo red solution (100 μ M) and a peptide solution (2 mg/mL) were mixed in PBS at room temperature for 24 h, and UV spectra were measured from 300 to 700 nm. (B) A4G107 in PBS (5 mg/mL) was pipetted onto a glass slide. After 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 μ L), and the resulting gel was kept at 4 $^{\circ}$ C for 1 week. The amyloid-like fibrils were stained with a 5% aqueous solution of uranyl acetate and observed using an electron microscope.

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% paraformaldehyde 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 $^{\circ}$ C overnight. The cells were then incubated with Alexa 488-conjugated donkey anti-

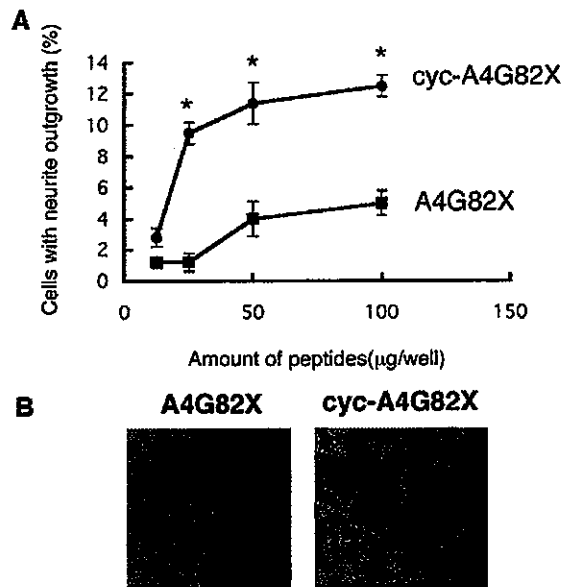


FIGURE 4: Neurite outgrowth activity of the cyclic peptide. (A) Various amounts of A4G82X and cyc-A4G82X peptides were coated on 24-well plates. PC12 cells (2×10^4 cells/well) were seeded in the plates. After being incubated for 24 h, cells were fixed and stained. The percentage of neurite outgrowth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

ostained images were analyzed on a confocal laser microscope (Carl Zeiss LSM510 instrument).

RESULTS

Neurite Outgrowth Activity of the Rec- α 4G Protein. A recombinant protein (rec- α 4G) containing the mouse laminin α 4 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- α 4G protein was tested using PC12 cells. Laminin 1 as a control promoted neurite outgrowth as described previously (4). The rec- α 4G protein also promoted neurite outgrowth activity in a dose-dependent manner (Figure 1A,B). These results indicated that the laminin α 4 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 α 4 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 α 1 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 similar to that of AG73, 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 α 1, was recently

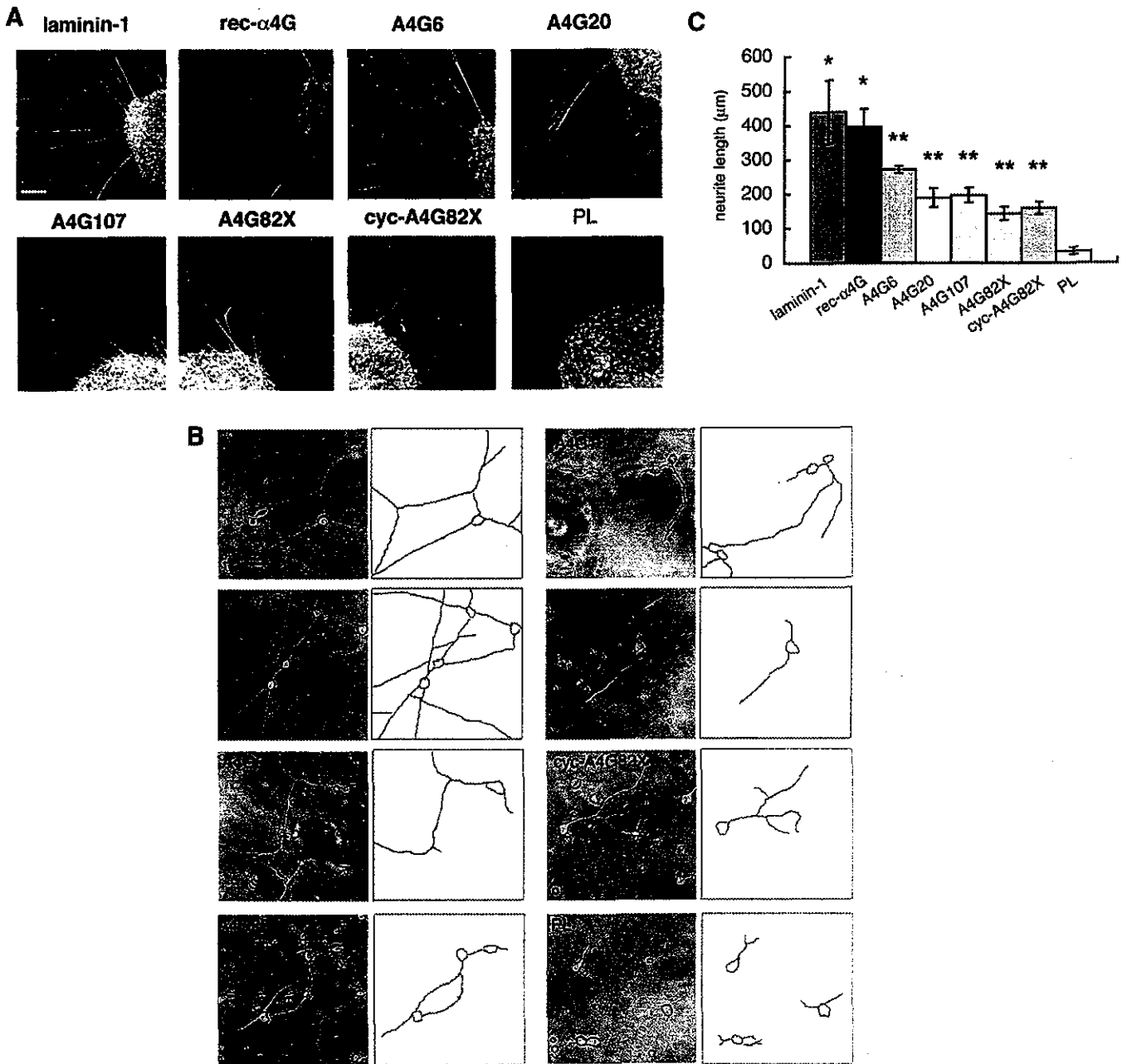


FIGURE 5: Neurite outgrowth of DRG explants (A) and cells (B) on peptide-, rec- $\alpha 4$ G-, and laminin 1-coated coverglasses. DRG explants (A) and cells (B), prepared as described in Materials and Methods, were cultured for 24 h on poly-L-lysine (PL) (10 $\mu\text{g}/\text{mL}$)-, peptide (50 $\mu\text{g}/\text{well}$)-, rec- $\alpha 4$ G (1.2 $\mu\text{g}/\text{mL}$)-, and laminin 1 (1.2 $\mu\text{g}/\text{mL}$)-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 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 $\alpha 4$ chain G domain, had biological activity (22, 34). A4G82X, which is located in the loop region of the laminin $\alpha 4$ 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-

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

Neurite Outgrowth Activity of the Rec- α 4G Protein and Synthetic Peptides on DRG Explants and Cells. The effects of rec- α 4G 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- α 4G 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 α 4 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 α 4 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 α 4 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 exhibited 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 α 1 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 α 4 chain may also interact with amyloid precursor proteins and be involved in amyloid plaque formation.

domain structure in all laminin α chains, its biological activities appear to be chain-specific (20). The laminin α 4 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 α 4 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 α 4 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 α 2 and α 4 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 α 3 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 α 4 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- α 4G protein, containing the whole G domain of the laminin α 4 chain.

ACKNOWLEDGMENT

We thank Drs. M. Hoffman and Y. Yamada for valuable comments on the manuscript.

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BI0476228