Articles

Identification of Neurite Outgrowth Promoting Sites on the Laminin α3 Chain G Domain†

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ABSTRACT: Laminins are expressed in specific tissues and are involved in various biological activities including promoting cell adhesion, growth, migration, neurite outgrowth, and differentiation. The laminin α3 chain is mainly located in the skin and is also expressed in the floor plate of the developing neural tube. Previously, we showed that the human laminin α3 chain LG4 module binds to syndecan-2/4, a membrane-associated proteoglycan, and promotes human fibroblast adhesion. Here, we have evaluated the neurite outgrowth activity of the laminin α3 chain LG4 and LG5 modules. Three overlapping recombinant proteins, which contained LG4 and/or LG5 modules of the human laminin α3 chain, were prepared using a mammalian cell expression system. Two proteins, rec-α3LG4-5 and rec-α3LG4, promoted cell attachment and neurite outgrowth of rat pheochromocytoma PC12 cells, but rec-α3LG5 was inactive. Twenty-two peptides covering the entire LG4 module were synthesized and tested for cell attachment and neurite outgrowth activity to identify active sites of the LG4 module. A3G75 (KNSFMALYLSKG, α3 chain 1411−1422) and A3G83 (GNSTISIRAPVY, α3 chain 1476−1487) promoted PC12 cell attachment and neurite outgrowth. Additionally, A3G75 and A3G83 inhibited PC12 cell attachment to rec-α3LG4. These results suggest that the A3G75 and A3G83 sites are important for PC12 cell attachment and neurite outgrowth in the laminin α3 chain LG4 module. We also conjugated the A3G75 and A3G83 peptides on chitosan membranes to test their potential as bio-materials. These peptide-conjugated chitosan membranes were more active for neurite outgrowth than the peptide-coated plates. These results suggest that the A3G75- and A3G83-conjugated chitosan membranes are applicable as bio-medical materials for neural tissue repair and engineering.

Laminin, a multifunctional glycoprotein of the basement membrane, consists of three different subunits, α, *β*, and *γ* chains (*1*). So far, five α, three *β*, and three *γ* chains have been identified (*2*−*4*). At least 15 isoforms (laminin-1 to -15) are formed by various combinations of each subunit (*2*−*5*). The laminin α3 chain, a subunit of laminin-5, -6, -7, and -13, promotes skin regeneration and wound healing (*6*−*9*). The C-terminus of the α3 chain contains a large globular domain (G domain), which is composed of five globular modules (LG1−LG5). The G domain is also involved in various additional biological functions of the α3 chain (*7*, *8*, *10*−*12*). The laminin α3 chain in the laminins is secreted as a mature form (200 kDa) and then processed to 165 kDa (*13*). The laminin α3 chain is cleaved between the LG3 and LG4 modules (*9*, *11*, *12*).

Laminin-5 has been found to promote neurite outgrowth (*14*−*16*). In addition, adhesion, migration, and invasion of glioma cells are stimulated by laminin-5 via integrin α3*β*1 (*17*). These assays employed laminin-5 which contains the processed form of α3 chain (165 kDa), i.e., lacking the LG4 and LG5 modules (*14*−*17*). Laminin-5, containing the unprocessed form of the α3 chain (containing the LG4 and LG5 modules), is expressed in the floor plate of the developing neural tube (*18*). This observation suggests a potential role of LG4 and LG5 modules in neural cell activity, although none has been investigated.

Previously, we identified biologically active sites in the laminin α3 chain G domain using human fibroblasts (*19*). The LG4 module was found to be the major active module for fibroblast adhesion and heparin binding in the laminin α3 chain G domain. We also identified two cell adhesive sequences, A3G75 (positions 1411−1422) and A3G83 (positions 1476−1487), on the LG4 module, and syndecan-2/4 as the cellular receptor(s) for A3G75 (*19*).

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Here, we focus on the neural cell activity of the LG4 and LG5 modules of the laminin α3 chain. We prepared three recombinant proteins, containing LG4−5, LG4, and LG5, and tested their cell attachment and neurite outgrowth activities using PC12 cells. We further identified neurite outgrowth promoting sequences on the LG4 module using a set of overlapping synthetic peptides. Finally, we covalently conjugated active peptides on chitosan membranes and evaluated their biological activity.

MATERIALS AND METHODS

Culture of Cells. PC12 cells (*20*) were cultured in Dulbecco's modification of 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). 293T cells (*21*) were cultured in DMEM containing 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin.

Preparation of Recombinant Proteins. Recombinant LG4−5 (rec-α3LG4−5) was prepared using a mammalian cell expression system as described previously (*19*). An expression vector, which encoded the laminin α3 chain LG4−5 module (1348−1713) with the signal peptide of the laminin γ2 chain (1−27) and human IgG Fc, was transfected into 293T cells. Conditioned medium (CM) with 1.5% FBS in DMEM was collected for 3 days at 12 h intervals, and 1 mM PMSF (Sigma, St. Louis, MO) was added. CM (70 mL) was applied to a heparin affinity column (HiTrap, Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated in 10 mM Tris-HCl (pH 7.4) containing 150 mM NaCl, 2 mM EDTA, and 0.5 mM *N*-ethylmaleimide (buffer A). The bound protein was eluted with buffer A in the presence of 1 M NaCl. The rec-α3LG4−5 protein was detected with biotinylated anti-human IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) and streptavidin-peroxidase (Sigma) in Western blotting. Protein concentration was determined by 8% SDS−PAGE and Coomassie Brilliant Blue staining with BSA as standard.

The rec-α3LG4 and rec-α3LG5 proteins were prepared using a mammalian cell expression system as described previously (*19*).

Synthesis of Peptides. Peptides were synthesized manually by the 9-fluorenylmethoxycarbonyl-based solid-phase method with a C-terminal amide as described previously (*22*). Peptides were generally 12 amino acids in length and overlapped with neighboring peptides by 4 amino acids. Cysteine residues were omitted. The respective amino acids were condensed manually in a stepwise manner using diisopropylcarbodiimide-*N*-hydroxybenzotriazole on a Rink amide resin (Novabiochem, San Diego, CA). The following side chain protecting groups were used: Asn, Cys, Gln, and His, trityl; Asp, Glu, Ser, Thr, and Tyr, *tert*-butyl; Arg, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; and Lys, *tert*-butoxycarbonyl. Resulting protected peptide resins were deprotected and cleaved from the resin using trifluoroacetic acid/thioanisole/*m*-cresol/ethanedithiol/H_2O (80:5:5:5:5, by volume) at 20 °C for 3 h. Crude peptides were precipitated and washed with ethyl ether and then purified by HPLC (using a Vydac 5C18 column with a gradient of H_2O/acetonitrile containing 0.1% TFA). Purity and identity of the synthetic peptides were confirmed by HPLC and by fast atom bombardment mass spectral analysis.

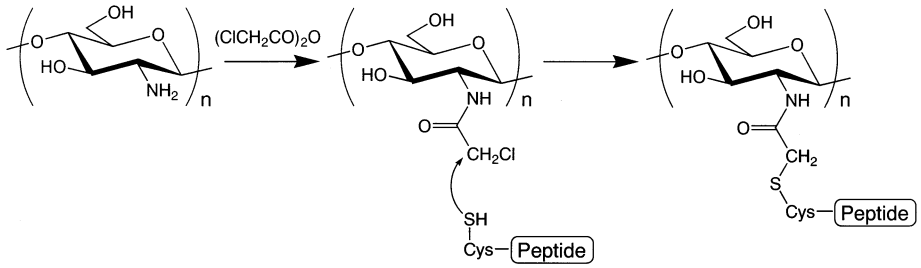
Cell Attachment Assay Using Recombinant Proteins and Synthetic Peptides. Cell attachment was assayed in 96-well plates (Nunc, Naperville, IL). The recombinant proteins in PBS (50 μL) were added to 96-well plates and incubated overnight at 4 °C. Synthetic peptides were dissolved in Milli-Q water, and the solution (50 μL) was added to each well, followed by drying overnight. After coating proteins or peptides, the plates were blocked with 1% heat-denatured bovine serum albumin (BSA, Sigma) in DMEM at 37 °C for 1 h and then washed with 0.1% BSA in DMEM. PC12 cells were harvested by agitation and allowed to recover in cultured medium for 30 min at 37 °C. After washing 3 times with 0.1% BSA in DMEM, cells were resuspended in 0.1% BSA in DMEM, plated at 2.0×10^4 cells/well, and incubated at 37 °C for 1 h in 5% CO_2. Medium was gently removed, and the attached cells were stained with 0.2% crystal violet aqueous solution in 20% methanol for 10 min. After washing with water, 1% SDS solution was used to dissolve the cells, and the optical density at 570 nm was measured in a model 550 microplate reader (Bio-Rad Laboratories, Hercules, CA).

In peptide inhibition experiments, 96-well plates were coated with rec-α3LG4 (0.2 μg/well) as described above. PC12 cells were preincubated with peptides (100 μg/mL) in 0.1% BSA in DMEM at 37 °C for 10 min, and then PC12 cells (2.0×10^4 cells/well) were added to the plates and incubated at 37 °C for 30 min in 5% CO_2. The attached cells were quantitated as described above.

Neurite Outgrowth Assay Using Recombinant Proteins and Synthetic Peptides. The neurite outgrowth assay using proteins and peptides was performed in 96- and 24-well plates (Nunc), respectively. The 96-well plates were coated with proteins the same as described above. The peptides in Milli-Q water (500 μL) were added to each well, followed by drying overnight. After coating proteins or peptides, the plates were washed with DMEM/F12 (Gibco). PC12 cells were primed with 100 ng/mL of 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 3 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 (Wako, Osaka, Japan), 5 μg/mL insulin (Gibco), and 100 ng/mL NGF. The cells were added to 96-well plates and 24-well plates at 3.0×10^3 and 2.0×10^4 cells/well, respectively. 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. In each well (three fields), 100 cells were viewed, and the percent which had neurites that extended 2 times the cell diameter in length or longer was determined and averaged for each peptide amount tested.

Coating Efficiency. Coating efficiency was determined as described previously (*23*). The synthetic peptides (5 μg/well) were added to 6 wells of the 96-well plates and dried at room temperature overnight. Half of the peptide-coated wells were gently washed 3 times with Milli-Q water (200 μL each). Coated peptides were extracted twice with PBS (200 and 100 μL) containing 3% Triton X-100. Fluorescamine in acetone (20 μL, 1 mg/mL) was added to the extracted solutions (total 300 μL), and fluorescence emission at 475 nm was determined using a F-4500 fluorescence spectro-

Scheme 1: Preparation Scheme of Peptide-Conjugated Chitosan Membrane



photometer (Hitachi, Tokyo, Japan), 400 μL cuvette, with excitation at 396 nm. Coating efficiencies (%) were determined using the fluorescence intensity of Mill-Q water-washed samples relative to nonwashed samples from the wells.

Preparation of Chloroacetylchitosan. Chitosan-10 (0.5 g, M_w = 100 000, 80% deacetylation, Wako) was dissolved in 10% AcOH solution (10 mL) and methanol (40 mL). Then, 4-(dimethylamino)pyridine (0.1 g, 0.8 mmol) and monochloroacetic anhydride (1.35 g, 8 mmol) were added and stirred overnight at room temperature. The solution was suspended in Milli-Q water (100 mL) and was neutralized at pH 6.5 by 10% NaOH solution. The resulting precipitate was collected by centrifugation and washed with Milli-Q water (30 mL × 5 times) (yield: 0.38 g). The precipitate was dissolved in 4% AcOH solution, filtrated using a syringe filter (0.45 μm, Iwaki, Tokyo, Japan), and lyophilized. The modification of a chloroacetyl group onto chitosan was confirmed by the KBr method using a RT-210 Fourier transform infrared spectrometer (Horiba, Kyoto, Japan) (*24*). Chloroacetylation and peptide coupling reactions are described in Scheme 1.

Cell Attachment Assay Using Peptide-Conjugated Chitosan Membranes. For the conjugation of peptides to chitosan membranes, we synthesized Cys-peptides (Cys-A3G75, CKNSFMALYLSKG; Cys-A3G83, CGNSTISIRAPVY; Cys-A3G75S, CLSKGKNSFMALY; Cys-A3G83S, CYVPARISITSNG). Cys at the N-terminus was used for coupling with chloroacetylchitosan (Scheme 1). Chloroacetylchitosan in 4% AcOH solution (50 μL, 0.2 mg/mL) was added to 96-well plates and dried overnight at room temperature. The plates were washed with 1% $NaHCO_3$ solution and PBS. The Cys-peptides in 0.1% TFA solution (50 μL) and 1% $NaHCO_3$ solution (50 μL) were added to each well and incubated for 2 h at room temperature. In the wells with chitosan membrane alone, for capping of the chloroacetyl group on the chitosan membrane, 0.1% TFA solution (50 μL) including 2% mercaptoethanol and 1% $NaHCO_3$ solution (50 μL) were added and incubated for 20 min. Resulting peptide-conjugated chitosan membranes in 96-well plates were used for cell attachment assays as described above. The amounts of coupled peptide were determined by amino acid analysis. The maximum peptide amount was 31 μmol/g of chloroacetylchitosan.

In heparin and EDTA inhibition experiments, 2.5 μg/well of Cys-peptide was added to the chloroacetylchitosan membrane (10 μg/well). PC12 cells were preincubated for 10 min at 37 °C with 0.1−10 μg/mL heparin or 5 mM EDTA prior to plating in the wells. After a 30 min incubation, the attached cells were measured as described above.

Neurite Outgrowth Assay Using Peptide-Conjugated Chitosan Membranes. Chloroacetylchitosan in 4% AcOH solution (500 μL, 0.1 mg/mL) was added to 24-well plates. After drying overnight, the plates were washed with 1% $NaHCO_3$ solution and PBS. The Cys-peptides in 0.1% TFA solution (500 μL) and 1% $NaHCO_3$ solution (500 μL) were added to each well, and the plates were incubated for 2 h at room temperature. Resulting peptide-conjugated chitosan membranes on 24-well plates were used for neurite outgrowth assays as described above.

RESULTS

PC12 Cell Attachment and Neurite Outgrowth Activities of Recombinant Proteins. Three recombinant proteins, which contained LG4 and/or LG5 modules of the human laminin α3 chain, were prepared using a mammalian cell expression system. The rec-α3LG4−5 protein showed a single band at 80 kDa in SDS−PAGE (Figure 1A). The rec-α3LG4 and rec-α3LG5 proteins also showed a single band in SDS−PAGE as described previously (*19*). Cell attachment activity of the recombinant proteins was evaluated using rat pheochromocytoma PC12 cells. PC12 cells attached to rec-α3LG4−5 and rec-α3LG4 in a dose-dependent manner but did not attach to rec-α3LG5 (Figure 1B). The rec-α3LG4−5 and rec-α3LG4 proteins also promoted neurite outgrowth of PC12 cells, but rec-α3LG5 was not active (Figure 1C). These results indicate that the laminin α3 chain LG4 module promotes PC12 cell attachment and neurite outgrowth, and that there are active sequences contained in the module. We used the rec-α3LG4 protein for further experiments in this study.

PC12 Cell Attachment Activity of Synthetic Peptides. We prepared 22 overlapping peptides, which covered the human laminin α3 chain LG4 module to identify PC12 cell attachment sites (Figure 2). PC12 cell attachment to the 22 peptides was evaluated on peptide-coated plastic plates. A3G75 (KNSFMALYLSKG, α3 chain 1411−1422) showed strong cell attachment activity in a dose-dependent manner (Figure 3A). A3G83 (GNSTISIRAPVY, α3 chain 1476−1487) also promoted PC12 cell attachment but was weaker than that of A3G75. The remainder of the peptides did not promote cell attachment in this assay (data not shown). A3G75S (LSKGKNSFMALY) and A3G83S (YVPARISITSNG), scrambled peptides of A3G75 and A3G83, respectively, were also inactive.

Next, the effect of the active peptides on PC12 cell attachment to the rec-α3LG4 protein was examined (Figure 3B). A3G75 and A3G83 inhibited the cell attachment mediated by rec-α3LG4. The scrambled peptides A3G75S and A3G83S did not affect the cell attachment mediated by rec-α3LG4. These results suggest that the cell attachment activity of A3G75 and A3G83 was sequence-specific and

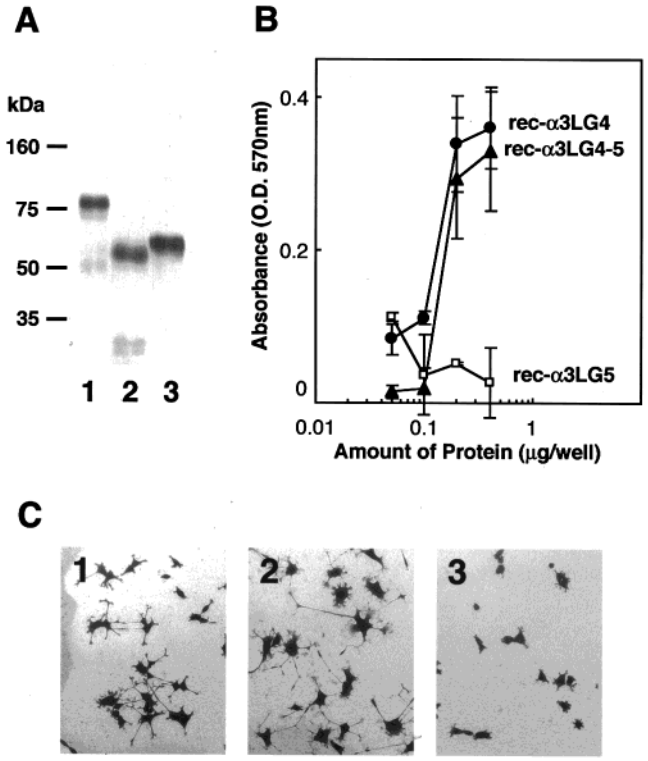


FIGURE 1: Recombinant proteins and their PC12 cell attachment and neurite outgrowth activity. (A) Recombinant proteins were prepared using a mammalian cell expression system as described under Materials and Methods. Recombinant proteins were detected with biotinylated anti-human IgG and streptavidin-peroxidase in Western blotting. Lane 1, rec-α3LG4−5; lane 2, rec-α3LG4; lane 3, rec-α3LG5. (B) 96-well plates were coated with various amounts of proteins. PC12 cells (2.0×10^4 cells/well) were incubated for 1 h, and the number of attached cells was assessed by staining with crystal violet. Data are expressed as mean ± SD of triplicate results. Triplicate experiments gave similar results. (C) Neurite outgrowth of PC12 cells on recombinant proteins. PC12 cells (3.0×10^3 cells/well) were cultured on various recombinant proteins (0.4 μg/well) for 24 h, stained with crystal violet, and photographed with a 200× objective on a microscope. 1, rec-α3LG4−5; 2, rec-α3LG4; 3, rec-α3LG5.

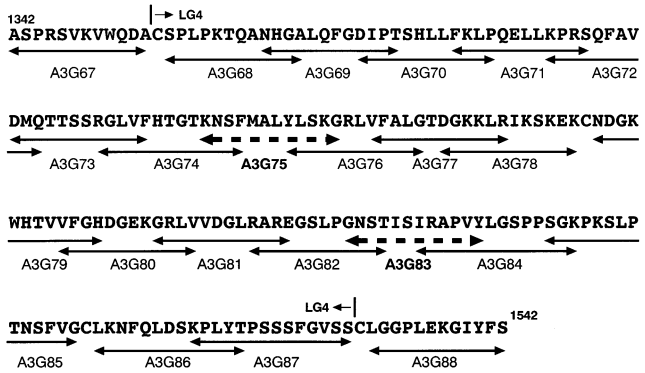


FIGURE 2: Sequence and peptides from the laminin α3 chain LG4 module. Sequences were derived from the human laminin α3 chain (*6*). Locations of peptides are indicated by arrows. Active peptides are shown by boldface dashed lines.

that these sites may be involved in PC12 cell attachment to the laminin α3 chain LG4 module.

Neurite Outgrowth Activity of Synthetic Peptides. A3G75 and A3G83 peptides were tested for their neurite outgrowth activity (Figure 4). As a positive control, AG73 (RKRLQVQLSIRT), a neurite outgrowth promoting peptide located in the

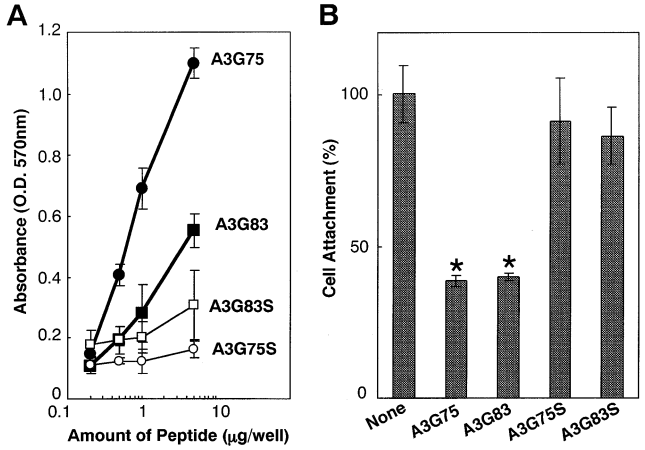


FIGURE 3: Attachment of PC12 cells to synthetic peptides (A) and inhibitory effect of synthetic peptides on PC12 cell attachment to rec-α3LG4 (B). (A) Various amounts of peptides were coated on 96-well plates. PC12 cells (2.0×10^4 cells/well) were seeded in the plates, and the number of attached cells (after 1 h) were assessed by staining with crystal violet. Data are expressed as mean ± SD of triplicate results. Triplicate experiments gave similar results. (B) 96-well plates were coated with rec-α3LG4 (0.2 μg/well). PC12 cells (2.0×10^4 cells/well) were preincubated with each synthetic peptide (100 μg/mL) for 10 min and added to the rec-α3LG4-coated plates. After a 30 min incubation, the number of attached cells was assessed by staining with crystal violet. Rec-α3LG4 attachment levels were normalized at 100%. Data are expressed as mean ± SD of triplicate results. Triplicate experiments gave similar results. *, $p < 0.01$.

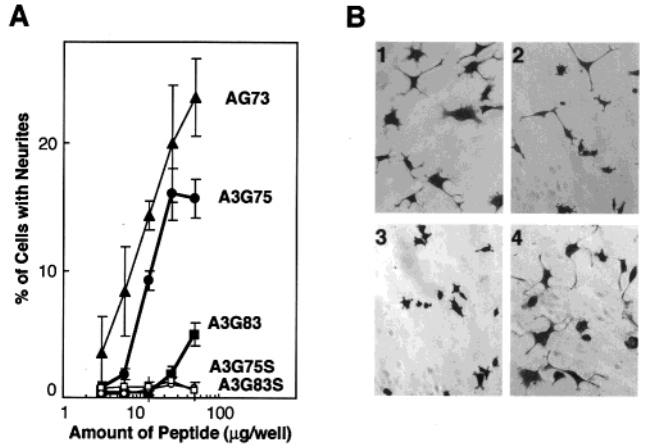


FIGURE 4: Neurite outgrowth of PC12 cells on synthetic peptides. (A) Various amounts of peptides were coated on 24-well plates. PC12 cells (2.0×10^4 cells/well) were seeded in the plates. After a 24 h incubation, cells were fixed and stained. The percentage of PC12 cells with neurites was determined as described under Materials and Methods. Data are expressed as mean ± SD of triplicate results. Triplicate experiments gave similar results. AG73 (RKRLQVQLSIRT), which is located on the laminin α1 chain LG4 module and has been found to promote PC12 cell neurite outgrowth (*25*−*28*), was used as a positive control. (B) The PC12 cells were cultured on various peptides (50 μg/well) for 24 h, stained with crystal violet, and then photographed with a 200× objective on a microscope. 1, A3G75; 2, A3G83; 3, A3G75S; 4, AG73.

α1 chain LG4 module, was used (*25*−*28*). A3G75 showed dose-dependent neurite outgrowth activity similar to that of AG73 (Figure 4). A3G83 also weakly promoted neurite outgrowth, but the scrambled peptides were inactive. These results suggest that the A3G75 and A3G83 sequences are important for neurite outgrowth activity in the laminin α3 chain LG4 module.

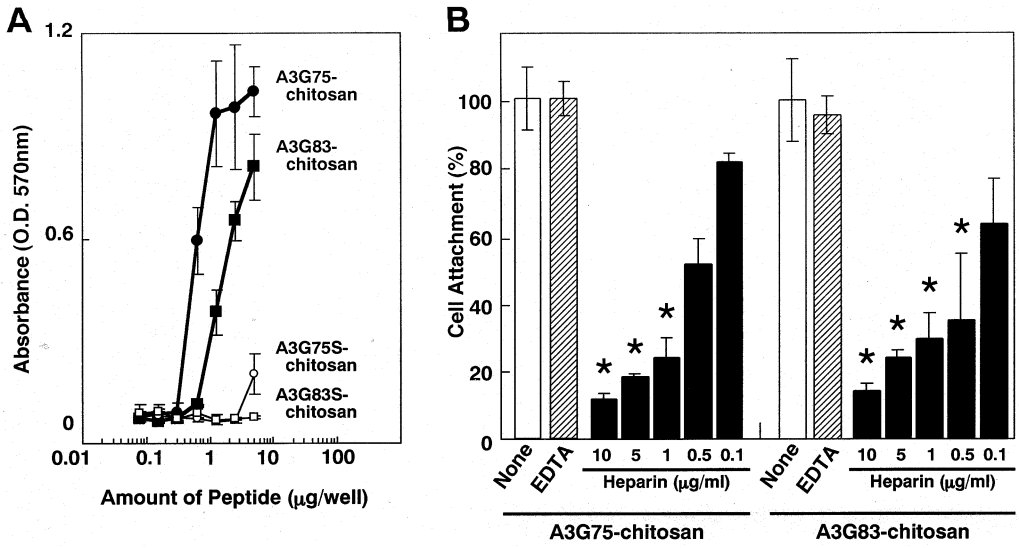


FIGURE 5: PC12 cell attachment to peptide-conjugated chitosan membranes (A) and inhibitory effect of heparin and EDTA on cell attachment (B). (A) The peptide-conjugated chitosan membranes on 96-well plates were prepared as described under Materials and Methods. PC12 cells (2.0×10^4 cells/well) were added to the plates, and the number of attached cells (after 1 h) was assessed by staining with crystal violet. Data are expressed as mean ± SD of triplicate results. Triplicate experiments gave similar results. (B) In heparin and EDTA inhibition experiments, 2.5 μg/well Cys-peptide was added to the chloroacetylchitosan membrane (10 μg/well) on 96 well plates. PC12 cells (2.0×10^4 cells/well) were preincubated with heparin (0.1−10 μg/mL) or EDTA (5 mM) for 10 min. After a 30 min incubation, attached cells were assessed by staining with crystal violet. Cell attachment activity in the absence of heparin and EDTA was taken as 100%. Data are expressed as mean ± SD of triplicate results. Triplicate experiments gave similar results. *, $p < 0.01$.

PC12 Cell Attachment Activity of the Peptide-Conjugated Chitosan Membranes. Peptides easily degrade in vivo and are difficult to localize in particular tissues. Modifications of the peptides are therefore required for therapeutic applications. We prepared peptide-conjugated chitosan membranes to further investigate the biological activities of peptides and to apply for tissue regeneration and engineering. PC12 cells attached to A3G75- and A3G83-chitosan membranes in a dose-dependent manner (Figure 5A). PC12 cells did not attach on chitosan membranes alone or on mercaptoethanol-coupled chloroacetylchitosan membranes (data not shown). The A3G75S- and A3G83S-conjugated chitosan membranes were also inactive (Figure 5A). These results indicate that the PC12 cell attachment activity of the A3G75 and A3G83 peptides was retained in the peptide-conjugated chitosan membranes. The peptide-conjugated chitosan membranes were more potent for cell adhesion than the peptide-coated plates (Figure 3A and Figure 5A).

Next, we tested the effect of heparin and EDTA on PC12 cell attachment to the peptide-conjugated chitosan membranes (Figure 5B). Cell attachment to both A3G75- and A3G83-chitosan membranes was inhibited by addition of heparin. Heparin inhibited the cell attachment to the peptide-chitosan membranes in a dose-dependent manner (Figure 5B). EDTA (5 mM) did not affect cell attachment to A3G75- and A3G83-chitosan membranes. These results suggest that heparin plays a critical role in PC12 cell attachment to A3G75- and A3G83-chitosan membranes.

Neurite Outgrowth Activity of the Peptide-Conjugated Chitosan Membranes. The A3G75- and A3G83-chitosan membranes were tested for neurite outgrowth activity. The A3G75-chitosan membrane promoted neurite outgrowth in a dose-dependent manner (Figure 6). The A3G83-chitosan membrane also weakly promoted neurite outgrowth. The scrambled peptide-conjugated chitosan membranes (A3G75S- and A3G83S-conjugated chitosan membranes) were inactive. Chitosan membrane and mercaptoethanol-coupled chloroacetylchitosan membrane also did not show the activity (data not shown). These results indicate that the neurite outgrowth promoting activity of the A3G75 and A3G83 peptides was maintained when they were conjugated to a chitosan membrane.

When 50 μg of A3G75 and A3G83 was dried on the plates and washed, the coating efficiencies of A3G75 and A3G83 were 6.4% (3.2 μg/well) and 3.8% (1.9 μg/well), respectively. A3G75 (3.2 μg/well) and A3G83 (1.9 μg/well) promoted 16% and 5% neurite outgrowth of PC12 cells, respectively (Figure 4A). In contrast, when Cys-peptide (3 μg/well) was added onto a chloroacetylchitosan membrane-coated well, 2.2 μg of peptide was found to be covalently conjugated (amount of peptide was determined by amino acid analysis as described under Materials and Methods). A3G75- and A3G83-chitosan membranes (2.2 μg of peptide/well) showed 30% and 7% neurite outgrowth activity, respectively (Figure 6A). These results indicate that the peptide-coupled chitosan membranes can promote PC12 cell neurite outgrowth more potently than that on the peptide-coated plates.

DISCUSSION

We have identified neurite outgrowth promoting sites on the human laminin α3 chain LG4 module using recombinant proteins and synthetic peptides. Laminin-5 contains the processed form of the α3 chain (165 kDa) which lacks the LG4 and LG5 modules. Processed α3 chain apparently is active for neurite outgrowth via integrin α3β1 (*14*). Interestingly, laminin-5 containing the unprocessed form of the α3 chain (190 kD) is present in the floor plate of the developing neural tube (*18*). This observation suggests that LG4 and LG5 modules may have functional roles for neural cells. Here, we found the rec-α3LG4−5 and rec-α3LG4 proteins promoted PC12 cell attachment and neurite outgrowth. Two LG4 peptides, A3G75 and A3G83, promoted PC12 cell

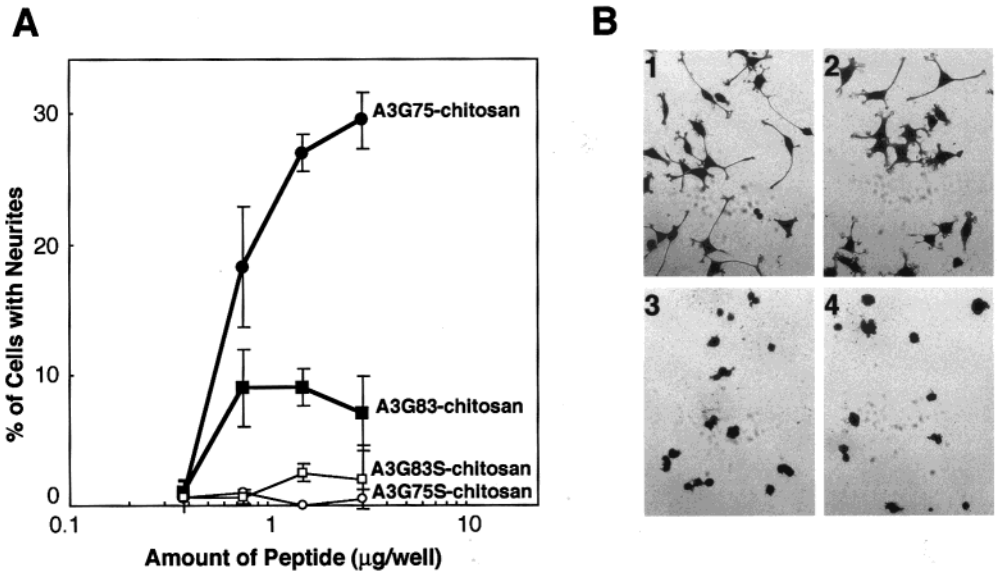


FIGURE 6: Neurite outgrowth of PC12 cells on peptide-conjugated chitosan membranes. (A) The peptide-conjugated chitosan membranes on 24-well plates were prepared as described under Materials and Methods. PC12 cells (2.0×10^4 cells/well) were added to the plates. After a 24 h incubation, cells were fixed and stained. The percentage of PC12 cells with neurites was determined as described under Materials and Methods. Data are expressed as mean ± SD of triplicate results. Triplicate experiments gave similar results. (B) The PC12 cells were cultured on peptide-conjugated chitosan membranes for 24 h, stained with crystal violet, and then photographed with a 200× objective on the microscope. In this assay, 3 μg/well of Cys-peptide was added to the chloroacetylchitosan membrane (50 μg/well) on 24-well plates. 1, A3G75; 2, A3G83; 3, A3G75S; 4, A3G83S.

attachment and neurite outgrowth in a dose-dependent manner. Both peptides also inhibited PC12 cell attachment to rec-α3LG4. These results suggest that the A3G75 and A3G83 sequences are involved in the cell attachment and neurite outgrowth activities of the laminin α3 chain LG4 module.

Previously, we demonstrated that the human laminin α3 chain LG4 module and A3G75 sequence bound to syndecan-2 on human fibroblasts (*19*). It is likely that PC12 cells also bind to A3G75 via cell surface syndecans, since heparin inhibited PC12 cell attachment to A3G75 and A3G83. Syndecan-2 induces the maturation of dendritic spines in rat hippocampal neurons (*29*, *30*). Syndecan-2 is also expressed in the floor plate of the neural tube (*31*). These observations suggest that syndecan-2 is involved in the promotion of neurite outgrowth. Furthermore, syndecans play a critical role in tissue repair and regeneration (*32*). The A3G75 and A3G83 peptides have the potential to interact with syndecans and promote nerve regeneration in vivo.

The IKVAV (α1 chain 2124−2128), P20 (RDNIAEJKDI, γ1 chain 1575−1584), LRE (β2 chain 1703−1705), and AG73 (RKRLQVQLSIRT, α1 chain 2719−2730) sequences have previously been found to promote neurite outgrowth (*25*−*28*, *33*−*36*). A3G75 and A3G83 also promoted cell attachment and neurite outgrowth, suggesting that these peptides are useful for development of therapeutic reagents. In this study, we prepared peptide-conjugated chitosan membranes for potential application in tissue regeneration and engineering. The chitosan conjugates were more potent than the peptides alone, and have several advantages. The peptides were covalently bound to the chitosan membrane and thus do not come off the dish. The coating efficiency of the peptides conjugated on the chitosan membranes was higher than that of the peptides coated on the plastic plates. These results suggest that peptides conjugated on chitosan membranes could remain at target tissue sites for longer times and sustain biological functions. The peptides on the chitosan membranes are highly flexible and may interact more effectively with cellular receptor(s) than free peptides. Chitosan membranes can form a gel and may serve as a matrix for cells. Chitosan membranes also promote wound healing (*37*), and have been used for medical applications, such as suture thread and artificial skin (*38*−*40*). We used monochloroacetic anhydride as a cross-linking reagent for coupling of Cys-peptide to chitosan membrane. The chloroacetyl group has low toxicity. Therefore, the A3G75- and A3G83-conjugated chitosan membranes have a potential ability to serve as bio-materials for tissue engineering and therapeutic use including nerve regeneration.

In summary, we have shown that there are neurite outgrowth promoting sites (A3G75 and A3G83) on the human laminin α3 chain LG4 module. These peptides have the potential to interact with syndecans on PC12 cells for cell attachment and neurite outgrowth. Furthermore, we have described the biological activity of the A3G75- and A3G83-conjugated chitosan membranes. A3G75- and A3G83-chitosan membranes may be useful for the development of bio-medical materials and therapeutic reagents.

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