An IKLLI-containing peptide derived from the laminin α 1 chain mediating heparin-binding, cell adhesion, neurite outgrowth and proliferation, represents a binding site for integrin α 3 β 1 and heparan sulphate proteoglycan

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We synthesized and characterized several peptides containing the IKLLI sequence in the α 1 chain of laminin-1. The IKLLI-containing peptides, such as LA4 (CSRNLSEIKLLISRARK), LA5 (EIKLLIS) and LA5L (SEIKLLIS), were found to mediate heparin binding and cell adhesion, while also promoting neurite outgrowth in PC12 cells. Furthermore, peptides LA4 and LA5 also mediated proliferation. However, a scrambled peptide, LA5S (ILEKSLI), did not show any of these activities. Anti-LA4 antibodies inhibited laminin- and LA5-mediated cell adhesion and neurite outgrowth, and anti-(integrin α 3) and anti-(integrin β 1) antibodies inhibited LA5-mediated cell adhesion and neurite outgrowth. Heparin and heparan sulphate inhibited LA5-mediated heparin binding and PC12 cell adhesion in a dose-

dependent manner. The IC₅₀ for inhibition of heparin binding and cell adhesion was observed with 9 μ M and 8 μ M heparin/heparan sulphate respectively. Furthermore, heparan sulphate proteoglycan also inhibited LA5-mediated PC12 cell adhesion with an IC₅₀ of 100 μ g/ml. However, chondroitin sulphate (dermatan sulphate) did not inhibit cell adhesion. These data suggest that an IKLLI-containing peptide derived from the laminin α 1 chain may be an active site of laminin and that its cell adhesion may thus interact with both integrin α 3 β 1 and cell-surface heparan sulphate proteoglycan.

Key words: cellular physiology, coiled-coil domain, flexibility, instability.

INTRODUCTION

Laminin is a large glycoprotein (molecular mass of 900 kDa) found only in the basement membrane [1,2] where it is a major component. Laminin-1 (Engelbreth–Holm–Swarm, EHS laminin) from the mouse EHS tumour consists of three chains designated α 1 (400 kDa), β 1 (210 kDa) and γ 1 (200 kDa), which are arranged in a cross-shaped structure [3–5]. Laminin mediates cellular interactions with the basement membrane including cell adhesion, migration, proliferation, differentiation, metastasis, collagenase IV activity, and neurite outgrowth [6–13]. Laminin-1 also has the ability to bind to heparin [12].

Laminin-1 contains a variety of potential cell-binding sequences, and seventeen different cell-adhesion sites have been reported in the α 1, β 1 and γ 1 chains (Figure 1) [14–24]. Several cell surface proteins binding to laminin-1 have been reported as follows: a non-conserved RGD site in the α 1 chain interacts with integrin α 3 β 1 [25] and α 6 β 1 [15]; the GD-6 peptide (KQNCLSSRASFRGCVRLRLSR) in the α 1 chain interacts with α 3 β 1 [19]; two peptides, YIGSR and LGTIPG, in the β 1 chain probably interact with a 67 kDa binding protein [21]; peptide IKVAV in the α 1 chain interacts with a 110 kDa protein [26].

The IKVAV-containing peptide, PA (SRNLSEIKLLISRARKQAASIKVAVSADR), stimulated thymidine incorporation at a concentration of 100 μ g/ml [27]. In addition, laminin and PA

rapidly and transiently induced c-Fos and c-Jun mRNA expression in PC12 cells [27]. PA also contains the IKLLI sequence. Tashiro et al. also reported that an IKLLIS peptide inhibited laminin-mediated cell adhesion in PC12 cells and thus suggested that the IKLLI sequence may be one of the adhesion sites of laminin [16]. However, an IKLLI-containing peptide has yet to be reported with other biological activities, especially heparin binding.

In the present study, four different sizes of IKLLI-containing peptides (LA4, LA5L, LA5 and LA6) were synthesized and tested for various biological activities. These results showed an IKLLI-containing peptide to be one of a number of sites in laminin that mediated heparin binding, cell adhesion, neurite outgrowth and neuronal cell proliferation, and that its cellular interaction may thus be related to both integrin α 3 β 1 and a cell-surface heparan sulphate proteoglycan.

MATERIALS AND METHODS

Synthetic peptides and laminin

Purified synthetic peptides were purchased from Takara Shuzo (Shiga, Japan). Amino acid analysis and automated amino acid sequence analysis of the peptides were performed with an HPG1005A protein sequencing system (Hewlett–Packard, Palo Alto, CA, U.S.A.).

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; EHS, Engelbreth–Holm–Swarm; EMEM, Eagle's minimal essential medium.

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Figure 1 Structural model of laminin and cell binding sites

The location of the IKLLI-containing peptide used in the present study is indicated by larger bold-face type and an arrow. In addition, the locations of other reported cell binding sites (and their references) are also indicated.

Table 1 Synthetic peptides and their amino acid sequence

C*, cysteine was included to facilitate coupling but is not part of the actual sequence; **, a cell adhesion peptide containing the IKVAV sequence reported previously [14].

Peptide	Residues	Sequences
LA4	2080–2095	C*SRNLSEIKLLISRARK
LA5L	2084–2091	SEIKLLIS
LA5	2085–2091	EIKLLIS
LA6	2086–2091	IKLLIS
LA5S	scrambled	ILEKSLI
PA22**	2116–2133	SRARKQAASIKVAVSADR

Each peptide was prepared in the amide form and contained amino acid compositions derived from the laminin $\alpha 1$ chain as shown in Table 1. The LA4 peptide included a cysteine residue to facilitate coupling. The average yields for all syntheses ranged from 95–98%. The peptides possessing biological activity were further purified using preparative HPLC when analytical HPLC indicated the product to be heterogeneous.

The amino acid composition of LA5 (EIKLLIS) was determined (calculated) to be: Ser 0.9 (1), Glu 1.05 (1), Lys 1.02 (1), Ile 1.85 (2) and Leu 2.0 (2). The molecular mass was estimated to be 815.01 Da. HPLC was carried out on an Ultrasphere column [Octadecylsilyl (ODS), 0.46 cm $\times$ 15 cm, C₁₈] with a linear gradient elution by 0–40% acetonitrile in 0.1% trifluoroacetic acid for

Table 2 Amino acid composition of LA5 derived from the $\alpha 1$ chain of laminin-1

Asn, Gln, Arg, Pro, Met, Trp were measured as 0.0; DPTU, diphenylthiourea.

Amino acid	Order of amino acid residues									
	1	2	3	4	5	6	7	8	9	10
Asp	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Glu	132.2	35.7	1.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Ser	0.6	0.0	0.0	0.0	0.0	0.0	20.3	12.5	5.1	2.5
Thr	3.1	1.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Gly	1.4	1.2	1.0	1.1	0.9	1.1	0.9	0.9	0.7	0.8
His	0.0	0.9	0.0	0.0	0.0	0.0	1.0	0.6	0.0	0.0
Ala	0.7	0.0	0.3	0.0	0.0	0.7	2.2	2.0	0.8	0.7
Tyr	0.0	0.0	4.1	1.0	0.0	0.0	0.0	0.0	0.0	0.0
Val	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.0
Lys	0.0	0.0	65.9	16.7	2.1	0.0	0.0	0.0	0.0	0.0
Phe	0.0	31.0	6.5	1.6	2.7	19.8	8.5	1.5	0.0	0.0
Ile	0.0	37.3	7.9	1.2	0.0	23.2	8.7	1.4	0.0	0.0
Leu	0.0	2.6	1.4	64.1	70.2	18.3	4.7	0.9	0.0	0.0
DPTU	18.2	15.0	14.0	14.8	15.7	15.3	16.9	21.2	16.2	19.8

20 min at a flow rate of 1 ml/min. The analysis data showed that the first peak was Glu (132.2), the second Ile (37.3), the third Lys (65.9), the fourth Leu (64.1), the fifth Leu (70.2), the sixth Ile (23.2), and the seventh Ser (20.3) (Table 2). The arrangement of LA5 was shown to be EIKLLIS.

Mouse laminin (laminin-1) was extracted and purified from the EHS tumour using methods described previously [1].

Preparation of an anti-LA4 antibody

Both the conjugation of LA4 and the preparation of antiserum were performed as described previously [16]. Furthermore, the antiserum against LA4 was purified to IgG with a Protein-G affinity column (HiTrap Protein G, 1 ml, Pharmacia Biotech).

Anti-LA4 IgG (1 μ g/ml) was reacted with native laminin-1 (1 ng) and LA5 (10 ng) in a dot blot analysis. Western blot analysis of laminin-1 and EHS tumour homogenate, using the anti-LA4 IgG antibody, detected a 400 kDa protein, which was the $\alpha 1$ chain of laminin-1 (results not shown).

Heparin-binding assay

The binding of [³H]heparin (NET-467, heparin sodium salt, [G-³H], DuPont NEN) to various peptides was measured by direct solid-phase binding assays as described previously [19,28]. Various amounts of the peptides (0.1, 1, 10 or 25 μ g) were added to each well in 50 μ l (final volume) of distilled water and then air dried. The wells were then treated for 2 h at 37 °C with 200 μ l of wash buffer (6 mM phosphate, pH 6.8/150 mM NaCl/60 μ M CaCl₂) containing BSA (2 mg/ml) to minimize non-specific binding.

[³H]Heparin (50 μ l; 1.11 $\times$ 10³ kBq/ml in wash buffer) was added to each well (50000 d.p.m./well) and the wells were incubated for 2 h at 37 °C. The wells were rinsed three times with wash buffer containing 0.05% Triton X-100 and then incubated for 30 min at 60 °C with 200 μ l of 0.5 M NaOH and 1% (w/v) SDS. The amount of bound [³H]heparin was quantified in Ecolite scintillation fluid (Aquasol-2; NEN Research Products), and counted in a liquid-scintillation counter (TRI-CARB 460 CD; Packard).

Effect of peptides on laminin-mediated heparin-binding

In order to inhibit laminin-mediated heparin-binding using the peptides, each well of the 96-well polystyrene plates was coated with 2 μg of laminin-1 as described earlier. The peptides were used at various dilutions ranging from 0.2 to 1 mg/ml. The percentage inhibition of bound [^3H]heparin was determined by, [^3H]heparin bound to laminin treated with peptide (d.p.m.)/[^3H]heparin bound to laminin (d.p.m.) $\times 100$.

Specificity of peptide binding to heparin

To determine the importance of the heparin structure for this interaction, 5 μg of LA4 or LA5 per well was dried on to a 96-well plate as described earlier. The wells were blocked for 2 h with 2 mg/ml of BSA in wash buffer, and 50 μl of [^3H]heparin (25000 d.p.m./well, 60 nM final concentration) was added to each well concomitantly with various concentrations (1 nM to 100 μM) of unlabelled heparin (heparin sodium salt, Sigma), heparan sulphate (Sigma) and chondroitin sulphate (dermatan sulphate, Sigma). After incubating for 2 h at 37 °C, the wells were washed and the radioactivity was determined as described earlier.

Cells and culture

Various cells were studied including B16-F10 mouse melanoma cells [29], HT-1080 human fibrosarcoma cells [30], NRK-49F rat kidney fibroblast cells (CRL 1570; A.T.C.C., Rockville, MD, U.S.A.), WI-38 human lung fibroblast cells (CCL 75; A.T.C.C.), PC12 rat pheochromocytoma cells, and NG108-15 murine neuronal-glia hybrid cells.

For the adhesion assays, B16-F10 cells, HT-1080 cells, WI-38 cells, NRK-49F cells and NG108-15 cells were washed with PBS, pH 7.4, lacking Ca^{2+} and Mg^{2+} , and then detached using 0.025 % trypsin/0.025 % EDTA (GIBCO). The cells were sedimented by low-speed centrifugation (100 g, 5 min), resuspended in serum-free Eagle's minimal essential medium (EMEM) containing 0.02 % BSA, and then used immediately in the cell adhesion assay.

Cell-adhesion assay

The cell-adhesion assays were performed in 96-well tissue culture dishes as described previously [14]. Synthetic peptides were used at 0.05, 0.1, 1, 5 and 10 μg /well. The attached cells were stained with 0.1 % Crystal Violet, extracted with 0.1 % TritonX-100 and measured in a spectrophotometer at 570 nm. Each assay was carried out in duplicate, and the results did not differ by more than 10 %. The effect of peptide, heparin, heparan sulphate and chondroitin sulphate on cell adhesion was assayed in a 96-well dish coated with 0.5 μg /well of laminin, as described earlier. Prior to the addition of cells, peptide (0.1, 0.2 and 0.3 mg/ml), heparin/heparan sulphate/chondroitin sulphate (1 nM to 100 μM), solubilized in serum-free EMEM containing 0.02 % BSA, were added to each well. The cells were incubated for 60 min at 37 °C and the number of attached cells was determined as described earlier. Each assay was performed in duplicate, and each peptide was assayed five times.

Effect of anti-integrin antibodies on laminin- and LA5-mediated cell adhesion in PC12 cells

The effect of polyclonal antiserum against the integrin $\alpha 1$ (AB1934), $\alpha 2$ (AB1936), $\alpha 3$ (AB1920), $\alpha 4$ (AB1924), $\alpha 5$ (AB1928) and $\beta 1$ (AB1938) (Chemicon) on cell adhesion was assayed. The epitope recognized by each antibody is on the cytoplasmic

domain of the protein. Each antibody showed no cross-reactivity with other integrins. In addition, PC12 cell lysate was sedimented with an anti-(integrin $\alpha 3$) and anti-(integrin $\beta 1$) antibody and the findings were compared with control rabbit IgG.

A Western blot analysis of the precipitate was carried out using the same antibody. Each well of a 96-well dish was coated with either laminin (0.5 μg) or LA5 (5 μg) as described earlier. PC12 cells (5×10^3 /well) were incubated with 1:10 dilutions of each anti-integrin antibody for 15 min prior to adding the cells to the substrate-coated wells. The cells were incubated at 37 °C for 20 min and the attached cells were stained with 0.1 % Crystal Violet and counted using a microscope.

Neurite-outgrowth assay

The neurite-outgrowth assay on PC12 cells was performed as described previously [14]. PC12 cells (2.5×10^3) primed with 50 ng/ml nerve growth factor (Calbiochem) were added to each well of a 24-well dish (Costar) which had been coated with either laminin (1 μg /well) or synthetic peptides (20 μg /well). After incubation overnight, the cells were photographed and fixed with 3.7 % (w/v) formalin. The percentage of the cells with neurite processes greater than two cell-body lengths was determined for duplicate wells for each concentration. At least 100 cells per data point were counted.

Effect of an anti-LA4 antibody on laminin- and LA5-mediated cell adhesion and neurite outgrowth in PC12 cells

For the cell-adhesion assay, an antibody against LA4 was prepared as described earlier. Laminin (0.2 μg /well) and LA5 (2 μg /well) were used to coat 96-well tissue culture dishes as described earlier. The unbound surfaces were blocked with 100 μl of EMEM containing 1.5 % (w/v) BSA for 30 min at 37 °C. The wells were rinsed with PBS and then incubated at 37 °C for 3 h with three different concentrations (0.1, 1 and 10 μg /ml) of purified anti-LA4 IgG. The antibody was removed and the wells were washed with PBS. PC12 cells (4×10^4 cells/well) were added to the wells and incubated at 37 °C for 30 min. The attached cells were then counted using the staining method described earlier.

For the neurite-outgrowth assay, the wells of 24-well tissue culture dishes (Costar) were coated with either laminin (1 μg /well) or LA5 (10 μg /well) and then evaporated to dryness. Anti-LA4 IgG (312 μg in 250 μl) was added to each well and incubated at 37 °C for 3 h. Pre-immune IgG (312 μg /well) was added to substrate-coated control wells. After 3 h of incubation, PC12 cells (1×10^3) were added to each well. The PC12 cells had been primed for 24 h with nerve growth factor previously, and the neurite-outgrowth assay was performed as described earlier. After 18 h of incubation, the neurite outgrowth was evaluated.

Assay of neuronal cell proliferation

Proliferation assays were performed in 48-well tissue-culture dishes (Costar). For both dose responses and time courses of PC12 cell proliferation, [^3H]thymidine incorporation was assessed as reported previously [31].

For the dose response assay, PC12 cells (1×10^5) were added to each well in culture medium overnight. The cells were rinsed once with serum-free medium and then cultivated for 2 days in Dulbecco's modified Eagle's medium (DMEM) containing 0.1 % BSA. The medium was exchanged for freshly prepared medium, peptides LA5 and LA5S (50, 100, 200 and 300 μg /ml) and [^3H]thymidine (20000 d.p.m./well, 37 kBq/ml in DMEM containing 0.1 % BSA) were added, and then the cells were incubated for an additional 36 h. The wells were washed once

with PBS and twice with cold 5% (v/v) trichloroacetic acid (Sigma). NaOH (0.25 M, 200 μ l) was added to the wells and then incubated for 40 min at 37 °C. Incorporated [3 H]thymidine was quantified in Ecolite scintillation fluid and counted in a liquid-scintillation counter.

For the time course assay, PC12 cells (1×10^5 /well) were allowed to attach for 15 h in standard media containing fetal-calf serum and horse serum. The unattached cells were then removed and the attached cells were starved in DMEM containing 0.1% BSA for 2 days. The medium was exchanged for the same freshly prepared medium, peptides LA5 and LA5S (each 200 μ g/ml) were added, and then the cells were incubated for an additional 48 h. [3 H]Thymidine (50 μ l, 37 kBq/ml) was added 4 h after the addition of the peptides. The first time-course sample was assayed 2 h after the addition of [3 H]thymidine. The incorporated [3 H]thymidine was counted as described earlier.

RESULTS

Binding of [3 H]heparin to synthetic peptides

The direct solid-phase binding of [3 H]heparin to peptides that had been air dried on to polystyrene plates was determined. LA4, LA5L and LA5 mediated heparin binding in a dose dependent manner, while LA6 (IKLLIS) was less active, and the scrambled peptide, LA5S, was not active (Table 3). Furthermore, an inhibition assay showed that LA4, LA5L and LA5 inhibited [3 H]heparin binding to laminin, but the scrambled peptide, LA5S, did not demonstrate any inhibition, thus confirming the results of the direct-binding assays (results not shown).

We studied the possibility that LA6 did not adhere well to plates, because this peptide is short and may not bind well to plastic, using the Ninhydrin assay [32]. These results showed that the coating ratios of LA4, LA5 and LA6 were 50%, 48% and < 3% respectively. Therefore there may be a discrepancy between data with LA6 immobilized and in solution.

The [3 H]heparin binding activity of peptides is intriguing as the short forms (LA5 and LA5L) have only a single positive charge. The binding assay was initially performed in hypotonic salt (100 mM NaCl) so we next assessed it in 150 mM NaCl. The results showed that heparin binding activity was decreased by 13% from 100 mM to 150 mM NaCl (Figure 2). Furthermore, we considered the effect of NaCl concentration (0–500 mM) on peptide–heparin binding. At a concentration of 400 mM, NaCl completely dissociated the peptide–heparin complex (Figure 2). These results gave a concentration similar to that reported by Delacoux et al. (350 mM NaCl) [33].

Figure 2 The effect of NaCl concentration on LA4 binding to [3 H]heparin

Peptide (5 μ g/well) was coated on to a 96-well plate. The range of NaCl concentration in the wash buffer (6 mM phosphate, 60 μ M CaCl₂, pH 6.8) used was from 10 to 500 mM. The heparin binding assay was performed as described in the Materials and methods section. The activity of peptide bound to [3 H]heparin in free NaCl was taken as 100%. The peptide–heparin complex was dissociated at a concentration of 400 mM NaCl.

Specificity of the interaction between LA4/LA5 and heparin

To understand the structural features of LA4/LA5 and heparin that are crucial for their interaction, structurally altered molecules were tested for their functional activity.

Heparin is composed of a number of negatively charged groups, in particular, sulphate groups. It is also important to establish whether binding occurs only to heparin, and thus we tested other glycosaminoglycans, particularly heparan sulphate and chondroitin sulphate. The results showed that both unlabelled heparin and heparan sulphate had an IC₅₀ for peptide binding to [3 H]heparin, of 9.0 μ M. However, chondroitin sulphate did not inhibit peptide binding to [3 H]heparin even at 100 μ M (Figure 3).

Effect of synthetic peptides, glycosaminoglycans and heparan sulphate proteoglycan on cell adhesion

Five different peptides (LA4, LA5, LA5L, LA5S and LA6) were tested for their ability to mediate cell spreading and adhesion of

Table 3 Effect of synthetic peptides and laminin-1 on cell attachment, spreading, neurite outgrowth and heparin binding

A, cell attachment; N, neurite outgrowth; S, spreading. Scoring based on the following subjective scale: ++, comparable with that on laminin-1; +, apparent but weaker than on laminin-1; –, not apparent.

Peptide	Cell line ...	PC12		NG108-15		B16-F10		HT-1080	NRK-49F		WI-38	Heparin binding
		A	N	A		A	S	A	A		A	
LA4		++	++	++		++	+	++	+		++	++
LA5L		+	+	+		+	+	+	+		+	+
LA5		+	+	+		+	+	+	+		+	+
LA5S		–	–	–		–	–	–	–		–	–
LA6		–	–	–		–	–	–	–		–	–
Laminin-1		++	++	++		++	++	++	++		++	++

Figure 3 Specificity of the interaction between LA4 and heparin

Peptide (5 $\mu\text{g}/\text{well}$) was coated on to a 96-well plate. [^3H]Heparin (50 μl , 25000 d.p.m./well, 60 nM final concentration) was added to each well concomitantly with various concentrations (1 nM to 100 μM) of unlabelled heparin, heparan sulphate and chondroitin sulphate. A heparin-binding assay was performed as described in the Materials and methods section. Values are means $\pm$ S.D., expressed as a percentage of the control values, for duplicate assays. Significant differences from the control (* P < 0.05, ** P < 0.01) were determined by the Student's t test. $\circ$, heparin; $\blacktriangle$, heparan sulphate; $\square$, chondroitin sulphate; uM, μM .

PC12 cells, NG108-15 cells, B16-F10 cells, HT-1080 cells, NRK-49F cells, and WI-38 cells. LA4, LA5 and LA5L were found to be biologically active for various types of cell adhesion (Table 3). LA4, LA5 and LA5L mediated cell spreading and adhesion of B16-F10 melanoma cells, and cell adhesion of PC12 cells, NG108-15 cells, HT-1080 cells, NRK-49F cells and WI-38 cells, while LA6 and LA5S did not mediate. LA4, LA5 and LA5L mediated cell adhesion of PC12 cells in a dose-dependent manner (Figure 4A). The cells attached to the peptides were expressed as a percentage of the cells attached to laminin (1 $\mu\text{g}/\text{well}$). The cell-adhesion activity with LA4 was the same or greater than that observed with laminin, and LA5L and LA5 showed approx. 80 % of cell-adhesion activity. However, LA6 and LA5S mediated neither cell spreading nor cell adhesion.

We then assessed the ability of these peptides to block laminin-mediated cell adhesion (Figure 4B). LA6 showed the same activity as LA4 and LA5 in inhibiting laminin-mediated PC12 cell adhesion by 40 %, LA5S showed no inhibition.

We investigated the physiological significance for the interaction of heparin/heparan sulphate and cells. We studied the effect of heparin/heparan sulphate/chondroitin sulphate on cell adhesion to adsorbed LA5. Secondly, we investigated whether or not heparan sulphate proteoglycan inhibits cell adhesion to the adsorbed peptide. The results showed that heparin/heparan sulphate and heparan sulphate proteoglycan inhibited peptide-mediated cell adhesion in a dose dependent manner. The IC_{50} of cell adhesion was 8 μM for heparin/heparan sulphate and 100 $\mu\text{g}/\text{ml}$ for heparan sulphate proteoglycan (results not shown). However, chondroitin sulphate did not inhibit cell adhesion even at 100 μM (results not shown).

There is only one negative control in the scrambled peptide, so we compared LA4 with an IKVAV-containing peptide (PA22) in the cell-adhesion and inhibition assays. Both LA4 and PA22 mediated PC12 cell adhesion (Figure 4A) and inhibited laminin-mediated cell adhesion by 40 % in the same manner (Figure 4B).

Figure 4 The effect of synthetic peptides on (A) cell adhesion in PC12 cells and on (B) laminin-mediated cell adhesion

(A) Tissue culture dishes (96-well) were coated with 0.05, 0.1, 1, 5 and 10 $\mu\text{g}/\text{well}$ of synthetic peptides. The cell-adhesion assay was performed as described in the Materials and methods section. (B) Various concentrations of the peptides, solubilized in serum-free EMEM containing 0.02 % BSA, were added to the wells before the addition of PC12 cells. The adhesion assay was performed as described in the Materials and methods section. Values are the means $\pm$ S.E.M., expressed as a percentage of the control values, for 4–8 determinations. Significant differences from the control (* P < 0.05, ** P < 0.01) were determined by the Student's t test. $\bullet$, LA4 + PA22; $\circ$, LA4; $\blacktriangle$, PA22; $\blacksquare$, LA5L; $\triangle$, LA5; $\square$, LA6; $\times$, LA5S; μg , μg .

We compared the effect of PA22 plus LA4 on cell adhesion. Cell adhesion mediated by the mixture of peptides showed the same dependent manner as that of each peptide individually (Figure 4A). PA22-mediated cell adhesion was inhibited by LA4 by approx. 40 %, and LA4-mediated cell adhesion was also inhibited by PA22 by approx. 40 % (results not shown). Thus these findings showed that one of the receptors for these peptides was the same as cell-surface heparan sulphate proteoglycan.

Effect of anti-integrin ($\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$ and $\beta 1$) antibodies on LA5-mediated cell adhesion in PC12 cells

An anti-(integrin $\alpha 3$) antibody (1:10 dilution) inhibited LA5-mediated cell-adhesion activity by 74.7 % compared with control rabbit IgG. Moreover, an anti-(integrin $\beta 1$) antibody inhibited

Table 4 Effect of anti-integrin antibodies on LA5-mediated cell adhesion

Each well of a 96-well dish was coated with 5 μ g of LA5. PC12 cells were incubated with a 1:10 dilution of anti-integrin serum for 15 min, and the cell-adhesion assay was performed as described in the Materials and methods section. The activity of LA5-mediated cell adhesion that was treated with control rabbit IgG (10 μ g/ml) was taken as 100%. Values, expressed as a percentage of the control, are means $\pm$ S.E.M. Significant differences from the control (* P < 0.01) were determined by the Student's *t*-test.

Antibody	Cell adhesion (% activity)
Anti-(integrin α 1)	97.1 $\pm$ 1.9
Anti-(integrin α 2)	98.0 $\pm$ 1.8
Anti-(integrin α 3)	25.3 $\pm$ 2.0*
Anti-(integrin α 4)	97.2 $\pm$ 2.0
Anti-(integrin α 5)	97.9 $\pm$ 1.5
Anti-(integrin β 1)	40.7 $\pm$ 2.1*

LA5-mediated cell-adhesion activity by 59.3%. However, other antibodies against integrin α 1, α 2, α 4 and α 5, did not inhibit LA5-mediated cell adhesion (Table 4).

To ascertain which anti-integrin antibodies detected cell-surface proteins, we sedimented PC12 cell lysates using anti-(integrin α 3) and anti-(integrin β 1) antibodies and compared these with control rabbit IgG. Western blot analysis of these precipitates was carried out using the same antibodies. These results showed that both anti-(integrin α 3) and anti-(integrin β 1) antibodies detected one band at approx. 130 kDa when the precipitates were separated by SDS/PAGE under reducing conditions. The protein size was the same as that for integrin α 3 or β 1 as reported previously [34]. However, these antibodies did not detect any protein precipitated by control rabbit IgG (results not shown). Therefore we demonstrated that the anti-(integrin α 3) and anti-(integrin β 1) antibodies bound to the rat protein. Thus we concluded that LA5 mediated adhesion by interacting not only with cell-surface heparan sulphate proteoglycan but also with integrin α 3 β 1.

Effect of LA4, LA5 and LA5S on neurite outgrowth

LA4, LA5 and LA5L promoted neurite outgrowth in PC12 cells as well as laminin did, but peptide LA6 and the scrambled peptide LA5S did not (Table 3). The neurite outgrowth of PC12 cells promoted by laminin was observed 2 h later after the addition of cells, while that promoted by peptide LA4 and LA5 was observed 15 h later.

Effect of an anti-LA4 antibody on laminin- and LA5-mediated cell adhesion and neurite outgrowth in PC12 cells

The anti-LA4 IgG antibody inhibited cell-adhesion activity and neurite-outgrowth activity, mediated by LA5, by 80% and 77% respectively (Table 5). However, this antibody only inhibited cell-adhesion activity and neurite-outgrowth activity, mediated by laminin-1, by 36% and 33% respectively (Table 5). Pre-immune IgG did not block cell adhesion and neurite outgrowth mediated by either laminin or LA5.

Effect of synthetic peptides on neuronal cell proliferation

We tested the effect of synthetic LA5 and LA5S on the growth of PC12 cells using [3 H]thymidine incorporation. The dose response assay, using [3 H]thymidine labelling, showed that LA5 increased DNA synthesis in PC12 cell culture in a dose-dependent manner with a maximum concentration of 50 μ g/ml (Figure 5A). PA22

Table 5 Effect of an anti-LA4 antibody on laminin- and LA5-mediated cell adhesion and neurite outgrowth in PC12 cells

Laminin (0.2 μ g/well) and LA5 (2 μ g/well) were coated on to a 96-well tissue culture dish for the cell-adhesion assay, while (1 μ g/well) and (10 μ g/well), respectively, were used to coat a 24-well tissue culture dish in the neurite-outgrowth assay. The wells were incubated at 37 °C for 3 h with 10 μ g/ml of anti-LA4 IgG, and cell-adhesion and neurite-outgrowth assays were performed as described in the Materials and methods section. The activities of laminin- or LA5-mediated cell adhesion and neurite outgrowth, which were treated with pre-immune IgG (10 μ g/ml), were taken as 100%. Values, expressed as a percentage of the control, are means $\pm$ S.E.M. for triplicate assays. Significant differences from the control (* P < 0.01) were determined by the Student's *t* test.

Antibody	Cell adhesion (% activity)		Neurite outgrowth (% activity)	
	Laminin-1	LA5	Laminin-1	LA5
Pre-immune IgG	98 $\pm$ 2	99 $\pm$ 1	97 $\pm$ 2	97 $\pm$ 1
Anti-LA4 IgG	64 $\pm$ 2*	20 $\pm$ 2*	67 $\pm$ 3*	23 $\pm$ 2*

also increased DNA synthesis in PC12 cell culture in the same dose-dependent manner as that of LA5 (results not shown). However, the scrambled peptide LA5S did not increase DNA synthesis.

In the time course assay, [3 H]thymidine incorporation reached a maximum at 36 h after the addition of LA5 into the medium (Figure 5B). Furthermore, LA5 stimulated the proliferation of NG108-15 cells as well as PC12 cells (results not shown). These results showed LA5 to be active in stimulating neuronal cell proliferation.

DISCUSSION

Many cell binding sites of laminin have been reported previously, using synthetic peptides [14–24]. However, the interaction of conformational structure and biological activities for laminin has not been sufficiently elucidated.

In the present study, we show an IKLLI-containing peptide to be one of the biologically active sites of laminin-1. However, an IKLLI-containing peptide from the coiled-coil region of the α 1 chain of laminin is thought to be present in a non-physiological conformation. Our findings show that an antibody against an IKLLI-containing peptide inhibits laminin-mediated cell adhesion and neurite outgrowth in PC12 cells. These results gave important data confirming the role of the peptide.

The discrepancy that a biologically active site was present in a non-physiological region may have occurred as a result of the specificity of chain recognition in laminin. Laminin consists of three polypeptide chains α 1, β 1, and γ 1 [35] which are connected in a long α -helical coiled-coil region located in the long arm of the cruciform molecule [36].

The specificity of chain recognition with its preference for heteroassembly was investigated by Hunter et al. [37,38] and Kammerer et al. [39]. They reported that the stability of the disulphide-linked α -helical coiled-coil structure, β - γ and β - β , formed from approx. 100-residue-long recumbent fragments of the β - and γ -chains, was comparable with the values determined for structures that did not show the disulphide-linked complex [40].

The recombinant α -fragments also formed a disulphide-linked complex under the reoxidation conditions described by Kammerer et al. [39]. However, this complex did not show any α -helix formation as revealed by circular dichroism [40]. In

Figure 5 (A) Dose dependence of synthetic peptides on DNA synthesis in PC12 cells, and (B) the effect of peptides on the cell cycle kinetics of PC12 cells

(A) PC12 cells were cultured in DMEM containing 0.1% BSA for 2 days, and then cultured for 36 h with [3 H]thymidine and various concentrations of LA5 and LA5S. The incorporated [3 H]thymidine were assayed as described in the Materials and methods section. (B) PC12 cells were cultured in DMEM containing 0.1% BSA for 2 days, and then cultured for 48 h with LA5 and LA5S (each 200 μ g/ml). The time-course was started by the addition of [3 H]thymidine 4 h after the addition of the peptides as described in the Materials and methods section. Values are means $\pm$ S.D. for duplicate assays. Significant differences from the control (* P < 0.05, ** P < 0.01) were determined by the Student's t test. (A) $\circ$, LA5; $\bullet$, LA5S; μ g, μ g. (B) $\circ$, LA5; $\square$, LA5S.

addition, very little or no coiled-coil formation was found for α and γ [39].

A possible advantage of disulphide bonds for stabilization compared with strong non-covalent interactions is conformational flexibility. Interaction of the α -fragment with double-stranded β - γ coiled-coils, reflected the formation of a three-stranded coiled-coil in laminin, but for the small recombinant fragments, association between α - and β -homoassociates was also observed [39]. The C-terminal 100 residues in the coiled-coil domain were therefore not solely responsible for the high specificity of chain selection in laminin [39]. Furthermore, the characteristic feature of the sequence in a chain with a potential

for coiled-coil formation is a heptad repeat a-b-c-d-e-f-g in which hydrophobic and non-polar residues occur in positions 'a' and 'd' [41]. These residues were involved in the stabilization of the coiled-coil domains by hydrophobic interactions [42] but ionic interactions between residues of different chains also played an important role [42,43]. The IKVAV and IKLLI sequences derived from the $\alpha 1$ chain of laminin were contained within the C-terminal 100 residues, and these residues were included in positions 'a' and 'd'. These findings may also support the active role for these peptides in cellular physiology. The α and γ -chains alone did not form stable α -helical coiled-coil structures. The instability and flexibility of the $\alpha 1$ chain may interfere with the cell biological activities.

An antibody against an IKLLI-containing peptide partially inhibited laminin-1-mediated cell adhesion and neurite outgrowth. These results show that the site of IKLLI residues of the $\alpha 1$ chain may be unstable and flexible for the cells while other cell-adhesion sites may also exist in laminin. In addition, an antibody against an IKLLI-containing peptide reacted with the $\alpha 1$ chain of human and EHS laminin preparations in Western blots. However, an antibody against the IKVAV-containing peptide did not react with the $\alpha 1$ chain (K.-i. Tashiro, unpublished work). These findings show that the site of the IKLLI sequence (residues 2080–2095) may appear on the surface of the coiled-coil region in laminin but that the site of the IKVAV sequence (residues 2116–2133) may be involved or masked in the denatured condition. For an immunoblot assay, therefore, an antibody against an IKLLI-containing peptide may be more useful for recognizing the laminin $\alpha 1$ chain than an antibody against an IKVAV-containing peptide.

Previous reports have suggested that cell-surface heparin-like molecules or heparan sulphate proteoglycans interact with specific regions on laminin [19]. The interaction with heparin is thought to be important for both the modulation of the basement membrane assembly and adhesion to cells.

The glycosaminoglycan contribution to PC12 cell adhesion was examined by plating the cells on to laminin and LA4 substrates in the presence of heparin, heparan sulphate, and chondroitin sulphate. LA4 promoted cell adhesion, and LA4-mediated adhesion was specifically inhibited by heparin or heparan sulphate but not by chondroitin sulphate, thus indicating that this region might behave as an adhesive binding site through a cell-surface heparan sulphate proteoglycan.

The laminin and heparin-binding proteins of the brain have also been shown to enhance neurite outgrowth. Several synthetic peptides from the G-domain containing positively charged regions were found to promote heparin binding, cell adhesion and neurite outgrowth [44]. These results indicate that laminin–heparin interaction may thus play an important role in both cell adhesion and neurite outgrowth. Since the activity of heparin binding has been connected to the activity of neurite outgrowth [12], our data also showed that LA4 mediated both heparin-binding and neurite outgrowth. Furthermore, PA22 mediated not only neurite outgrowth but also heparin binding.

The neurotoxicity of another neurite outgrowth-promoting peptide derived from laminin (RNIAEIIKDIGC) has been reported previously [24]. PA22 has been reported as having no neurotoxicity to cells [45]. LA4 did not affect neuronal survival up to a concentration of 1 mg/ml in the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] cell assay (K.-i. Tashiro, unpublished work). Therefore, the possibility that LA4 was simply toxic to neurons or altered neuronal metabolism could be ruled out.

The proliferation of many cells is thought to depend on cell adhesion to the extracellular matrix components [46,47]. A

recent study has shown that the adhesion of suspension-arrested cells rapidly induces growth-associated (c-Fos, c-Myc, and actin) gene expression [48]. An IKVAV-containing peptide stimulated the metastasis of B16F10 to the mouse lung [49] and was also reported to induce c-Fos and c-Jun mRNA expression in PC12 cells [27]. LA4 has not yet been examined for whether it induces metastasis or growth-associated genes. However, the present study showed that LA5 mediated neuronal cell proliferation as well as PA22. These findings suggest that IKLLI-containing peptides may interact with growth-associated genes as well as IKVAV-containing peptides.

In conclusion, an IKLLI-containing peptide was present in a coiled-coil region which is a non-physiological site of laminin. However, this peptide showed several biological activities for the cells. This may occur as a result of the instability and flexibility of this domain. In the present study, an antibody against an IKLLI-containing peptide showed this possibility. The domain containing the IKLLI and IKVAV sequences was one of the heparin-binding sites in laminin, and thus this function may interact with many cells and mediate many biological activities. Our findings suggest that an IKLLI-containing peptide interacts with both integrin $\alpha 3 \beta 1$ and cell-surface heparan sulphate proteoglycan. However, only a mixed IKLLI-containing peptide with an IKVAV-containing peptide failed to increase cell adhesion. Thus this result shows that the conformational structure of this domain plays a significant role in the biological activity.

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