A synthetic peptide deduced from the sequence in the cross-region of laminin A chain mediates neurite outgrowth, cell attachment and heparin binding

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Synthetic peptides from the cross-region of the laminin A chain were prepared and tested for their biological activities, especially for neurite outgrowth. A synthetic 8-mer peptide (designated LMA-5) in the cross-region of the laminin A chain was found to promote neurite outgrowth in PC12 cells and cerebellar micro-explant cultures. Furthermore, this peptide mediated cell attachment and heparin binding. In addition, an antibody against

peptide LMA-5 inhibited laminin- and LMA-5-mediated cell attachment. This antibody also inhibited more than half of the LMA-5-promoted neurite outgrowth in cerebellar microexplant cultures. These data suggest that peptide LMA-5 is one of the active sites in laminin that regulate cell behaviour including neurite outgrowth, cell attachment and heparin binding.

INTRODUCTION

Laminin (M_r 900 000) is a large glycoprotein specific to the basement membrane [1,2]. It is composed of three chains, A (M_r 400 000), B1 (M_r 210 000) and B2 (M_r 205 000), which are arranged in a cross-shaped structure [3–5]. Laminin mediates cellular interactions with the basement membrane including cell attachment, migration, growth, differentiation, metastasis, collagenase IV activity and neurite outgrowth [6–12]. Two active domains for cell attachment have been reported in laminin using proteolytic fragments: the three short arms minus the globules (designated E1-4 fragment) [13] and the end of the long arm including the large globule (designated E8 fragment) (see Figure 3) [14]. The latter fragment also promotes neurite outgrowth [8] and mediates heparin-binding [15]. Recently, the pepsin fragment of laminin (P-LAM) has been found to contain a part of the cross-region of laminin, and this fragment has been shown to promote neurite outgrowth in PC12 cells and NG108-15 cells [16]. Three peptides have been observed in the cross-region of laminin, namely YIGSR (Tyr-Ile-Gly-Ser-Arg), F9 and RGD-containing peptide PA21, but only PA21 conjugated with either keyhole limpet haemocyanin (KLH) or BSA has been reported to promote neurite outgrowth in NG108-15 cells [17–20]. However, no active site(s) for neurite outgrowth in either PC12 cells or other neuronal cells that respond to laminin or P-LAM fragment has yet been detected in the cross-region of laminin.

We have detected several active sites for cell attachment and neurite outgrowth in laminin using synthetic peptides [21–23]. Two peptides, PA22-2 and PA57, which are located in the E8 fragment, mediate not only cell attachment but also neurite outgrowth [21,22]. Repeats of hydrophobic amino acid sequences have been reported in the I-II domain of the laminin A chain (Figure 2) [24]. Both PA22-2 and PA57 were included in these repeats. In the present work, we have synthesized several peptides

with sequences similar to PA22-2 and PA57 in this domain, and detected a site that is especially active for neurite outgrowth in the cross-region of laminin which has not been previously reported.

MATERIALS AND METHODS

Synthetic peptides and laminin

Peptides were synthesized using an automated model 430A synthesizer (Applied Biosystems, Foster City, CA, U.S.A.), and prepared as previously described [25]. Synthetic peptides were purified by h.p.l.c. [model LC-8A (Shimadzu, Tokyo, Japan) using a reversed phase column (3 cm $\times$ 25 cm) of YMC Pack AQ-AM-363-ODS (YMC, Tokyo, Japan)] and were prepared as acetic acid salts. Analytical h.p.l.c. was performed with a model LC-4A reversed phase column (0.6 cm $\times$ 15 cm) of YMC Pack AQ-312. All peptides were prepared in the amide form and included a cysteine residue to facilitate coupling, except for peptide LMA-8. Each peptide contained amino acid sequences derived from the laminin A chain as follows: LMA-1, CSIKVAVS (residues 2099–2105); LMA-2, CSGKVAVS (Ile changed to Gly in LMA-1); LMA-3, CLKNLTS (residues 1956–1961); LMA-4, CAGSKTQ (residues 1754–1759); LMA-5, CYFQRYLI (residues 1583–1589); LMA-6, CEQLHANIK (residues 1645–1652); LMA-7, CDLLSRIQ (residues 1711–1717); LMA-8, IKLLIS (residues 2086–2091).

Mouse laminin was extracted and purified from the Engelbreth-Holm-Swarm (EHS) tumour using methods previously described [2].

Cell and culture

The two cell lines studied were HT-1080 human fibrosarcoma cells [26] and PC12 rat pheochromocytoma cells. PC12 cells

Abbreviations used: KLH, keyhole limpet haemocyanin; FBS, fetal bovine serum; NGF, nerve growth factor; DMEM, Dulbecco's modified Eagle's medium; EMEM, Eagle's minimal essential medium; MBS, *M*-maleimidebenzoylsulphosuccinimide ester; P-LAM, pepsin fragment of laminin.

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obtained from Dr. G. Guroff (NIH, Bethesda, MD, U.S.A.) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 7.5% (v/v) fetal bovine serum (FBS), 7.5% (v/v) horse serum and penicillin (100 U/ml)/streptomycin (100 U/ml). For assay of neurite-process formation, PC12 cells were primed with 50–100 ng/ml 7S nerve growth factor (NGF) (Calbiochem) 24–48 h before assay. For the attachment assay, PC12 cells were grown to 80% confluency, washed with serum-free Eagle's minimal essential medium (EMEM) containing 0.02% BSA, detached mechanically by pipetting and then used for the assay.

HT-1080 cells obtained from Dr. Y. Iwamoto (Faculty of Medicine, Kyushu University, Fukuoka, Japan) were maintained in DMEM supplemented with 10% (v/v) FBS and grown to 80% confluency. For the attachment assays, the cells were washed with PBS (pH 7.4), lacking Ca^{2+} and Mg^{2+} , and then detached with 0.025% trypsin/0.025% EDTA (Gibco). The cells were then sedimented by low-speed centrifugation, suspended in serum-free EMEM containing 0.02% BSA, and then used immediately in the assay.

Assay of neurite-process formation

PC12 cell layers were rinsed with serum-free 50% Hams F12/50% DMEM containing 100 $\mu\text{g}/\text{ml}$ transferrin, 100 μM putrescine hydrochloride, 20 nM progesterone, 30 nM Na_2SeO_3 , 5 $\mu\text{g}/\text{ml}$ insulin and 50–100 ng/ml NGF (defined medium). The cells were dislodged with a stream of serum-free medium and were pelleted, resuspended in defined medium and counted using an electronic cell counter (ZBI; Coulter Electronics). Approx. 2500 cells were added to each well of a 48-well dish (Costar) which had been coated with either laminin or synthetic peptide. After incubation overnight, the cells were fixed with 3.7% formalin. The percentage of cells with processes greater than two cell-body lengths was determined for duplicate wells for each concentration. At least 100 cells per data point were counted.

The neurite-outgrowth assay in cerebellar microexplant cultures was performed as described previously [27]. Laminin (0.2 $\mu\text{g}/\text{coverglass}$) was further coated after the coating of poly-L-lysine on a cleaned round coverglass (15 mm diam.) for 1 h at 37 °C. LMA-1, LMA-5 and LMA-2 [10 μl of each dissolved in distilled H_2O (1 mg/ml)] were coated directly on to coverglasses and evaporated to dryness. Small tissue specimens from the cerebellar grey matter of 6-day-old postnatal mice were prepared. About 15 samples of tissue specimens (microexplants) were placed on either a laminin- or peptide-coated coverglass and then cultured in serum-free hormone-supplemented medium [27]. After cultivation for 2 days at 37 °C in a 5% CO_2 incubator, the cultures were rinsed with PBS (pH 7.2) and fixed with 4% paraformaldehyde/PBS solution. Neurite outgrowth was evaluated by taking photographs of all the fixed explants and measuring the neurite extension from the explants.

Conjugation of peptides

KLH (30 mg) was dissolved in 2 ml of 0.1 M NaHCO_3 (pH 8.3) containing 6M urea (coupling buffer) and stirred for 15 min. *m*-Maleimidebenzoylsulphosuccinimide ester (MBS, 3 mg) was added to the KLH and the mixture stirred for 15 min. Then 10 mg of peptide LMA-5 was added to the KLH/MBS solution and stirred for 2 h in the coupling buffer at 25 °C. Conjugates were dialysed against 10% acetic acid for 18 h at 4 °C and then lyophilized.

Preparation of antibody against peptide LMA-5

After conjugation, 10 mg of KLH-conjugated peptide LMA-5 was dissolved in 2.5 ml of 0.1 M PBS (pH 7.4), and 0.5 ml of the solution was emulsified in the same volume of complete Freund's adjuvant and used to immunize a white rabbit (about 2500 g). After three boosts, antiserum was obtained which was allowed to react with 1 ng of native laminin and 10 ng of peptide LMA-5 at a 1:18000 dilution. This antiserum against peptide LMA-5 was purified to IgG by Protein A-Sepharose affinity column chromatography. A control IgG fraction was prepared from preimmune serum of the immunized rabbit, and neither 1 μg of laminin nor 10 μg of peptide LMA-5 reacted with the control IgG until a concentration of 1 mg/ml was reached.

Inhibition of neurite outgrowth by anti-LMA-5 IgG

The wells of 24-well tissue culture dishes (Costar) were either coated with 1 μg of laminin, 10 μg of LMA-1 or 50 μg of LMA-5 or were left uncoated and then evaporated to dryness. Next 312 μg of anti-LMA-5 IgG in 250 μl was added to each substrate and incubated at 37 °C for 3 h. Preimmune IgG (312 μg) was added to the substrate-coated control wells. After 3 h of incubation, 1000 PC12 cells were added per well. These cells had been primed for 24 h with NGF, and the neurite-outgrowth assay was performed as described above. After 18 h of incubation, neurite outgrowth was evaluated.

Two poly-L-lysine-coated coverglasses were further coated with 0.2 μg of laminin for 1 h at 37 °C [27]. Two uncoated coverglasses were coated with 5 μl of LMA-5 (1 mg/ml) and evaporated to dryness. Then 0.2 μg of anti-LMA-5 IgG was added to either laminin- or LMA-5-coated substrate and incubated at 37 °C for 3 h. As a control, 1 μg or 0.2 μg of preimmune IgG was added to either the laminin- or LMA-5-coated coverglass respectively. After incubation for 3 h at 37 °C and washing twice with PBS, a small portion of cerebellar tissue, prepared as described above, was placed on IgG-treated substrates, cultured for 2 days and fixed with 3% paraformaldehyde/PBS. A neurite-outgrowth assay was carried out as described above.

Cell attachment

A cell-attachment assay was performed in 96-well tissue culture dishes (Costar) as previously described [21] with some minor modifications. Synthetic peptides were dissolved in distilled H_2O to a concentration of 1 mg/ml. Three different volumes (1, 5 and 10 μl) of peptide solution were added to 96-well dishes in a final volume of 50 μl , and evaporated to dryness. The unbound surfaces of the wells were subsequently blocked with 100 μl of EMEM containing 1.5% (w/v) BSA for 30 min at 37 °C before addition of cells. The cells (4×10^4), prepared as described above, were added to each well and incubated for 1 h at 37 °C in 5% CO_2 and 95% air [28]. At the end of this period, the wells were gently washed with PBS to remove any unattached cells. The attached cells were fixed with 100 μl of 100% ethanol for 10 min and stained with 50 μl of 0.1% Crystal Violet for 25 min at room temperature. The stained cells were washed once with distilled H_2O , and 50 μl of 0.1% Triton X-100 was added for 30 min to extract the Crystal Violet. After the addition of 150 μl of H_2O , the absorbance of the extracted solution was measured in a spectrophotometer at 570 nm. Each assay was carried out in duplicate, and the results did not differ by more than 10%. Each peptide was tested in five separate experiments and at least three different concentrations.

Peptide inhibition of cell attachment was assayed in 96-well

dishes coated with 2 μ g of laminin as described above. Before the addition of the cells, various amounts of peptide solubilized in serum-free EMEM containing 0.02 % BSA were added to each well at a concentration of 0.4, 0.7 or 1 mg/ml. The cells were incubated for 30 min at 37 °C and the number of attached cells was determined as described above. Each assay was performed in duplicate, and each peptide was assayed at least five times.

Inhibition of cell attachment by anti-LMA-5 IgG

Antibody against peptide LMA-5 was prepared as described above. In this assay, laminin (0.2 μ g/well) and peptides (2 μ g/well) were coated to 96-well tissue culture dishes as described above. The unbound surfaces were blocked with 100 μ l of EMEM containing 1.5 % (w/v) BSA for 30 min at 37 °C. The wells were then rinsed with PBS and incubated at 37 °C for 3 h with three different concentrations (10, 1 and 0.1 μ g/ml) of the purified anti-LMA-5 IgG. The antibody was removed, and the wells were washed with PBS. PC12 cells (4×10^4) were added to the well and incubated for 37 °C for 30 min. The attached cells were then counted by the staining method as described above.

Heparin-binding assay

The binding of [^{3} H]heparin (NET-467, heparin sodium salt, [^{3} H(G)]-, NEN Research Products) to various peptides was measured by direct solid-phase-binding assays. These assays were performed in 96-well tissue culture dishes (Costar) as previously described [17,29]. Stock solutions of peptides at a maximum concentration of 2 mg/ml were prepared in PBS. Various amounts of peptide (1, 12.5 or 25 μ l) were added to the well at a final volume of 50 μ l, and the wells were evaporated to dryness. The wells were then treated for 2 h at 37 °C with 100 μ l of wash buffer (2 mg/ml BSA in 6 mM phosphate, 100 mM NaCl, 60 mM CaCl₂, pH 6.8) to minimize any non-specific binding. Next, 50 μ l of [^{3} H]heparin (3 μ Ci/ml in the wash buffer) was added to each well (1×10^5 d.p.m./well) and incubated for 2 h at 37 °C in 5 % CO₂ and 95 % air. 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/1 % SDS. The amount of [^{3} H]heparin bound was quantified in Ecolite scintillation fluid (Aquasol-2; NEN Research Products) and determined in a liquid-scintillation counter (TRI-CARB 460CD; Packard).

Specificity of peptide binding to heparin

To determine the importance of the heparin structure for this interaction, 3 μ g of peptide/well was dried in 96-well plates as described above. The wells were blocked for 2 h with 2 mg/ml BSA in the wash buffer, and 50 μ l of [^{3} H]heparin (50000 d.p.m./well) was added to each well concomitantly with various amounts of unlabelled heparin. After incubation for 2 h at 37 °C, the wells were washed and the radioactivity was determined as described above.

RESULTS

Peptide synthesis

The average yields for all syntheses ranged from 80 to 90 %. The composition of all peptides was verified by amino acid analysis. Peptide LMA-5 which possesses biological activity was further

purified using preparative h.p.l.c. when analytical h.p.l.c. indicated that the product was heterogeneous. The purity of this peptide was then verified by h.p.l.c., the yield of the monomer being 89 % and that of the dimer 11 % (Figure 1). The amino acid composition of peptide LMA-5 (CYFQRYLI amide) was determined by the Pico-Tag system (Waters) and the peptide content was determined free form. The amino acid composition was found (calculated) to be: Glx 1.1 (1), Arg 1.0 (1), Tyr 2.0 (2), Ile 0.9 (1), Leu 0.9 (1), Phe 1.0 (1), Cys 1.4 (1) [secondary ion mass spectrometry [29a]: m/z ($M+H$)⁺ 1104 (C₅₃H₇₇N₁₃O₁₁S formula 1103, M_r 1104.335, peptide content 49.6 %)].

Neurite outgrowth in PC12 cells and cerebellar microexplant cultures

To locate the active sites of laminin, we synthesized eight peptides related to the sequences of peptides PA22-2 and PA57 in the I-II domain of the laminin A chain and tested for their ability to promote neurite outgrowth (Figure 2). These positions in the laminin A chain sequence are indicated by arrows in Figure 3. LMA-1 and laminin efficiently promoted neurite outgrowth in PC12 cells used as control (Figures 4c and 4d). Other than LMA-1, the only peptide that promoted neurite outgrowth in PC12 cells was LMA-5 (Figure 4b). When the activity of laminin was taken as 100 %, those of peptides LMA-1 and LMA-5 were about 60 % and 30 % respectively (Table 1). Uncoated and poly-L-lysine-coated wells did not show any neurite outgrowth (Figure 4a). Peptide LMA-8, which had no cysteine residue, did not show any neurite-promoting activity. This may have been because this peptide was not so well coated on the dishes as LMA-1 because

Figure 1 Analytical h.p.l.c. of peptide LMA-5

Analytical h.p.l.c. was performed with a model LC-4A using a reversed phase column (0.6 cm $\times$ 15 cm) of YMC Pack AQ-312. The flow rate was 2.4 ml/min. The monomer of LMA-5 was detected as peak 1 and the dimer as peak 2.

1	defgabcdefgabcdefgabcdefgabc	1590
2	(L TGVSPAPY GILENLENTTKYFORVLLIK	1618
3	ENAKKIRAEIQLEGIAEQTENLQKELTR	1646
4	VLARHQKVNAEMERTSNGTQALATFIEQ	1674
5	LHANIKETITEKVATLNQTARKDFQPPVS	1704
6	ALQSMHQNISSLLGLIKERNFTMQQNATL	1732
7	ELKAAKDILLSRIQKRFPQKPQEKLKALKE	1760
8	ANSLLSNHSSEKLQAAEELLK)EAGSKTOE	1789
9	SNLLLLLVKANLKKEEFQEKLRVQEEQNV	1817
10	TSELIAGKREWVDAAGTHTAAAQDTLTQ	1847
11	LEHHRDELLWARKIRSHVDDLVQMMSKRR	1878
12	ARDLVHRAEQHASELQSRAGALDRDLENVRN	1906
13	VSLNATSAA(HVHSNIQTTLTEEAEMLAAD	1934
14	AHKTANKTDLISESLASRGKAVLQRSSR	1962
15	FLKESVGTTRKQGGITMKLDELKNLITSQ	1991
16	FQESVDNITKQANDSLAMLRESPGGMREX	2019
17	GRKARELAAAANESAVKTLEDVLALSRLR	2050
18	VFNTSEDLSRVNATVQETNDLLHNSTMTTL	2078
19	AGRKMKDMEMQANLLLDRLKPLKLTLEEN	2106
20	LSRNLSEIKLLISRRARKQAASIKVAVSA	
21	defgabcdefgabcdefgabcdefgabc	

Figure 2 Hydrophobic region of laminin A chain

The square bracket shows the start of the E8 fragment (from residue 1989); curly brackets show the cross-region of the laminin A chain (residues 1563–1752).

Figure 3 Structural model of laminin and location of active sites

The roman numerals designate the domains in the A chain. G is the A-chain terminal domain. The location of the peptides used in this study are indicated by arrows. All peptides except for LMA-8 contained a cysteine residue. Active sites and fragments reported previously are also indicated by arrows [17,18,20]. Both the P-LAM and E8 proteolytic fragments described previously are active in neurite outgrowth and cell attachment [8,14,16].

Figure 4 Photographs of neurite outgrowth in PC12 cells

A neurite-outgrowth assay was performed as described in the Materials and methods section. Process formation is apparent with laminin (4 µg coated/well) (d), LMA-1 (20 µg coated/well) (c) and LMA-5 (50 µg coated/well) (b), but is not apparent when no treatment was administered (a). The photographs were taken after 2 days of incubation.

Table 1 Effect of laminin and synthetic peptides on neurite outgrowth in PC12 cells and cerebellar microexplant cultures

Neurite outgrowth of PC12 cells was apparent with laminin (1 µg coated/well) and some synthetic peptides (20 µg coated/well), and that of cerebellar cultures was also apparent with laminin (0.2 µg coated/coverglass) and some synthetic peptides (10 µg coated/coverglass). Neurite extension caused by laminin was taken as 100%. The values are expressed as means ± S.E.M. (%) of the control values for triplicate assays. Significant differences from the control (*P < 0.01, **P < 0.05) were determined by Student's *t* test. n.d., not determined.

Coated substrates	Neurite extension (%)	
	PC12 cells	Cerebellar cultures
Lamini	100.0 ± 3.1*	100.0 ± 4.2*
LMA-1	59.2 ± 4.0**	40.1 ± 3.1**
LMA-2	2.1 ± 2.3	2.4 ± 2.2
LMA-3	5.3 ± 2.8	n.d.
LMA-4	1.8 ± 2.0	n.d.
LMA-5	31.4 ± 1.9*	28.5 ± 3.2*
LMA-6	2.0 ± 2.7	n.d.
LMA-7	1.7 ± 2.0	n.d.
LMA-8	4.3 ± 2.1	n.d.
YIGSR	n.d.	3.2 ± 2.1
Non-coated control	2.1 ± 1.8	2.0 ± 1.5

of the lack of a cysteine residue. However, it inhibited both laminin- and LMA-1-mediated neurite outgrowth in PC12 cells as well as cell attachment (results not shown). These findings suggest that the IKLLI sequence, as well as the IKVAV sequence, is significant for the biological activity of laminin. Peptide LMA-3 did not inhibit neurite outgrowth mediated by laminin, LMA-1 and LMA-5. As peptides LMA-1 and LMA-5 could not be dissolved in the defined medium (pH 7.4), they were not tested in this assay.

To determine the relationship between laminin peptides and neurite outgrowth of the primary neurons, we also examined the neurite outgrowth of granule cells in cerebellar microexplant cultures on laminin and its peptides. Besides LMA-1, LMA-5 showed about 30% of laminin-mediated activity for neurite

Table 2 Effect of anti-LMA-5 IgG on neurite outgrowth in cerebellar microexplant cultures

Laminin (0.2 μ g) or LMA-5 (10 μ g) was coated on a poly-L-lysine-precoated round coverglass. Then 1 μ g of preimmune IgG and anti-LMA-5 IgG were added to the laminin- and LMA-5-coated coverglasses respectively. Neurite outgrowth on either the laminin or LMA-5 substrate pretreated with preimmune IgG was taken as 100%. The values are expressed as means $\pm$ S.E.M. (%) of the control values for triplicate assays. Significant differences from the control (* P < 0.01, ** P < 0.05) were determined by Student's *t* test.

Coated substrates	Neurite length from explants (%)	
	+ Preimmune IgG	+ Anti-LMA-5 IgG
Laminin	100.0 $\pm$ 6.2	101.1 $\pm$ 8.2**
LMA-5	100.0 $\pm$ 4.1	40.3 $\pm$ 3.1*

outgrowth (Table 1). In contrast, non-, YIGSR- and LMA-2-coated substrate showed no significant neurite outgrowth.

Cell attachment

Laminin and peptide LMA-1 containing the IKVAV sequence appeared to mediate PC12 cell attachment as reported previously (Figure 5a) [23]. Peptide LMA-5 also mediated cell attachment in PC12 cells in a dose-dependent manner, and showed 90% of the LMA-1-mediated activity (Figure 5a). Furthermore, this peptide also mediated cell attachment in HT-1080 cells (results not shown). LMA-2, LMA-4, LMA-6 and LMA-7 did not mediate cell attachment. Peptide LMA-3 showed about 20% of the LMA-1-mediated activity in PC12 cells only (Figure 5a).

We also assessed the ability of these peptides to inhibit laminin-mediated cell attachment. Peptide LMA-8 containing the IKLLI sequence blocked PC12 cell attachment to laminin in a dose-dependent manner, and inhibited about 40% of the laminin-mediated activity. Peptides LMA-2, LMA-3, LMA-4, LMA-6 and LMA-7 did not block laminin-mediated cell attachment (Figure 5b). Peptides LMA-1 and LMA-5 did not dissolve in the assay media and were therefore not used in this assay.

Laminin did not inhibit laminin-mediated cell attachment but instead promoted cell attachment. It was considered possible that small amounts of type-IV collagen might have contaminated the laminin used which would have caused laminin precipitation to occur in the cell-attachment assay. This was a possibility because purified laminin is a soluble protein that does not precipitate when incubated alone, but the addition of either heparin sulphate proteoglycan or type-IV collagen stimulates its precipitation [30].

Effect of the anti-LMA-5 IgG on neurite outgrowth and cell attachment

An antibody against LMA-5 was developed and used to test the specificity of the peptide activity. It was found that 1 μ g/ml of anti-LMA-5 IgG reacted with 1 ng of native laminin and 10 ng of peptide LMA-5 in dot-blot analysis, and it also reacted specifically with the A chain in Western-blot analysis (results not shown). Preimmune IgG did not block neurite outgrowth and cell attachment on either laminin or LMA-5 (results not shown).

Anti-LMA-5 IgG did not inhibit LMA-5-mediated neurite outgrowth in PC12 cells, nor did it inhibit laminin- and LMA-1-mediated neurite outgrowth (results not shown). This antibody inhibited 60% of neurite-outgrowth activity in cerebellar micro-

Figure 5 Effect of synthetic peptides on cell attachment in PC12 cells (a) and on cell attachment to a laminin substrate (b)

Tissue culture dishes (96-well) were coated with 2 μ g of laminin per well. Before addition of PC12 cells, various concentrations of the peptide solubilized in serum-free EMEM containing 0.02% BSA were added to the wells for the peptide-inhibition assay. The attachment assay was performed as described in the Materials and methods section. The values are expressed as means $\pm$ S.E.M. of the control values in four to eight determinations. Significant differences from the control (* P < 0.01, ** P < 0.05) were determined by Student's *t*-test. (a) ■, Laminin; □, LMA-1; ○, LMA-5; △, LMA-3; ▲, LMA-2. (b) □, LMA-8; ◇, LMA-7; ■, LMA-6; ◆, LMA-4; □, LMA-3; ■, LMA-2.

Table 3 Effect of anti-LMA-5 IgG on cell attachment to either laminin or peptide substrates

Laminin (0.2 μ g), LMA-1 or LMA-5 (20 μ g each) was coated on a 96-well tissue culture dish. Coated dishes were treated with 1 μ g/ml preimmune IgG or anti-LMA-5 IgG at 37 °C for 3 h. Laminin-mediated cell attachment after treatment with preimmune IgG was taken as 100%. The values are expressed as means $\pm$ S.E.M. (%) of the control values for triplicate assays. Significant differences from the control (* P < 0.01, ** P < 0.05) were determined by Student's *t* test.

Coated substrates	Attached PC12 cells (%)	
	+ Preimmune IgG	+ Anti-LMA-5 IgG
Laminin	100.0 $\pm$ 2.2	62.0 $\pm$ 3.3*
LMA-1	85.0 $\pm$ 4.2**	83.1 $\pm$ 3.3**
LMA-5	73.1 $\pm$ 3.0*	15.2 $\pm$ 5.2*

explant cultures mediated by LMA-5, but did not inhibit the activity mediated by laminin (Table 2). These results suggest that laminin may contain other active sites for neurite outgrowth.

Figure 6 Binding of heparin to peptides dried on to culture wells (a) and the specificity of peptide binding to heparin (b)

The amount of $[^3\text{H}]$ heparin bound was determined in a liquid-scintillation counter. For specificity of peptide binding to heparin, 3 μg of peptide/well was coated in a 96-well plate. A heparin-binding assay was performed as described in the Materials and methods section. The values are expressed as means $\pm$ S.E.M. of the control values for triplicate assays. Significant differences from the control (* $P < 0.01$, ** $P < 0.05$) were determined by Student's *t*-test. $\square$, LMA-1; $\bullet$, LMA-5; $\circ$, LMA-2.

Anti-LMA-5 IgG inhibited 80% of LMA-5-mediated cell attachment and 30% of laminin-mediated cell attachment in PC12 cells, but did not inhibit LMA-1-mediated cell attachment because this antibody did not bind to peptide LMA-1 (Table 3). In addition, this antibody did not inhibit cell attachment mediated by any other peptides such as YIGSR, F9 and PA21 (results not shown). These results indicate that laminin has multiple sites for cell attachment in the molecular structure.

Heparin binding

Peptides LMA-5 and LMA-1 mediated heparin binding in a dose-dependent manner. LMA-5 showed about 80% of the LMA-1-mediated activity in 25 $\mu\text{g}/\text{well}$ coated plates. Peptides LMA-2 (Figure 6a), LMA-3, LMA-4, LMA-6, LMA-7 and LMA-8 did not bind to $[^3\text{H}]$ heparin. To determine the importance of the heparin structure for this interaction, we tested the specificity of peptide binding to $[^3\text{H}]$ heparin. The heparin-binding activity of LMA-5 was the same as that of LMA-1, and

the binding was inhibited completely by 100 $\mu\text{g}/\text{ml}$ unlabelled heparin (Figure 6b).

DISCUSSION

Laminin has multiple biological activities, including promoting the attachment of epithelial cells, the migration of tumour cells and process formation by neural cells. By proteolytic cleavage of laminin, two separate domains have been reported to promote cell attachment and neurite outgrowth (Figure 3): fragment E8 [8] and fragment E1-4/P-LAM [13,16]. The data obtained from sequencing recombinant DNA clones have allowed the primary structures of the A, B1 and B2 chain to be deduced [24,31,32], and have also clarified the relationship between the molecular structure and biological activity. Three peptides (YIGSR, F9 and PA21) have been previously reported in the E1-4/P-LAM fragment [17–20]. On the other hand, several peptides, including PA22-2, PA57 and P-20, have been found in the E8 fragment [21,25,33]. These studies suggested that the active sites for neurite outgrowth were located in the E8 fragment alone. We also found that the significant sequences of peptides PA22-2 and PA57 were IKVAV and IKLLI respectively [21,22]. These peptides contain four hydrophobic amino acids and one basic amino acid. Furthermore, both amino acid sequences (IKVAV and IKLLI) are included in the repeats of hydrophobic amino acid sequences reported in the I-II domain of the laminin A chain (Figure 2) [24]. Therefore from these data we thought that peptides containing the IKVAV or IKLLI sequence, even in the E1-4/P-LAM fragment, may show biological activity including neurite outgrowth. We therefore synthesized several peptides containing hydrophobic amino acid sequences and analysed their biological activities.

Peptide LMA-1 containing the IKVAV sequence mediated neurite outgrowth, cell attachment and heparin binding, as reported previously [23]. Peptide LMA-2, with the isoleucine of LMA-1 changed to glycine, mediated neither neurite outgrowth nor cell attachment. This finding suggests that the isoleucine residue may be important for the activity of the IKVAV-containing peptide.

Peptide LMA-5 has the sequence CYFQRYLI, which is completely different from the IKVAV sequence. Whereas its cell-attaching activity was almost the same as that of peptide LMA-1, it showed about 50% of the LMA-1 activity for neurite outgrowth. It was not tested in the peptide-inhibition assay because it contained many hydrophobic amino acids and therefore could not be solubilized in the assay medium. Therefore, to discover the significance of this peptide for the biological activity, we investigated whether the antibody against it could inhibit cell attachment and neurite outgrowth. Anti-LMA-5 IgG indeed inhibited LMA-5-mediated neurite outgrowth in cerebellar neurons. It also inhibited both laminin- and LMA-5-mediated cell attachment in PC12 cells. However, it did not inhibit LMA-5-mediated neurite outgrowth in PC12 cells. The discrepancy between the inhibition of cell attachment and the inhibition of neurite outgrowth may be the result of the longer time required for the neurite-outgrowth assay which may decrease the effectiveness of the antibody. It is also possible that the antibody may be removed by the cells or the peptide may be released from the well so that blocking becomes ineffective. These possibilities are suggested by the fact that the sprouting of the PC12 cells in the neurite-outgrowth assay was initially delayed (for about 3 h) by the antibody, but after 18 h of incubation, the neurite extension of the antibody-treated PC12 cells was the same as that of the untreated cells (results not shown). These results suggest that the anti-LMA-5 antibody may recognize the active sites for cell

Table 4 Effect of peptides on neurite outgrowth, cell attachment and heparin binding

The activity of laminin (1 µg coated/well) in mediating neurite outgrowth, cell attachment and heparin binding was taken as 100%. Each peptide (20 µg) was coated on a 96-well tissue culture dish. The values are expressed as means ± S.E.M. (%) of the control values for triplicate assays. Significant differences from the control (*P < 0.01, **P < 0.05) were determined by Student's *t* test.

Coated peptide	Neurite outgrowth (%)	Cell attachment (%)	Heparin binding (%)
LMA-5	52.2 ± 5.1*	85.1 ± 4.0*	82.3 ± 4.1*
YIGSR	2.0 ± 3.1	22.3 ± 4.2**	2.0 ± 3.1
F9	3.2 ± 3.1	51.2 ± 5.4*	75.3 ± 5.2*
PA21	10.1 ± 4.2†	2.0 ± 3.1	3.2 ± 3.0

† This activity was determined in NG108-15 cells with KLH- or BSA-conjugated PA21, but PA21 did not mediate neurite outgrowth in PC12 cells.

attachment of laminin or peptide LMA-5 as well as active sites for neurite outgrowth in cerebellar neurons, but the site may be different from that for neurite outgrowth in PC12 cells. Furthermore, the activity of peptide LMA-5 for neurite outgrowth was not as strong (about 50%) as that of peptide LMA-1, which contains the IKVAV sequence, suggesting that there may be other regions involved in neurite outgrowth in the cross-region of the laminin A chain. Recently, we analysed the three-dimensional structures of the peptides, which showed that peptides such as PA22-2, PA57 and LMA-5 active for neurite outgrowth commonly have a β -sheet structure (K. Tashiro, I. Nagata, N. Yamashita, K. Okazaki, K. Ogomori, N. Tashiro and M. Anai, unpublished work). Peptide LMA-5 also mediated heparin binding, which indicates that it may be a heparin-binding domain in the cross-region of laminin, as reported previously [29].

We also investigated the LMA-5-mediated activities by comparing three different peptides (YIGSR, F9 and PA21) which had been reported previously in the cross-region of laminin [17–20]. When the activity of LMA-1 was taken as 100%, LMA-5 showed 50% activity for neurite outgrowth in PC12 cells. The RGD-containing peptide PA21 promoted neurite outgrowth in NG108-15 cells when it was conjugated with KLH, but it did not show any such activity in PC12 cells. These results suggest that the mechanism of neurite outgrowth in PC12 cells may be different from that in NG108-15 cells. Peptide LMA-5-mediated cell attachment in PC12 cells had exhibited stronger heparin binding than did the other three peptides (Table 4).

These results suggest that peptide LMA-5 may be an active site for not only neurite outgrowth but also cell attachment and heparin binding in the cross-region of laminin.

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