Active peptides from the carboxyl-terminal globular domain of laminin α2 and *Drosophila* α chains

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Received 10 July 1996; revised version received 10 September 1996

Abstract The laminin α1 chain carboxyl-terminal globular domain (G domain) contains multiple biological activities. Recently, we identified five cell binding sequences from the G domain by screening with overlapping 12-mer peptides encompassing the entire domain. The structures of these five sequences in the α1 chain are conserved in the corresponding regions of the different laminin α chains. Here we characterize the adhesion activities of the corresponding peptide segments from both the mouse laminin α2 chain and *Drosophila* laminin α chain using peptide-coated plastic plates and peptide-conjugated Sepharose beads. Using several cell lines, the laminin α2 chain peptides showed cell attachment and/or spreading activities with cell type specificities. Cell spreading on MG-10 was inhibited by integrin antibodies. Four of the *Drosophila* laminin peptides showed cell attachment activities. These results suggest that biologically active regions in the G domain are conserved in the laminin α1 and α2 chains, and that these regions in laminin play an important role in cell surface receptor interactions.

Key words: Laminin; Synthetic peptide; Cell attachment; Basement membrane; Integrin

1. Introduction

Laminins are major components of basement membrane, the thin extracellular matrix which underlies all epithelia and surrounds muscle, peripheral nerve, and fat cells [1–3]. Laminin-1 consists of α1, β1 and γ1 chains, which assemble into a triple-stranded coiled-coil structure in the long arm to form a cross-like structure (Fig. 1) [3]. Laminin-1 has diverse biological activities including promotion of cell adhesion, spreading, growth, neurite outgrowth, tumor metastasis, and collagenase IV activity [1]. Several laminin isoforms have been identified with at least eight genetically distinct chains [4]. Laminin-2 consisting of α2, β1 and γ1 chains is located in the basement membranes of nervous tissue, Schwann cells, muscle, trophoblast and placenta [5,6]. The laminin α1 chain carboxyl-terminal globular domain (G domain) has been shown to be important for biological activities. The E8 fragment containing the C-terminal α-helical triple stranded coiled-coil domain and the N-terminal G domain possesses the major cell binding activity which is mediated, in part, through α6β1 integrin [7–9]. Recombinant and reconstitution experiments have suggested that this activity may be dependent, in part, on protein conformation [10,11].

Several active sequences on laminin chains have been identified using synthetic peptides [12,13]. Recently we identified five cell binding sites (AG-10, AG-22, AG-32, AG-56 and AG-73) from the laminin α1 chain G domain by screening 113 overlapping synthetic peptides attached to beads followed by further assays of the free peptides (Fig. 1) [14]. Several additional in vitro biological activities of the five peptides were evaluated and one of the peptides (AG-73) was active for neurite outgrowth [15]. Cell spreading on AG-10 and AG-32 was inhibited by α6β1 integrin antibodies whereas the activities of the these other peptides were not blocked [14]. These five active peptides have sequence homologies with their corresponding regions on the human laminin α1 and α2 chains, the mouse laminin α2 chain and the *Drosophila* laminin α chain. Positive amino acids (arginine residues) in the minimum active sequences of AG-10, AG-32 and AG-73 (SIYITRF, IAFQRN and LQVQLSIR, respectively) are completely conserved among the different species and different laminin α chains. Since the sequences are structurally conserved, we determined if the biological activities of the different laminin α chains were conserved. Using peptide-coated plates and peptide-conjugated Sepharose beads, synthetic peptides from the mouse α2 chain and *Drosophila* α chain which correspond to known active sequences in the mouse α1 chain were evaluated for cell adhesive activities.

2. Materials and methods

2.1. Synthetic peptides and laminins

All peptides were manually synthesized by the 9-fluorenylmethoxycarbonyl (Fmoc) based solid-phase strategy and prepared as the C-terminal amide form as described previously [14]. The respective amino acids were condensed manually in a stepwise manner using a tris(alkoxy)benzylamine resin. For condensation, diisopropylcarbodiimide/*N*-hydroxybenzotriazole was employed, and for deprotection of N^{α}-Fmoc groups, 20% piperidine in dimethylformamide was employed. The following side chain protecting groups were used: Asn, Gln and His, trityl; Asp, Glu, Ser, Thr and Tyr, *t*-butyl; Arg, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; Lys, *t*-butoxycarbonyl. Protected peptide resins were treated with trifluoroacetic acid-thioanisole-*m*-cresol-ethanedithiol-H_2O (80:5:5:5:5, v/v) at 20°C for 4 h. The resulting crude peptides were precipitated and washed with diethyl ether, then purified by reverse-phase high-performance liquid chromatography. Purity of the peptides was confirmed by analytical high-performance liquid chromatography. Identity of the peptides was confirmed by amino acid analysis. Amino acid analyses were performed at the Faculty of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan. Mouse laminin-1 was prepared from the Engelbreth-Holm-Swarm tumor as described previously [16].

2.2. Antibodies

The rat monoclonal antibodies used were as follows: mAb 13, an antibody to the β1 integrin subunit [17], was a generous gift from Dr. S. K. Akiyama (NIH, Bethesda, MD) and GoH3, an anti-α6 integrin subunit antibody, was purchased from AMAC (Westbrook, ME).

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PII S0014-5793(96)01060-5

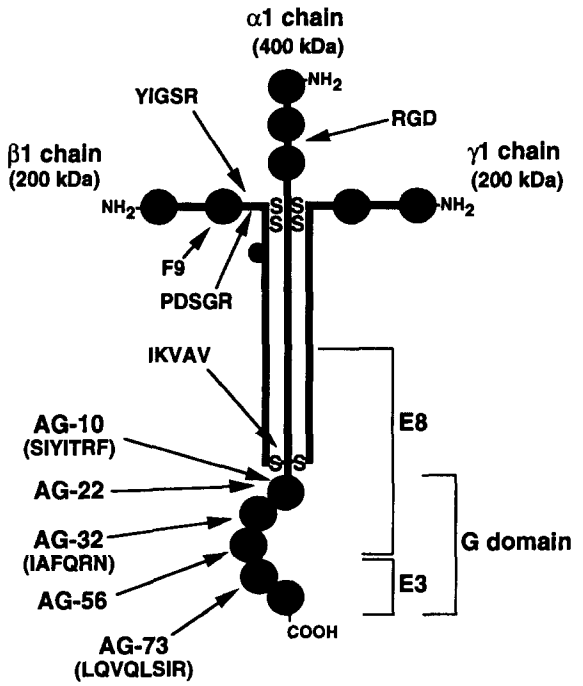

Fig. 1. Structural model of laminin-1. The location of cell binding sites are indicated by arrows. YIGSR from Graf et al. [27], PDSGR from Kleinman et al. [28], IKVAV from Tashiro et al. [29], F-9 (RYVVLPR) from Charonis et al. [30] and Skubitz et al. [31], RGD from Tashiro et al. [32], AG-10, AG-22, AG-32, AG-56, AG-73 from Nomizu et al. [14]. E8 and E3 designate previously described proteolytic fragments active in cell adhesion [33]. The E8 and E3 fragments and the G domain are indicated by square brackets.

Mouse monoclonal antibodies used were: P1E6, an anti-α2 integrin subunit antibody, and P1B5, an anti-α3 integrin subunit antibody. These antibodies and mouse pre-immune IgG were purchased from Life Technologies, Inc. (Gaithersburg, MD) and Sigma (St. Louis, MO).

2.3. Preparation of peptide-beads

The synthetic peptides, RGD sequence containing fibronectin peptide segment (FIB-1: YAVTGRGDSPAS) [14], and laminin-1 were coupled to CNBr-activated Sepharose 4B (Pharmacia Biotech AB, Uppsala, Sweden) according to the manufacturer's instructions. The peptide solutions (1 ml, 1 mg/ml in PBS) or laminin-1 solution (1 ml, 0.1 mg/ml in PBS) were mixed with 50 mg of the activated Sepharose beads. Ethanolamine-coupled beads were prepared as a control. Amounts of coupled peptide were determined by amino acid analysis and the all peptide-beads were coupled at 10–20 μmol peptides per g of Sepharose beads.

2.4. Cells and culture

The following cells were used for this study: B16-F10 mouse melanoma cells [18], HT-1080 human fibrosarcoma cells [19], C2C12 mouse skeletal myoblasts (ATCC, CRL-1772), and RPMI 7951 human melanoma cells (ATCC, HTB66). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS, Hyclone, Logan, UT), 100 units/ml penicillin, and 100 μg/ml streptomycin (Gibco, Grand Island, NY).

2.5. Attachment assay

Cell attachment was assayed in a round bottom 96-well plate (Immulon 2, Dynatech, Chantilly, VA) coated with either various amounts of synthetic peptides or laminin-1. The peptides or laminin-1 were dissolved in Milli-Q water and 50 μl of the solution was added to each well, followed by drying overnight. The wells were blocked by addition of 200 μl of 3% bovine serum albumin (BSA) in DMEM at 37°C for 1 h, then washed three times with DMEM containing 0.1% BSA. Cells, detached by 0.02% EDTA in PBS and resuspended in DMEM containing 0.1% BSA, were added (2×10^4/0.2 ml) to each well and incubated at 37°C for 1 h in 5% CO_2. The attached cells were stained with 200 μl of 0.2% crystal violet aqueous

Table 1
Synthetic peptides and their cell attachment activities

Peptide[a]	Sequence[b]	Residue[c]	Cell attachment activity[d]					
			Plate coat			Sepharose beads		
			HT-1080	B16-F10	C2-C12	HT-1080	B16-F10	C2-C12
AG-10	NRWH**SIYITR**FG	α1: 2183–2194	++	++	++	++	++	++
MG-10	SYWYRIEASRTG	α2: 2290–2301	+	+	++	+	++	++
DG-10	GRWYQAVVDRMG	Dα: 2761–2772	+	+	+	+	+	+
AG-22	SSFHFDGSGYAM	α1: 2290–2301	+	+	+	–	–	–
MG-22	GTIQFDGEGYAL	α2: 2335–2346	+	+	+	–	–	–
DG-22	TGLRFKGNGYVQ	Dα: 2877–2888	+	+	+	+	++	++
AG-32	TWY**KIAFQRN**RK	α1: 2369–2380	+	++	++	++	++	++
MG-32	KWKAFTLSRIQK	α2: 2417–2428	+	+	++	++	++	++
DG-32	QWHKVQAERENR	Dα: 2959–2970	+	+	+	+	+	+
AG-56	SLVRNRRVITIQ	α1: 2570–2581	+	+	+	+	+	+
MG-56	HVERTRGIFTVQ	α2: 2623–2634	+	+	+	+	+	+
DG-56	TVQHTQGELRLT	Dα: 3038–3049	–	–	–	–	–	–
AG-73	**RKRLQVQLSIR**T	α1: 2719–2730	+++	+++	+++	+++	+++	+++
MG-73	KNRLTIELEVRT	α2: 2780–2791	+++	+++	+++	+++	+++	+++
DG-73	RRHHDIGISFRT	Dα: 3370–3381	+	+	+	++	++	++
MG-73S	RTLEVINTKLER		–	–	–	–	–	–
FIB-1	YAVTGRGDSPAS		–	–	–	+++	+++	+++
Laminin-1			+++	+++	+++	+++	+++	+++

[a]AG-10, 22, 32, 56 and 73: mouse laminin α1 chain peptide segments. MG and DG peptides: mouse laminin α2 and *Drosophila* laminin α chains, respectively.
[b]Sequences of the synthetic peptides are given in the single-letter code. Minimum active sites of laminin-1 peptides previously determined are shown in bold. All peptides have C-terminal amides.
[c]Residues of peptides are described. α1, mouse laminin α1 chain [34]; α2, mouse laminin α2 chain [23]; Dα, *Drosophila* laminin α chain [24].
[d]For cell attachment assays, various amounts of laminin-1 and peptides were coated on the wells as described in Section 2. In all cases, the biological activities were quantitated and peptide activities were evaluated relative to the activity observed with laminin-1, as shown in Fig. 2. Cell attachment on the following subjective scale: +++, adhesion comparable to that on laminin-1; ++, adhesion apparent but weaker than on laminin-1; +, adhesion level is low; –, no adhesion.

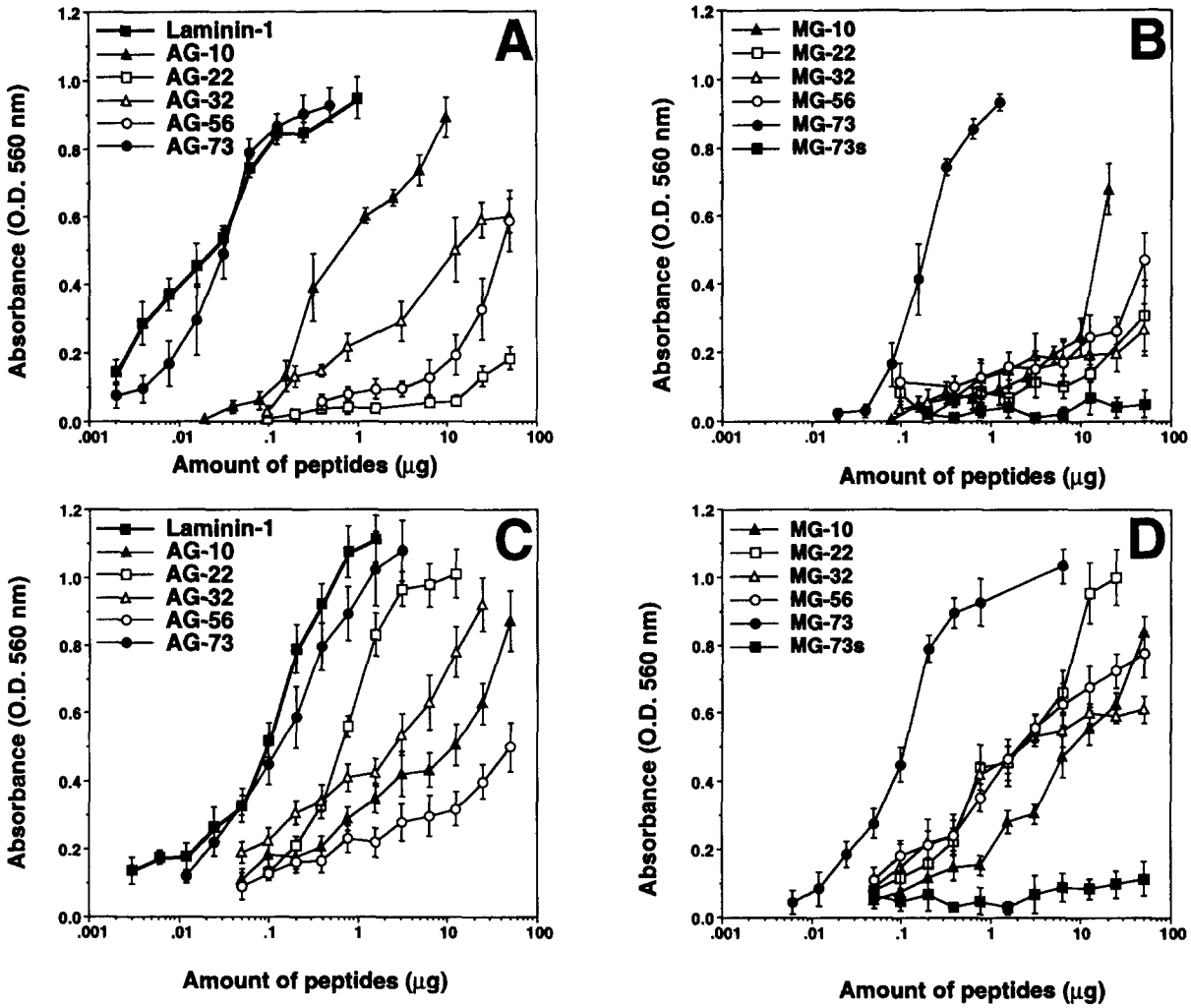

Fig. 2. Attachment of B16-F10 cells and C2C12 cells to peptide-coated plates. (A,B) B16-F10 mouse melanoma cell attachment to AG-peptides and MG-peptides, respectively. (C,D) C2C12 mouse skeletal myoblast cell attachment to AG-peptides and MG-peptides, respectively. Peptides and laminin-1 were dissolved in H_2O, added to 96-well tissue culture plates, and dried overnight. B16-F10 mouse melanoma cells and C2C12 mouse skeletal myoblasts were added and the number of attached cells was assessed by crystal violet staining. Each value represents the mean of three separate determinations+S.E.M. Duplicate experiments gave similar results.

solution in 20% methanol for 10 min. After washing, 200 μl of 1% SDS was used to dissolve the cells and the optical density at 560 nm was measured in a Titertek Multiskan. Peptide-coated wells without cells were processed simultaneously to subtract the background because some peptides at higher concentrations were stained with crystal violet. Coating efficiencies (%) of the synthetic peptides were determined using fluorescamine as shown previously [20,21]. Coating efficiencies of laminin-1 (5 μg/well) and the synthetic peptides (50 μg/well) were 12.1% (laminin-1), 6.7% (AG-10), 0.1% (AG-22), 0.1% (AG-32), 0.2% (AG-56), 12.5% (AG-73), 5.9% (MG-10), 3.9% (MG-22), 0.2% (MG-32), 0.2% (MG-56), 14.5% (MG-73), 0.2% (MG-73S), 0.2% (DG-10), 0.2% (DG-22), 0.1% (DG-32), 0.1% (DG-56), 0.3% (DG-73), 0.1% (FIB-1).

2.6. Cell attachment assay using peptide-beads

Cell attachment to peptide-beads was assayed in 96-well plates which were blocked with DMEM containing 3% BSA. Peptide-beads (20 μl, 100 mg bead/ml in PBS) and cells (100 μl, 1×10^5/ml in DMEM containing 0.1% BSA) were added to the 96-well plates and incubated at 37°C in 5% CO_2 for 2 h. The beads were stained with crystal violet and washed with H_2O (200 μl×2 times). Cell attachment to the peptide-beads was analyzed under the microscope.

2.7. Cell spreading assays

Wells of a flat bottom 96-well plate (Immulon 2, Dynatech, Chantilly, VA) were coated with various amounts of either laminin-1 or synthetic peptides, then incubated for 2 h or dried overnight. Wells were subsequently blocked with 3% BSA in DMEM for 1 h at room temperature. RPMI 7951 human melanoma cells were detached with trypsin and allowed to recover in the presence of 10% FBS for 30 min at 37°C in 5% CO_2. After washing twice with DMEM containing 0.1%BSA, cells were placed in the coated wells at 5×10^3 cells/well in 100 μl of DMEM containing 0.1%BSA. After 45 min of incubation at 37°C, the cells were fixed with 3% formaldehyde and stained with 0.004% crystal violet, and the percentage of spreading cells was counted using phase contrast microscopy [14].

3. Results

The segments of the five laminin α1 chain-derived peptides previously identified as active for cell attachment (AG-10, AG-22, AG-32, AG-56, and AG-73) [14] are listed in Table 1. Laminin α2 chain peptides (MG-10, MG-22, MG-32, MG-56 and MG-73) and *Drosophila* α chain peptides (DG-10, DG-22, DG-32, DG-56 and DG-73), which was corresponding segments of the active sequences in laminin α1 chain, were prepared (Table 1). As controls, the Arg-Gly-Asp (RGD) sequence containing fibronectin peptide segment (FIB-1:

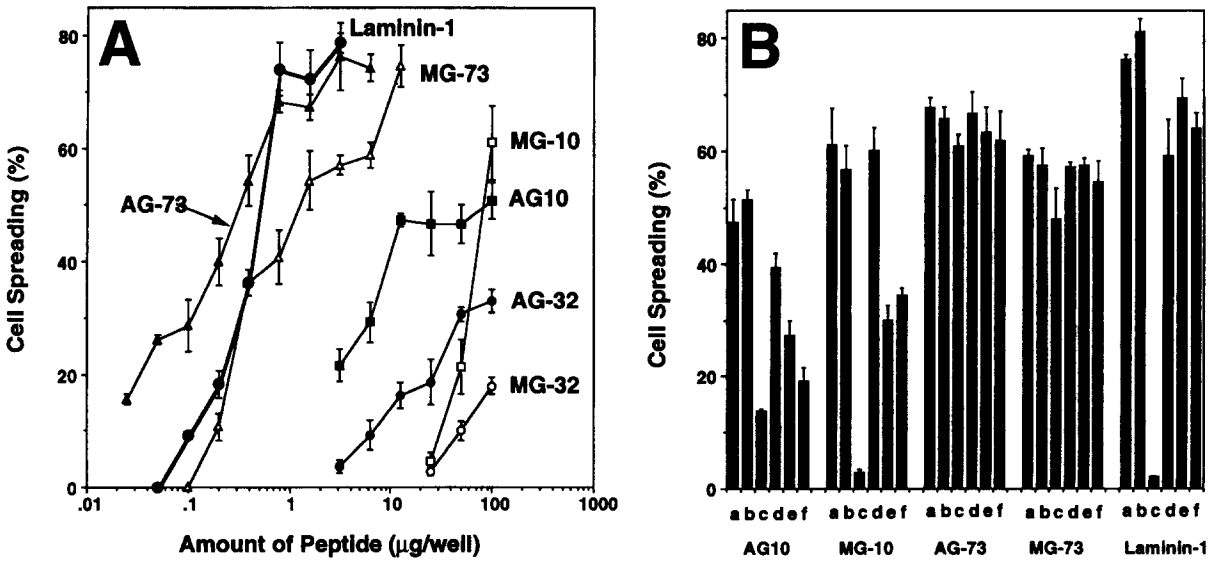

Fig. 3. Effect of peptides and integrin antibodies on RPMI 7951 human melanoma cell spreading. (A) Effect of different concentrations of laminin-1 and synthetic peptides on cell spreading. (B) Inhibitory effect of anti-integrin antibodies. Cell spreading assays were performed on untreated controls (a), or in the presence of mouse pre-immune IgG (2 μg/ml) (b), anti-β1 integrin monoclonal antibody (mAb 13, 67 μg/ml) (c), anti-α2 integrin monoclonal antibody ascites (P1E6, 1: 33 dilution) (d), anti-α3 integrin monoclonal antibody ascites (P1B5, 1: 33 dilution) (e), and anti-α6 integrin monoclonal antibody (GoH3, 2 μg/ml) (f). The amounts of coated peptides were 2 μg/well for laminin-1 AG-73 and MG-73,50 μg/well for AG-10, and 100 μg/well for MG-10. Each value represents the mean of three separate determinations ± S.E.M. Duplicate experiments gave similar results.

YAVTGRGDSPAS) [14], and a scrambled MG-73 peptide, MG-73S, were prepared.

Cell attachment activities of the laminin α1 and α2 chain G domain peptides were evaluated on peptide-coated plastic plate using B16-F10 mouse melanoma cells and C2C12 mouse skeletal myoblasts (Fig. 2). The laminin α1 chain peptides, AG-peptides, showed dose-dependent cell attachment activities for both B16-F10 and C2C12 cells as expected (Fig. 2A,C). C2C12 cells adhered equally well to MG- (Fig. 2D) and to AG peptides, 10 and 73, better to AG-22 than MG-22 and less well to MG-32 and 56 relative to the AG peptides. However, B16-F10 cells were found to adhere weakly to MG peptides (Fig. 2B) except for MG-73 when compared to AG-peptides. AG-22 and AG-56 had weak activity relative to the other AG peptides for B16-F10 cells. Both B16-F10 and C2C12 cells did not attach to a sequentially scrambled MG-73 peptide, MG-73S (Fig. 2B,D). MG-10, MG-22, MG-32 and MG-56 showed dose-dependent cell attachment activity to both B16-F10 and C2C12 cells, and C2C12 cells showed stronger attachment activities on these peptides compared to those of B16-F10 cells. The MG-peptides interacted more favorably with muscle cells rather than melanoma cells.

Cell attachment activities of the synthetic peptides from the mouse α1 and α2 chains and from the *Drosophila* α chain (Table 1) were tested using HT-1080 human fibrosarcoma cells, B16-F10 mouse melanoma cells and C2C12 cells (Table 1). Four of the *Drosophila* α chain peptides, DG-10, DG-22, DG-32 and DG-73 showed cell attachment activities, whereas DG-56 was not active. An RGD sequence containing fibronectin peptide segment, FIB-1, did not show cell attachment activity in this assay (Table 1), possibly due to conformation changes when dried on the dish.

Cell spreading activity of AG and MG peptides was assessed using RPMI 7951 human melanoma cells. RPMI 7951 cells attached and spread in a dose-dependent manner on AG-10, AG-73, MG-10, and MG-73 peptide-coated plates (Fig. 3A). RPMI 7951 cells spread weakly on AG-32- and MG-32-coated plates (Fig. 3A) and did not spread on AG-22-, MG-22-, AG-56-, MG-56-coated plates (data not shown). The effects of integrin antibodies on cell spreading mediated by AG-10, MG-10, AG-73 and MG-73 were tested using anti-β1, α2, α3 and α6 integrin antibodies (Fig. 3B). The anti-β1 integrin antibody inhibited laminin-1-mediated cell spreading. Cell spreading mediated by AG-10 and MG-10 was inhibited by anti-β1 integrin antibodies. Anti-α3 and α6 integrin antibodies partially inhibited AG-10- and MG-10-mediated cell spreading. While anti-β1, α2, α3 and α6 integrin antibodies did not affect AG-73- and MG-73-mediated cell spreading.

Next, we evaluated cell adhesion to covalently conjugated peptides on Sepharose beads (Fig. 4). All synthetic peptides listed in Table 1 were conjugated to Sepharose beads and their cell attachment activities were tested using HT-1080 human fibrosarcoma cells, B16-F10 mouse melanoma cells and C2C12 cells (Table 1). B16-F10 cells attached and spread on the Laminin-1 and FIB-1 conjugated Sepharose beads (Fig. 4I,J), but the cells did not attach on control ethanolamine conjugated beads or on the scramble MG-73 (MG-73S) beads (Fig. 4K,L). AG-10, AG-32 and AG-73 conjugated Sepharose beads showed cell attachment and spreading (Fig. 4A,B,D). The cells weakly attached on AG-56 beads (Fig. 4C), and none of the cells attached on AG-22 beads. MG-10, MG-32 and MG-73 beads showed cell attachment and spreading (Fig. 4E,F,H) and MG-56 beads showed weak cell attachment activity (Fig. 4G), while few attached cells were observed on MG-22 beads. DG-10, DG-22 and DG-73 showed cell attachment and spreading activities, however, few cells were observed on DG-32 and DG-56 conjugated Sepharose beads. These data demonstrate that the α2 and *Drosophila* α homologues of the laminin α1 chain active peptides are active in attachment.

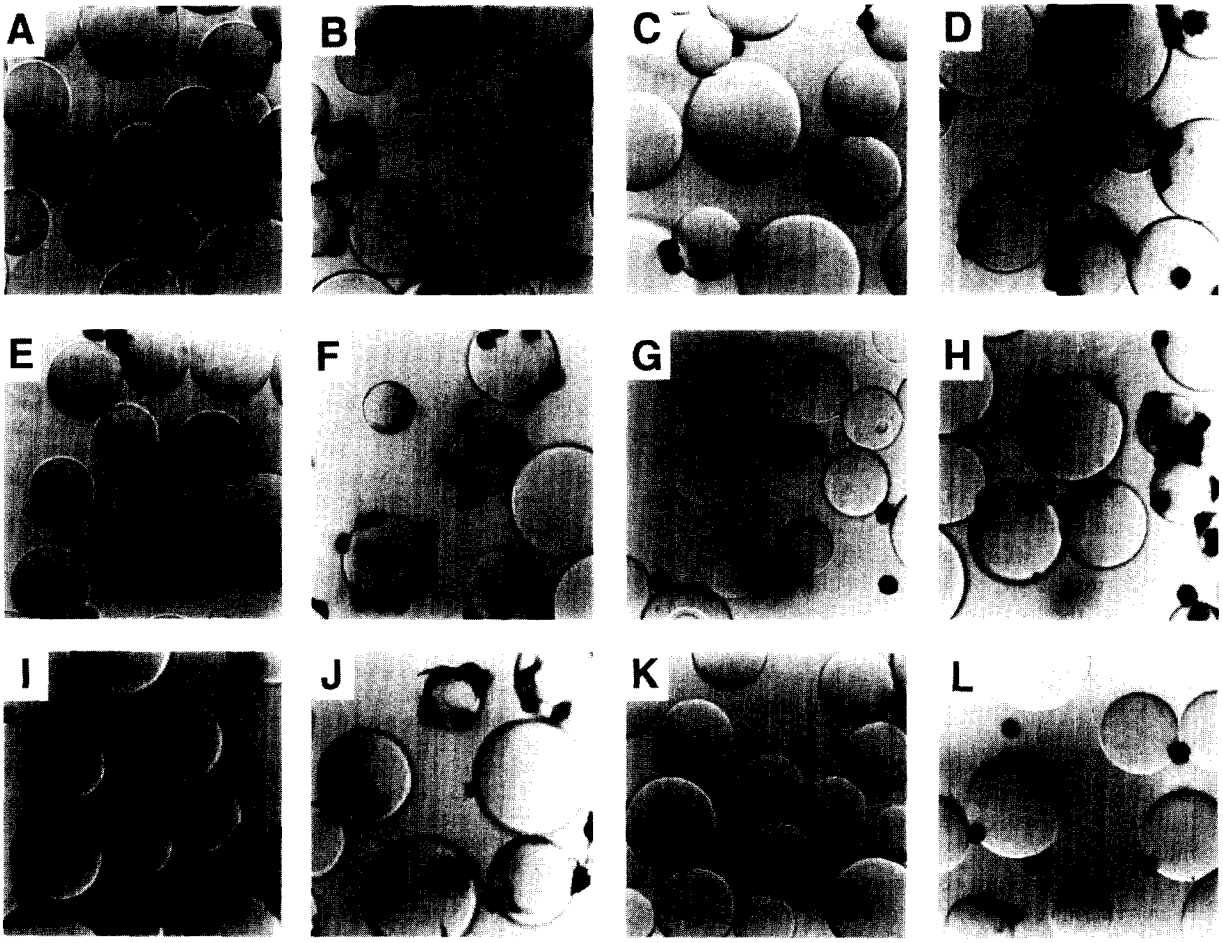

Fig. 4. Adhesion of B16-F10 cells on peptide-conjugated Sepharose beads. B16-F10 mouse melanoma cells were allowed to attach to peptide-conjugated Sepharose beads for 2 h and stained with crystal violet. A, AG-10; B, AG-32; C, AG-56; D, AG-73; E, MG-10; F, MG-32; G, MG-56; H, MG-73; I, laminin-1; J, FIB-1; K, ethanolamine; L, MG-73S.

4. Discussion

Here we have shown cell attachment activities of small peptide segments on peptide-coated plates and on peptide-conjugated Sepharose beads. The peptide-conjugated Sepharose bead assay was originally used for evaluating the biological activities of fibronectin-derived active segments [22]. An RGD-containing fibronectin segment, FIB-1, did not display cell attachment activity in the peptide-coated plate assay, but did show strong cell attachment and spreading activity in the peptide-conjugated Sepharose bead assay. The RGD peptide is active in solution to inhibit cell attachment and spreading on a fibronectin-coated dish. It seems that peptides aggregate on the plate and may be less likely to form active conformations, whereas peptides are flexible on Sepharose beads and able to form active conformations [23]. The cell attachment activities on peptide-coated plates are also dependent, in part, on the coating efficiencies of the peptides. It is difficult to compare directly the cell attachment activities among all the peptides since they showed different coating efficiencies (from 0.1 to 14.5% depending of the peptide). The peptide-coated plate assay is quite useful for comparing cell attachment activity among peptides having similar coating efficiencies. It is also possible to perform dose-response type assays using the peptide-coated dishes whereas such a dose-response test would be difficult with the beads. The Sepharose bead method is useful for evaluating biological activities of short peptides which require active conformations and which show low coating efficiency to plastic dish. In this study, we use both a peptide-coating assay and a peptide-conjugated bead assay for evaluating cell adhesive activities of the laminin-derived synthetic peptides to address these questions of conformation and coating efficiencies of the various peptides. The data as noted below indicate that coating may not have been a significant factor since AG-22 showed low activity with B16-F10 cells and high activity with C2C12 cells. These data demonstrate an important effect due to cell type specificity in adhesion rather than peptide coating. Additional examples of cell type specificity are noted with the MG peptides as well.

We previously identified five different cell binding sequences from the mouse laminin α1 chain G domain by systematic screening using a large set of overlapping synthetic peptide-beads and free peptides [14]. These mouse laminin α1 chain active sequences are highly conserved in the corresponding regions of the different laminin α chains (human α1, human α2, mouse α2, and *Drosophila* α chains). Here we have characterized the cell adhesive activities of the corresponding peptide segments from the mouse laminin α2 chain [24] and *Drosophila* laminin α chain [25]. The laminin α2 chain peptides (MG-10, MG-22, MG-32, MG-56 and MG-73) showed cell attachment activity in the peptide-coated plate assay and in the peptide-bead assay with the exception of MG-22 which

was not active in the bead assay. The AG peptides attached similarly to B16-F10 and C2C12 cells, however, the MG peptides except for MG-73 showed weaker B16-F10 cell attachment activities compared to those of C2C12 cell attachment. Laminin α2 chain is mainly located in basement membranes of nervous tissue, Schwann cells, muscle, trophoblast and placenta [5,6]. MG-10, MG-32 and MG-73 peptides showed cell attachment and spreading activities on both peptide-coated plates and peptide-conjugated Sepharose bead assay with cell type specificities. The MG peptides seem to have more affinity to the muscle-derived cell line, C2C12, compared to melanoma cells. These data suggest that the MG peptides may have cell type specificities for interactions with cell surface receptors.

Cell spreading of AG-10 and AG-32 was previously shown to be mediated by α6β1 integrin [14]. In the present study, cell spreading of AG-10 and MG-10 was inhibited by β1 integrin antibody and weakly inhibited by α3 and α6 integrin antibodies. While AG-73 and MG-73 showed strong cell spreading activity, α2, α3, α6, and β1 integrin antibodies did not inhibit the activity. These results suggest that cell spreading on AG-10 and MG-10 is mediated by similar integrin subunits and the adhesion and spreading on AG-73 and MG-73 probably is mediated by either a different integrin and/or a non-integrin receptor.

Four *Drosophila* laminin α chain peptides (DG-10, DG-22, DG-32 and DG-73) were also found to have cell attachment activity, but these activities were weaker than those of the corresponding mouse α1 and α2 peptides. Recently, we found that the DG-22 peptide promoted neurite outgrowth in *Drosophila* primary cell cultures, whereas the corresponding mouse laminin α1 chain peptide, AG-22, did not show this activity [26]. These results suggest that the biological activities of the five sites are conserved in the laminin α chain family with cell type specificities, and that these five regions may play an important role in laminin interactions with cell surface receptors.

Acknowledgements: We thank Dr. Akira Otaka, Faculty of Pharmaceutical Sciences, Kyoto University, for performing the amino acid analyses of the synthetic peptides.

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