Identification of Cell Binding Sequences in Mouse Laminin γ1 Chain by Systematic Peptide Screening*

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Laminin-1, a major component of basement membranes, consists of three different chains designated α1, β1, and γ1 and has diverse biological functions. We have identified cell binding sites on the mouse laminin γ1 chain, using systematic screening of 165 overlapping synthetic peptides covering the entire chain. We identified 12 cell binding sequences using HT-1080 human fibrosarcoma and B16-F10 mouse melanoma cells in two independent assays employing peptide-conjugated Sepharose beads and peptide-coated dishes. Four peptides (C-16, C-28, C-64, and C-68) located on the globular domains of the γ1 chain were the most active and showed dose-dependent cell attachment. Cell attachment to C-68 was inhibited by EDTA and by anti-α,b, and integrin antibodies. Cell attachment to C-16 and C-64 was partially inhibited by EDTA but was not inhibited by anti-integrin antibodies. EDTA and anti-integrin antibodies did not affect cell attachment to C-28. The four peptides were tested in adhesion and differentiation assays with endothelial, neuronal, and human salivary gland cells. C-16 was the most active for all of the cells, whereas the other three peptides showed cell type specificity in their activities. The active core sequences of C-16, C-28, C-64, and C-68 are YVRL, IRVTLN, TTVKYIFR, and SIKIRGTY, respectively. These sequences are highly conserved among the different species and in the laminin γ2 chain. These results suggest that the specific sequences on the laminin γ1 chain are biologically active and interact with distinct cell surface receptors.

Laminin-1, a major component of the basement membrane matrix, has multiple biological activities including promotion of cell adhesion, spreading, growth, neurite outgrowth, tumor metastasis, and collagenase IV secretion (1–4). There are at least 11 isoforms of laminin, each consisting of three different chains (5). The most extensively characterized laminin, laminin-1 (Mr = 900,000) from the mouse Engelbreth-Holm-Swarm tumor consists of α1, β1, and γ1 chains, which assemble into a triple-stranded cross-like structure with a coiled-coil domain at the long arm (6).

Several active sequences on laminin-1 have been identified using proteolytic fragments, recombinant proteins and synthetic peptides (7, 8). The YIGSR sequence located on the β1 chain promotes cell adhesion and migration, and inhibits angiogenesis and tumor metastasis (9–11). The PDSGR and F-9 (RYVVLPR) sequences located on the β1 chain also promote cell adhesion (12, 13). An IKVAV sequence located on the C-terminal end of the long arm of the α1 chain promotes cell adhesion, neurite outgrowth, experimental metastasis, collagenase IV activity, angiogenesis, plasminogen activator activation, cell growth, and tumor growth (14–18). Recently, we identified five cell binding sequences in the mouse laminin α1 chain C-terminal globular domain, G domain (positions 2111–3060), by systematic peptide screening using overlapping peptide-poly styrene beads and free peptides.

Although the laminin γ1 chain is present in most laminin isoforms except for laminin-5 (5), little is known about its functions or biologically active sites. One of the functions of the laminin γ1 chain is binding to entactin/nidogen, a basement membrane-specific protein, through epidermal growth factor (EGF)1-like repeats located on its short arm (19, 20). The laminin γ1 chain has also been found to be important for neurite outgrowth (21). Active sites on the γ1 chain have not been well characterized, and only one sequence on its C-terminal region has been identified as active for neurite outgrowth (22, 23).

In this paper, we describe the identification of cell binding sequences on laminin γ1 chain using systematic peptide screening. We examined the cell attachment activities of 165 different peptides by two separate assays using peptide-conjugated beads and peptide-coated plastic plates. Twelve active sequences were identified from the two assays. Four of the peptides showed strong cell attachment activities. Several additional biological activities were also evaluated for the four most active peptides, and attempts were made to determine if the cellular receptors were integrins.

MATERIALS AND METHODS

Synthetic Peptides and Laminin-1—All peptides were manually synthesized by the 9-fluorenylmethoxycarbonyl (Fmoc) strategy and prepared in the C-terminal amide form. The respective amino acids were condensed manually in a stepwise manner using 4-(2′,4′-dimethoxybenzyl)-Fmoc-aminomethyl)-phenoxy resin (Bachem Inc., Torrance, CA; and Novabiochem, La Jolla, CA) (24). For condensation, diisopropylcarbodiimide/N-hydroxybenzotriazole was employed, and for deprotection of N-Fmoc groups, 20% piperidine in dimethylformamide was employed. The amino acid side chain protecting groups were the same as described above. Resulting protected peptide resins were deprotected and cleaved from the resin using trifluoroacetic acid-thioanisole-mercuric thiocresol-ethanediethyl-hydroxylamine at 25 °C for 3 h (25). Resulting peptides were purified by gel filtration. The purity of the final peptide was confirmed by the absence of impurities on reversed-phase HPLC, and mass spectrometry analysis confirmed that the molecular weight of the peptide was within 5% of the calculated value.

1 The abbreviations used are: EGF, epidermal growth factor; FBS, fetal bovine serum; Fmoc, 9-fluorenylmethoxycarbonyl; DMEM, Dulbecco’s modified Eagle’s medium; HUVEC, human umbilical cord vein endothelial cell; HSG, human submandibular gland; BSA, bovine serum albumin.

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crude peptides were precipitated and washed with ethyl ether, then purified by reverse-phase high performance liquid chromatography (using a Vydac 5C18 column with a gradient of water/acetonitrile containing 0.1% trifluoroacetic acid). Seven peptides (C-6, C-7, C-13, C-51, C-58, C-99, and C-151) were difficult to dissolve in aqueous solutions. The sequences were tested as peptides coupled to polystyrene beads as described previously (26). Purity of the peptides was confirmed by analytical high performance liquid chromatography.

All synthetic peptides were characterized by a Sciex API IIIE triple quadrupole ion spray mass spectrometer (27). Mouse laminin-1 was prepared from the Engelbreth-Holm-Swarm tumor as described previously (28).

Preparation of Peptide-conjugated Sepharose Beads—The synthetic peptides and laminin-1 were coupled to cyanogen bromide (CNBr)-activated Sepharose 4B (Pharmacia Biotech AB, Uppsala, Sweden) according to the manufacturer’s instructions. The peptide solutions (0.3 ml, 1 mg/ml in Milli-Q H2O) were mixed with 25 mg of the activated Sepharose beads. Ethanolamine-coupled beads were prepared as a control. Amounts of coupled peptide were determined by amino acid analysis (10–20 μmol of peptides/g of Sepharose bead) (29).

Antibodies—Mouse monoclonal antibodies used were: P4C10, an anti-β1 integrin subunit antibody; P1E6, an anti-α2 integrin subunit antibody; and P1B5, an anti-α3 integrin subunit antibody. These antibodies were purchased from Life Technologies, Inc. GoH3, rat anti-α6 integrin subunit monoclonal antibody, was purchased from Serotec Ltd. (Oxford, United Kingdom). Mouse preimmune IgG was purchased from Sigma.

Cells and Culture—B16-F10 mouse melanoma cells (30) and HT-1080 human fibrosarcoma cells (31) were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Life Technologies, Inc.) containing 10% fetal bovine serum (FBS) (HyClone, Logan, UT), 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Inc.).-PC-12

FIG. 1. Sequence and peptides from the laminin γ1 chain. Sequences were derived from the mouse laminin γ1 chain (35). Locations of peptides are indicated by arrows. Active peptides are shown by bold dotted arrows. Cell attachment activities are shown in parentheses as follows: (+), active in both peptide-conjugated bead assay and peptide-coated plate assay; (.), active in one assay either bead assay or coated plate assay; (−), not active in any assay. * peptide was not soluble. Cell attachment activity of the peptides was determined on peptide-conjugated polystyrene beads as described previously (26).
coated peptides (29).

In EDTA inhibition experiments, various concentrations of EDTA were added to the HT-1080 cell suspensions before plating the cells. In anti-integrin antibody inhibition experiments, HT-1080 cells were pre-incubated with antibodies for 15 min at room temperature and then plated. After a 50-min incubation, the attached cells were measured as described above. Student’s t test was used to determine statistical significance of the results.

Cell Spreading Assay—HT-1080 cells were detached by Versene, incubated with 10% FBS for 15 min, and then added to a flat-bottom 96-well plate (Immulon 2) (5,000/0.1 ml of DMEM containing 0.1% BSA) coated with either laminin-1 or synthetic peptides as described above. After the incubation at 37 °C for 45 min, the cells were fixed in 3% formaldehyde and stained with 0.02% crystal violet, and the percentage of spread cells was determined (26).

Tube Formation Assay—Disruption of in vitro tube formation was carried on 48-well plates that had been coated with 200 μl/well Matrigel prepared from Engelbreth-Holm-Swarm tumors as described previously (34). HUVECs were detached from confluent plates with 0.5% trypsin (Life Technologies, Inc.) and plated at 24,000 cells/well in 0.5 ml of HUVEC medium containing 10% defined and supplemented bovine calf serum and the test peptide at a concentration of 200 μg/ml. As controls, cells were plated on medium alone or in the presence of 100 μg/ml IKVAV peptide, a laminin peptide that disrupts tube formation. Endothelial cell tubes were allowed to form overnight at 37 °C. Cells were then fixed and stained with Diff-Quik (Baxter Scientific Products, Miami, FL) and observed under the microscope.

Neurite Outgrowth Assay—Assays were performed in 96-well plates without serum in the medium specified for each cell line. In all assays, cells were incubated for 24 h prior to and for the duration of the assay in serum-free medium containing 10 ng/ml nerve growth factor (Sigma). 1–50 μg of peptide in solution was added to each well of a 96 well plate, then dried overnight at room temperature in a laminar flow hood. Prior to plating cells, the wells were blocked by incubation with 100 μl of 3% BSA in DMEM/F12 for 1 h at 37 °C. Cells were resuspended in serum-free medium and plated at 2,500 cells/well. After 24–48 h, the cells were scored as positive for neurite outgrowth if the neurites were at least two cell body diameters in length.

Acinar Formation Assay—Assays were performed in 96-well tissue culture dishes coated with growth factor-reduced Matrigel (33) (155 μg in 50 μl of serum-free medium) and incubated at 37 °C for 1 h to form a gel. HSG cells (10,000 cells in 100 μl of medium) and peptides (20 μg in 50 μl of medium) were both added to the well. After 48 h, the cells were stained with Diff-Quik. The number and area of acini formed in the matrix were measured using an image analysis program (NIH image). Three fields/well in triplicate wells were assayed (70–150 acini total). The percent of acini with a surface area either less than 800 μm² or greater than 800 μm² was determined.

RESULTS

Cell Attachment Activities of Synthetic Laminin γ1 Chain Peptides Conjugated to Beads—One hundred and sixty-five peptides comprising overlapping sequences of the entire laminin γ1 chain (Fig. 1) (35) were screened for cell attachment activity. Peptides were generally 12 amino acids in length and overlapped with neighboring peptides by 4 amino acids. If the N-terminal amino acid was either glutamine or glutamic acid, one amino acid was extended at the N terminus to avoid pyroglutamine formation (36). If the N-terminal amino acid was proline, either one amino acid was extended or an alanine residue was added at the N terminus for conjugation with CNBr-activated Sepharose beads. Cysteine residues were omitted. All 155 soluble peptides were coupled to CNBr-Sepharose beads. The seven insoluble peptides were prepared directly on the peptide polystyrene beads (26). As a control, laminin-1- and ethanalamine-conjugated Sepharose beads were prepared. We also prepared laminin-1 chain G-domain peptides, AG-10 and AG-73 (26), conjugated to Sepharose beads as described controls as these had been shown previously to actively bind to cells (29). We evaluated cell adhesion to the covalently conjugated peptides on either Sepharose or polystyrene beads using both HT-1080 human fibrosarcoma cells and B16-F10 mouse melanoma cells. The cells attached and spread on laminin-1, AG-10-, and AG-73-conjugated Sepharose beads but they did
not bind to control ethanolamine-conjugated beads as expected (Fig. 2, A–D). Six of the 165 peptides showed cell attachment activity. C-16-conjugated Sepharose beads demonstrated very strong cell attachment and spreading activities comparable to that of the positive control AG-73 beads (Fig. 2F). Cells attached and spread on C-3-, C-35-, and C-57-conjugated Sepharose beads with weaker activity comparable to that of the AG-10 beads (Fig. 2, E, H, and I). C-18 and C-59 beads also showed cell attachment activities, but those activities were weaker than that observed with AG-10 beads (Fig. 2, G and J). The remaining 159 peptide-beads including C-64 and C-68 beads did not show cell attachment activity in this assay (Fig. 2, K and L).

Cell Attachment Activities of Synthetic Laminin γ1 Chain Peptides Coated to Plates—Cell attachment activities of the 158 soluble synthetic laminin γ1 chain peptides were evaluated on peptide-coated plastic plates using HT-1080 human fibrosarcoma cells and B16-F10 mouse melanoma cells. As positive controls, AG-10, AG-73, and laminin-1 were coated on plastic. Twelve of the synthetic peptides from the γ1 chain showed cell attachment activity, including the six peptides that were active in the bead assay (Table I). C-16 was found to have strong HT-1080 and B16-F10 cell attachment activity comparable to that observed with both AG-73 and laminin-1 (Table I). C-64 did not show cell attachment to the peptide-conjugated Sepharose beads (Fig. 2), but in the peptide-coated plate assay, this peptide showed activity similar to that of C-16. Although the cells did not attach to C-28-Sepharose beads and C-68-Sepharose beads, they showed cell attachment activity similar to that of AG-10 when these peptides were coated on plastic (Table I). Eight peptides (C-3, C-18, C-30, C-31, C-35, C-57, C-59, and C-118) showed cell attachment activities, but these activities were weaker than that of AG-10 (Table I). The other 146 peptides showed little or no cell attachment activity when tested as free peptides on plastic substrates.

**Dose-dependent Cell Attachment and Spreading Activities of Active Peptides**—We next performed dose-dependent cell adhesion assays with the 12 active free peptides using peptide-coated plastic plates and HT-1080 cells (Fig. 3). C-16 and C-64 promoted cell adhesion in a dose-dependent manner with activity comparable to that of AG-73 and of laminin-1 (Fig. 3A). C-28 and C-68 were also active in a dose-dependent manner but with activity comparable to that of the weaker AG-10 peptide (Fig. 3A). Eight peptides (C-3, C-18, C-30, C-31, C-35, C-57, C-59, and C-118) showed dose-dependent cell attachment activities, but these activities were weaker than that of AG-10 (Fig. 3B).

![Attachment of HT-1080 cells to peptide-coated plates.](image)

**Table I** Synthetic laminin γ1 peptides and their cell attachment activities

| Peptide | Sequence         | Cell attachment activity |
|---------|------------------|--------------------------|
|         |                  | Sepharose beads           | Plate coat       |
|         |                  | HT-1080 | B16-F10 | HT-1080 | B16-F10 |
| C-3     | LWPLLAVLAAPA     | ++     | +     | +     | +     |
| C-16    | KAFDITYVRLKF     | ++     | ++    | ++    | ++    |
| C-18    | RPESFAYRQTR      | --     | --    | ++    | ++    |
| C-28    | TDIRVTLNRLINTF   | --     | --    | ++    | ++    |
| C-30    | NEPKVLLLLYIAY   | --     | --    | +     | +     |
| C-31    | YYYAISDFAVGGR    | --     | --    | +     | +     |
| C-35    | LPFFNDPRWRNT     | ++     | ++    | ++    | ++    |
| C-57    | APYRFLGQNLGSLY  | ++     | ++    | ++    | ++    |
| C-59    |SSFPRVRRDROTR    | +      | +     | +     | +     |
| C-64    | SETVKYFIRLHE     | --     | --    | +     | +     |
| C-68    | TSIKIRGTYSER     | --     | --    | +     | +     |
| C-118   | TSEAAYNLLIRT     | --     | --    | +     | +     |
| AG-10   | NRWHSIYITRFG     | ++     | ++    | ++    | ++    |
| AG-73   | NFLQVQLSRT      | ++     | ++    | ++    | ++    |
| Laminin-1 |    | +++   | +++   | +++   | +++   |

*a* Cell attachment activity on peptide-conjugated Sepharose beads.

*b* For cell attachment assays, various amounts of peptides and laminin-1 were coated on the wells as described under “Materials and Methods.” In all cases, the biological activities were quantitated and peptide activities were evaluated relative to the activity observed with laminin-1, AG-73, and AG-10 as shown in Fig. 4. Cell attachment was evaluated on the following subjective scale: ++ +, adhesion comparable to that of laminin-1 and AG-73; ++ , adhesion similar to that of AG-10; +, apparent adhesion is low; -- , no adhesion. Triplicate experiments gave similar results.
active peptides (C-16, C-28, C-64, and C-68) were determined and compared with those of AG-10, AG-73, and laminin-1 using HT-1080 cells. The cells spread on low amounts of C-16 and C-64 in a dose-dependent manner when tested on coated plates, and the activity was similar to that of AG-73 and laminin-1 (Fig. 4). C-28 and C-68 showed dose-dependent cell spreading activity comparable to that observed with the weaker peptide AG-10 (Fig. 4).

Based on the two independent assays using peptide-conjugated Sepharose beads and peptide-coated plates, we identified four potent biologically active sequences corresponding to peptides: C-16, C-28, C-64, and C-68. We next focused on evaluating these four peptides to further define their cellular interactions.

**Active Core Sequences of C-16, C-28, C-64, and C-68**—We first determined the active core sequence of four of the peptides: C-16, C-28, C-64, and C-68. For these experiments, all peptides were systematically truncated by one amino acid from either their N- or C-terminal ends and then tested for activity (Table II). C-16c (DITYVRLKF), an N-terminal truncated peptide, still retained full activity, whereas a deletion of aspartic acid from C-16c (C-16d) resulted in decreased activity (Table IIA). C-16f (YVRLKF) showed activity whereas deletion of the tyrosine (C-16g, VRLKF) eliminated its activity. The C-terminal deletion peptides, C-16i (KAFDITYVRK) and C-16j (KAFDITYVR), showed cell attachment activity but weaker than that of C-16. Further deletion of leucine (C-16k, KAFDITYVR) eliminated its cell binding activity. These results indicate that the four-amino acid sequence, YVRL, is critical for activity in C-16.

Systematically truncated peptides of C-28, C-64, and C-68 were similarly evaluated for cell attachment activity. Respectively, minimal active sequences are IRVTLN for C-28 (Table II, B), TTVKYIFR for C-64 (Table II, C), SIKIRGTY for C-68 (Table II, D).

**Effects of EDTA and Integrin Antibodies on C-16, C-28, C-64, and C-68-mediated Cell Attachment**—The effects of EDTA on HT-1080 cell attachment to C-16, C-28, C-64, and C-68-coated plates were examined to determine the role of cations (Fig. 5). Cell attachment to laminin-1 was inhibited by EDTA at 3 mM or greater, whereas cell attachment to C-68 was inhibited by 2 mM EDTA. EDTA partially inhibited cell attachment to C-16 and C-64 peptides. In contrast, C-28-mediated cell attachment was not inhibited by EDTA. These results indicate that cell binding to C-68 is mediated by divalent cation-dependent cell surface receptors similar to that of laminin-1, suggesting that integrins are candidates for interacting cell surface molecules.

Next, we tested the effects of integrin antibodies on cell attachment mediated by the four active peptides using anti-β1, -α2, -α3, and -α6 integrin antibodies (Fig. 6). The anti-β1 inte-
grin antibody inhibited laminin-1-mediated HT-1080 cell attachment. Cell attachment to C-68 was inhibited by both anti-β1 and anti-α2 integrin antibodies. Anti-β1, -α3, and -α6 integrin antibodies did not affect C-16, C-28-, or C-64-mediated cell attachment. We conclude that cell attachment to C-68 is mediated by α3β1 integrin, whereas the other active peptides, C-16, C-28, and C-64, likely interact with non-integrin receptors.

Cell Type Specificity—We next determined if the four most active peptides were active with other cell types known to interact with laminin including primary endothelial cells from human umbilical vein (HUVEC), PC-12 pheochromocytoma cells, and HSG cells (Table III). We found that only two of the peptides, C-16 and C-64, were active with endothelial cells in promoting adhesion and affecting capillary-like tube formation on basement membrane Matrigel. PC-12 cells attached to C-16 and weakly attached to C-28 and C-64. C-16 promoted neurite outgrowth of PC-12 cells, whereas the other three peptides showed no activity with neurite outgrowth. HSG cells were active for adhesion with all of the peptides. C-16 decreased acinar formation on Matrigel, whereas the other three peptides had no activity in this assay. The activity of C-16 was similar to that of AG-73, a peptide from the G domain of laminin, shown previously to inhibit acinar formation on Matrigel (37). These studies demonstrate that there is cell type specificity in the response of various cells to the most active laminin sequences in the γ1 chain.

**DISCUSSION**

Here we have employed a systematic peptide screening for identification of cell binding sequences from the laminin γ1 chain. We prepared 165 overlapping peptides and tested their cell binding activities by two separate assays using peptide-conjugated Sepharose beads and peptide-coated plates. Six of the peptides showed cell attachment activities on both assays, while other six peptides were active only in the peptide-coated plate assay. C-64 and C-68 showed strong cell attachment activity on the peptide-coated plates, but these peptides were not active in the Sepharose bead assay. C-64 and C-68 contain a lysine residue within their active core sequences. It is possible that the peptides were coupled to CNBr-activated Sepharose beads through their ε-amino groups in the lysine residues and this eliminated the cell attachment activity. We showed previously an Arg-Gly-Asp (RGD) (38) containing 12-mer peptide derived from fibronectin was active in the Sepharose bead assay, but this peptide was not active in the peptide-coated plate assay (29). The peptide-conjugated Sepharose bead assay is advantageous for determining the cell binding activity of small peptides, which might not bind to the plastic or have the...
Active Peptides from Laminin γ1 Chain

Table III
Biological activities of the major active γ1 chain peptides

| Peptide | Endothelial cells (HUVEC) | Neuronal cells (PC-12) | Salivary gland cells (HSG) |
|---------|---------------------------|------------------------|---------------------------|
|         | Adhesion Tube formation*  | Adhesion Neurite outgrowth* | Adhesion Acinar formation* |
| C-16    | + +                       | + +                    | + +                       |
| C-28    | + +                       | + +                    | + +                       |
| C-64    | + +                       | + +                    | + +                       |
| C-68    | + +                       | + +                    | + +                       |

* Peptides at 200 μg/ml were assayed for ability to interfere with endothelial cell tube formation on Matrigel.

![Comparison of amino acid sequences of the active peptides](Image)

![Location of active sequences on the laminin γ1 chain](Image)

(NIHBI1KDI), derived from the C-terminal region of the γ1 chain was found to promote neurite outgrowth (22, 23). While the C-163 peptide contained most of that sequence except for the arginine residue, it was not active in our cell attachment assay using neuronal cells.2 It is possible that the terminal arginine is important for biological activity. The Leu-Arg-Glu (LREE) sequence from laminin β2 chain has been shown to bind to neuronal cells and inhibit neurite outgrowth (39, 40). In the laminin γ1 chain, there are two LREE sequences. Two peptides, C-103 and C-130, contain LREE sequence, but HT-1080 and B16-F10 cells did not attach to both the peptide-conjugated Sepharose beads and the peptide-coated plates. The C-103 and C-130 peptides may be able to interact with only certain types of neuronal cells.

The EGF-like repeats of the γ1 chain have been reported to bind to nidogen/entactin through short peptide sequences (19, 20). Peptides C-81, C-82, C-83, and C-84 (residues 829–878), which contained the corresponding region of the entactin/nido-
C-28, C-64, and C-68 showed cell type specificity. Previously, we also showed that the G domain peptides have cell type-specific activity for different neuronal cells (42, 43). These findings suggest that laminin interacts specifically with various cellular receptors through different domains.

Four major cell attachment active sequences of the mouse laminin γ1 chain are completely conserved in the human laminin γ1 chain (44) (Fig. 8). Furthermore, domain IV peptides (C-64 and C-68) are highly conserved among mouse (45) and human (46) laminin γ2 chains (Fig. 8). The active core sequences of C-16, C-28, C-64, and C-68 each contained one arginine residue, and these arginine residues were conserved among the corresponding peptides. These corresponding regions of C-16, C-28, C-64, and C-68 in the other laminin γ chains may also have similar cell binding activities.

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