Laminin-1, a multifunctional glycoprotein of the basement membrane, consists of three different subunits, α1, β1, and γ1 chains. Previously, we used synthetic peptides to screen for biologically active sequences in the laminin α1 chain C-terminal globular domain (G domain) and identified several cell binding sequences (Nomizu, M., Kim, W. H., Yamamura, K., Utani, A., Song, S. Y., Otaka, A., Roller, P. P., Kleinman, H. K., and Yamada, Y. (1995) J. Biol. Chem. 270, 20583–20590). Here, we identify new cell binding sequences on the remainder of the laminin α1 chain by systematic peptide screening, using 208 overlapping synthetic peptides encompassing the central and N-terminal portions of the α1 chain. HT-1080 cell attachment activity to the peptides was evaluated using peptide-coated plastic substrates and peptide-conjugated Sepharose beads. Twenty five peptides showed cell attachment activities on either the peptide-coated plastic substrates and/or the peptide-conjugated Sepharose beads. A-13 (RQVFQVAYIIIKA) showed strongest cell attachment activity in both the assays. Cell attachment to 14 of the peptides was inhibited by heparin. EDTA and integrin antibodies inhibited cell adhesion to two of the peptides, A-13 and A-25, suggesting that these sites likely bind to integrins. These peptides inhibited cell attachment to laminin-1 but not to collagen I, suggesting these active sites are available on the intact molecule. Most of active sequences were localized on globular domains suggesting that these structures play a critical role in binding to cell-surface receptors.

Laminin-1, a major component of the basement membrane matrix with diverse biological functions (1–4), is a trimeric glycoprotein composing of α, β, and γ chains. At least 11 isoforms of laminin, each consisting of three different chains, have been identified to date (5, 6). The most extensively characterized laminin, laminin-1 (M, 900,000) from the mouse Engelbreth-Holm-Swarm tumor, consists of α1, β1, and γ1 chains that assemble into a triple-stranded coiled-coil structure at the long arm to form a cross-like structure (7). Laminin-1 has multiple biological activities including the promotion of cell adhesion, spreading and growth, neurite outgrowth, tumor metastasis, and collagenase IV secretion (1). Proteolytic fragments, recombinant proteins, and synthetic peptides have been used to identify and characterize functional domains in laminin (8, 9). The YIGSR sequence located on the β1 chain has been shown to promote cell adhesion and migration and to inhibit angiogenesis and tumor metastasis (10–12). The PDSGR and F-9 (RYVVLPR) sequences located on the β1 chain were also found to promote cell adhesion (13–15). An IKVAV sequence located on the C-terminal end of the long arm of the α1 chain was found to promote cell adhesion, neurite outgrowth, experimental metastasis, collagenase IV induction, angiogenesis, cell growth, and tumor growth (16–19). These synthetic peptides have been used to understand the various biological functions of laminin and to develop therapeutic agents. However, biologically functional domains/sequences of laminin-1 have not been fully characterized, since it is a large molecule with complex conformations.

Recently, we began a systematic screening of cell binding sequences from the multifunctional molecule, laminin-1, using large numbers of overlapping synthetic peptides (20, 21). Several cell binding sequences in the laminin α1 chain C-terminal globular domain (G domain) have been identified by such an approach (20). Some of these G domain peptides have cell type-specific biological activities (22–24). AG-73, which showed the strongest cell attachment activity among the G domain peptides, promoted neurite outgrowth for some but not all laminin-responsive neuronal cells (23, 25) and acinar development of salivary gland cells (26, 27). In addition, this peptide promoted B16-F10 mouse melanoma cell metastasis to the liver (28, 29). Additionally two other G domain peptides promoted integrin-mediated cell invasion with induction of a tumor cell-specific protease (30, 31). Systematic peptide screening is a powerful and logical method for identification of bioactive peptide sequences from multifunctional large molecules such as laminin.

Here we describe a systematic peptide screening of the laminin α1 chain (positions 1–2110, not including the G domain) using a large set of overlapping peptides. We examined the cell attachment activities of 208 different peptides by two assay systems at the first stage of screening. Twenty five potential active sequences were identified and further evaluated for integrin binding and cell type specificity.

**EXPERIMENTAL PROCEDURES**

**Synthetic Peptides, Laminin-1 and Collagen I—All peptides were manually synthesized by the 9-fluorenylmethoxycarbonyl (Fmoc) strategy and prepared in the C-terminal amide form as described previously (20, 32). For the screening, 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 glutamate or glutamic acid...
Active Peptides from the Laminin α1 Chain

acid, one amino acid was extended at the N terminus to avoid pyrogualu-
tamine formation. (33). Cysteine residues were omitted. The respective
amino acids were condensed manually in a stepwise manner using 4-(2',4'-
dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy resin (34). For con-
densation, diisopropycarbodiimide/N-hydroxybenzotriazole was em-
ployed for preparation of Nα-Fmoc peptide resins. tert-Butanol and
N-methylpyrrolidone was employed. The amino acid side chain protect-
ing groups were the same as described previously (20). The resulting
protected peptide resins were deprotected and cleaved from the resin
with trifluoroacetic acid/thioanisole/CH2Cl2/ethanedithiol/H2O (80:5:
5:5:5, ν/v) for 3 h at room temperature. The resulting crude peptides
were precipitated and washed with ethyl ether and then purified by
reverse-phase high performance liquid chromatography (using Vydac
5C18 column and a gradient of water/acetonitrile containing 0.1% tri-
fluoroacetic acid). 6 peptides (A-2, -47, -69, -75, -147, and -181) were
not dissolved in aqueous solutions. These sequences were evaluated for
their biological activity as peptides coupled to polystyrene beads (20).

The purity of the peptides was confirmed by analytical high perfor-
mance liquid chromatography. The identity of the peptides was con-
figured by a Scion API IIIE triple quadruple ion spray mass spectrometer (35).

Mouse laminin-1 was prepared from the Engelbreth-Holm-Swarm
tumor as described previously (36). Collagen I was purchased from
Collagen Corp. (Palo Alto, CA).

Preparation of Peptide-Sepharose Beads—The synthetic peptides and
laminin-1 or 0.05
l was added to each well, followed by drying over-
night. The wells were blocked by the addition of 100 μl of PBS at room tem-
perature followed by washing with PBS. HT-1080 cells (3 × 104 cells/well) were
preincubated with various
amounts of peptide for 15 min in 0.02% BSA/DMEM and plated on
either laminin-1 or collagen I-coated dishes. After a 20-min incubation
at 37 °C, the attached cells were quantitated as described above.

RESULTS

Cell Attachment Activity on Laminin α1 Chain Peptide-con-
jugated Beads—Two hundred and eight peptides from the lami-
nin-1 α1 chain were prepared for screening cell attachment ac-
tivity (Fig. 1). First, we evaluated cell adhesion to covalently
conjugated peptides on Sepharose or polystyrene beads. Two
hundred and two soluble peptides were coupled with CNBr-
activated Sepharose beads. Six insoluble peptides were pre-
pared directly on the polystyrene beads (20). As a positive
control, the laminin α1 chain G domain peptides AG-10 and
AG-73 were conjugated to Sepharose beads (22). As a negative
control, ethanolamine-conjugated Sepharose beads were also
prepared. Cell attachment activities on the peptide-Sepharose
beads and peptide-polystyrene beads were tested using HT-
1080 human fibrosarcoma cells (Fig. 2 and Table I). The cells
attached and spread on the AG-10- and AG-73-conjugated
Sepharose beads as shown previously (22), but the cells did not
attach on control ethanolamine-conjugated beads (Fig. 2). Four
peptide-Sepharose beads (A-3, -12, -54, -55, and
-174) showed weak cell attachment activity. The remaining 191
peptide beads did not have significant cell adhesive activities in
this assay (Fig. 2). None of the insoluble peptides conjugated to
polystyrene beads showed cell attachment activity.

Cell Attachment Activity of Laminin α1 Chain Peptides—HT-
1080 cell attachment to the 202 soluble laminin α1 chain pep-
tides was further evaluated using peptide-coated plastic plates
(Fig. 3 and Table I). As a positive control, laminin-1 and the
active laminin G domain peptides, AG-10 and AG-73, were
used. Laminin-1 and AG-73 showed strong cell attachment
activity, and AG-10 showed moderate cell attachment activ-
ity (Fig. 3). Three peptides (A-13, -24, and -208) showed strong
cell attachment activity similar to that of laminin-1 and AG-73
(Table I). These peptides showed similar dose-dependent
curves to that observed with laminin-1 and AG-73 (Fig. 3).

Inhibition of cell attachment to laminin-1 and collagen I by
active peptides was analyzed to examine the availability of
these active sites on laminin-1 and substrate specificity of their
activity (Fig. 4). A-10 and A-13 significantly inhibited HT-1080
cell attachment to laminin-1 by about 90%. Inhibition of cell
attachment by A-24 and A-25 was much weaker than A-10 and

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FIG. 1. Sequence and peptides from the laminin α1 chain (position 24 to 2108). Sequences were derived from the mouse laminin α1 chain (51). Locations of peptides are indicated by arrows. Active peptides are shown by a dashed line. 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 insoluble peptides was determined on peptide-conjugated polystyrene beads as described previously (20).
A-13. A-4 did not inhibit HT-1080 cell attachment on a laminin-1 substrate. All these peptides showed little inhibition of cell attachment to collagen I, indicating laminin-specific activity. AA-13, a scrambled peptide of A-13, showed no activity on either laminin-1 or collagen I (data not shown). Also A-29, A-50, A-51, A-54, and A-64 showed no activity on either substrate (data not shown). A-55 inhibited only at highest concentrations on laminin and collagen I and was not specific (data not shown). Therefore, active sites for A-10, A-13, A-24 and A-25 are likely available on the intact laminin molecule. These results suggest that conformation is important in cell recognition with these peptides.

Effects of Heparin on Cell Attachment—Next, we evaluated the effects of heparin on HT-1080 cell attachment to the 20 active peptides (Fig. 5). As a control, laminin-1 and AG-73 were used. Cell attachment to AG-73 was inhibited by heparin, whereas attachment to laminin-1 was not affected as shown previously (21). Cell attachment to 5 peptides (A-4, -10, -50, -54, and -55) was slightly inhibited by heparin, whereas attachment to the remaining 15 peptides was significantly inhibited. Thus, many of the sequences may bind to negatively charged surface molecules.

Effects of EDTA and Integrin Antibodies on Cell Attachment to the Peptides—We next focused on evaluating the 20 peptides active in the plate assay to define further their cellular interactions. The effects of EDTA on HT-1080 cell attachment to the peptide-coated plates were examined to evaluate the role of cations (Fig. 6). Cell attachment to laminin-1 and to AG-73 was inhibited by 5 mM EDTA as shown previously. Attachment to 6 peptides, A-12, -13, -25, -64, -119, and -208, was significantly inhibited by EDTA. EDTA did not have an effect on cell attachment to the other peptides tested. These results suggest that HT-1080 cell attachment to some of the peptides is mediated via cation-dependent cellular receptors such as integrins.

Next, we tested the effects of integrin antibodies on cell attachment to very active peptides, A-13, A-25, and A-64, using anti-β1, -α2, -α3, and -α6 neutralizing integrin antibodies (Fig. 7 lanes c–f, respectively). The anti-β1 integrin antibody inhibited laminin-1-mediated cell attachment. None of the integrin antibodies affected AG-73-mediated cell attachment. Cell attachment to A-13 and A-25 was inhibited by the anti-β1 integrin antibody, whereas cell attachment to A-64 was not affected by any of the integrin antibodies. We conclude that cell attachment to A-13 and A-25 involved integrins, whereas the A-64 peptide likely interacts with either a different integrin and/or a non-integrin receptor.

Active Core Sequence of A-13—Since A-13 is very active for cell adhesion, we examined the structural requirements for biological activity. The active core sequence of A-13 was determined using systematically truncated N-terminal and C-terminal peptides (Table II). When we examined cell attachment activity in the peptide-coated plate assay, A-13b (FQVAYII-
IKA), an N-terminal truncated peptide, still retained full activity. A deletion (A-13c) of phenylalanine and glutamine from A-13b resulted in reduced activity in the plate assay but not in the bead assay. Since glutamine at the N terminus of the peptide is known to easily form a pyroglutamine (33), a deletion of phenylalanine from A-13b was not tested. A deletion (A-13d) of valine from A-13c resulted in complete loss of activity in the bead assay, but some activity was retained in the plate assay.

**Table I**

**Synthetic laminin α1 peptides and their cell attachment activities**

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. 3. Cell attachment was evaluated on the following subjective scale: +++, adhesion comparable to those on laminin-1 and AG-73; ++, adhesion similar to that of AG-10; +, apparent adhesion is low; –, no adhesion. Triplicate experiments gave similar results.

| Peptide | Sequence | HT-1080 cell attachment activity |
|---------|----------|----------------------------------|
|         |          | Sepharose beads | Plate coat |
| A-3     | LWTVRSQQRGLF | ++ | ++ |
| A-4     | RGLFPAILNLAT | – | – |
| A-10    | GINNWWSPSINQ | ++ | ++ |
| A-12    | WVTVDLDRQVFQ | ++ | ++ |
| A-13    | RQVFQVASYIIRA | +++ | +++ |
| A-15    | LTRYKTRRQPFT | ++ | – |
| A-24    | ILETSARVRL | – | +++ |
| A-25    | YIRRLQTRTL | ++ | + |
| A-50    | VLGGRQISINN | – | – |
| A-51    | SINNTAVMRKL | +++ | + |
| A-54    | LGLKLTAFGGFL | ++ | – |
| A-55    | GGEIKVTQVSVDI | ++ | ++ |
| A-64    | RDQLMTNLAVNT | ++ | +++ |
| A-65    | ANVTHLIRANY | – | – |
| A-99    | AGTFALRDGDPQG | +++ | – |
| A-112   | VLIKGKARHKV | +++ | – |
| A-119   | LSNIIVSNIKAS | – | ++ |
| A-121   | LQQRISANSM | – | – |
| A-167   | NLLLIVALKL | ++ | ++ |
| A-174   | HKDELLLARK | ++ | ++ |
| A-177   | KERRDILHRK | ++ | – |
| A-194   | PGGMREKGGKAR | – | ++ |
| A-203   | MEMQANLLRDL | – | + |
| A-206   | LSGIKLLISAR | – | ++ |
| A-208   | ASDSVVADR | – | ++ |
| AG-10   | NRHSIYITRFG | ++ | ++ |
| AG-73   | PRLQVQLSR | +++ | +++ |
| Laminin-1 | | +++ | +++ |

**Fig. 3.** Attachment of HT-1080 cells to peptide-coated plates. 96-Well plates were coated with various amounts of peptides. HT-1080 cells were added, and the number of attached cells after 30 min was assessed by crystal violet staining (A and B). Data are expressed as mean of triplicate results. Triplicate experiments gave similar results.
When the N-terminal alanine was deleted from A-13d, the peptide (A-13e) showed no activity even in the plate assay. The C-terminal deletion peptide, A-13g, showed full activity. A deletion of lysine (A-13h) reduced cell binding activity in the plate assay, but full activity was retained in the bead assay until all three isoleucines were deleted (A-13k). Further deletion (A-13m) of tyrosine and alanine from A-13k abolished activity in the plate assay as well. These results indicate that the alanine at position 7 of AG-13 is critical for cell attachment activity in the peptide-coated plate assay. In contrast, valine (position 6) and isoleucine (position 9) are important for activity in the bead assay. Based on these results from the two separate assays, we conclude that the active core sequence for A-13 is VAYI.

CellType Specificity—We next determined if the 25 peptides active with HT-1080 cells would interact with other cell types including PC-12 cells (rat pheochromocytoma cells), HSG cells (human salivary gland cells), and human umbilical vein endothelial cells, all of which bind well to laminin-1 (Table III). Several of the peptides including A-12, -18, -25, -50, -54, -99, -112, -177, -194, and -206 when coated on dishes were inactive with all three cell types. In contrast, A-10, -13, -55, -64, -167, -203, and -208 when coated on dishes or in solution were active with all three cell types. Surprisingly 2 peptides A-64 and A-119 were not active for endothelial cell adhesion but did reduce tube formation on Matrigel suggesting that soluble peptides could be recognized by these cells. The bound forms of these peptides were adhesive with PC-12 and HSG cells but not with endothelial cells. Two peptides, A-3 and -65, were not active with PC-12 cells but were active with HSG and endothelial cells. A-4 was only active with HSG cells, and A-119 was active with PC-12 cells. These data demonstrate considerable cell type specificity in the cellular interactions with laminin peptides suggesting specific receptor recognition.

**DISCUSSION**

Previously we screened the G domain of the laminin α1 chain and identified several active sites using systematic synthetic

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**Fig. 5. Inhibition of HT-1080 cell attachment to peptides by heparin.** HT-1080 cells were allowed to attach to the peptide-coated plates in the absence (black bars) or presence (stippled bars) of 10 μg/ml heparin. A 96-well plate was coated with 0.5 μg/well laminin-1, AG-73, A-13, A-24, and A-208, or 5 μg/well A-3, A-12, A-55, A-64, A-65, A-167, and A-206, or 25 μg/ml A-10, A-25, A-119, A-121, and A-174, or 50 μg/ml A-4, A-50, A-51, A-54, and A-203. Each value represents the mean of five separate determinations ± S.D. Duplicate experiments gave similar results. *1, p < 0.002; *2, p < 0.01; *3, p < 0.05.

**Fig. 6. Inhibition of HT-1080 cell attachment to peptides by EDTA.** The amounts of the coated peptide were the same as described in Fig. 4. 3 mM EDTA were added to cell suspensions, and after 10 min cells were added onto the plates. After a 30-min incubation, the attached cells were assessed by crystal violet staining. Each value represents the mean of five separate determinations ± S.D. Duplicate experiments gave similar results. *1, p < 0.0002; *2, p < 0.01; *3, p = 0.05.
The effect of peptides and integrin antibodies on HT-1080 cell attachment. Cell attachment assays using HT-1080 cells were performed on untreated controls (a), or in the presence of mouse preimmune IgG (2 μg/ml) (b), anti-αvβ3 integrin monoclonal antibody (P4C10, 1:33 dilution) (c), anti-αvβ5 integrin monoclonal antibody (P1E6, 1:33 dilution) (d), anti-αv integrin monoclonal antibody (P1B5, 1:33 dilution) (e), and anti-αv integrin monoclonal antibody (GoH3, 2 mg/ml) (f). The amounts of coated peptides were 0.1 μg/well for laminin-1, A-13, and AG-73, and 1 μg/well for A-64, and 10 μg/well for A-25. Each value represents the mean of three separate determinations ± S.D. Duplicate experiments gave similar results.

Table II

| Peptide | Sequence | Attachment on plates | Attachment on beads |
|---------|----------|----------------------|---------------------|
| A-13    | RQVFQVAYXIIKA | ++                  | ++                  |
| A-13a   | VQVFQVAYXIIKA | ++                  | ++                  |
| A-13b   | FQVFQVAYXIIKA | ++                  | ++                  |
| A-13c   | YAYXIIIKA     | ++                  | ++                  |
| A-13d   | AYXIIIKA      | +                   | +                   |
| A-13e   | YIIIIKA       | –                   | –                   |
| A-13f   | IIIIIKA       | –                   | –                   |
| A-13g   | RQVFQVAYXIIK | ++                  | ++                  |
| A-13h   | RQVFQVAYXI   | ++                  | ++                  |
| A-13i   | RQVFQVAYXI   | ++                  | ++                  |
| A-13j   | RQVFQVAY     | ++                  | ++                  |
| A-13k   | RQVFQVAY     | –                   | –                   |
| A-13l   | RQVFQV       | –                   | –                   |
| A-13m   | RQVFQ        | –                   | –                   |
| A-13n   | RQVFQ        | --                  | --                  |

Activity was scored on the following subjective scale: ++, activity comparable to that of original peptide; +, activity apparent but weaker than that of original peptide; –, no activity. Active core sequences are written in bold type. All peptides have C-terminal amides.

This site may be an important biological domain on laminin-1. Several peptides previously reported to be active with certain cells were not active in our screen further confirming cell type specificity within neuronal cells. A YFQRYLI peptide (position 1583–1589) was found previously to promote neurite outgrowth (43). A-144 and A-145 containing a part of the YFQRYLI did not show HT-1080 cell attachment activity. However, when a peptide containing the entire YFQRYL sequence was prepared, we confirmed it was active for cell attachment with PC-12 (data not shown). Previously an LRE (Leu-Arg-Glu) sequence was found to be active for neuronal cell attachment and neurite outgrowth (44–46). A-193 (SLAMLRESPGGM, position 2001–2012) contains an LRE sequence, but this peptide showed HT-1080 cell attachment activity using the peptide-conjugated Sepharose beads and peptide-coated plates. It was also not active with PC-12. The LRE sequence may have
cell binding activity specifically for certain neuronal cells. Such cell type specificity has been observed with some of the G domain peptides of laminin-1 (23).

The finding of additional cell type specificity in the remainder of the α1 chain is important. The differences observed cannot be due to coating efficiencies on the plastic or conformation of the peptides since various cells showed differences in attachment with the same peptides. The cell type specificity observed demonstrates unique cellular receptors. There may be specific receptors for each cell type. Alternatively, the observed differences could be the result of differences in the receptor levels and/or affinity to different cell types. Some of the receptors are in the integrin family, which can recognize multiple sequences (47).

We have now screened the entire laminin-1 component chains α1, β1, and γ1 using 673 overlapping synthetic peptides (20, 21). Approximately 11% of the peptides (74 out of 673) were active in either the Sepharose bead assay or the peptide-coated plate assay. Of the peptides active in either assay, approximately a third (28 peptides) or 4% of the total peptides tested were active in both assays. More than half of the active peptides (31 peptides) were localized in the globular domains. The globular domains contain few cysteines and thus are relatively less conformationally stable than either the epidermal growth factor-like repeats located between some of the globular domains in the N termini or the coiled-coil domain located centrally in the α1 chain. The presence of the majority of the cell-binding sites in the globular domains may be due to the less rigid structures of these regions. Our data with the bead assay and the peptide-coated plate assay suggest the importance of conformations regulating cellular interactions. Since the test peptides were attached to the beads in solution versus dried on the dishes, it is likely that different conformations occurred. Inhibition of cell attachment on a laminin-1 substrate by peptides suggests that active sites of A-10, A-13, A-24, and A-25 are available on the intact molecule and that their activity is specific to laminin-1. These results confirm the importance of conformation for cell interactions.

It is possible that some active sequences in laminin are always cryptic and are not exposed with different laminin-protein interactions. Such cryptic sites may be active only when laminin is cleaved. Recently, fragments of laminin-5 generated by MMP-9 were found to induce cell migration and tumor metastases (48). Likewise, certain other proteolytic fragments of some proteins have been found to possess activities not exhibited by the intact molecule. For example, the anti-angiogenesis factors angiotatin and endostatin are fragments of plasminogen activator and collagen XVIII, respectively (49, 50). Thus, while some of the active cell binding sequences may be dependent on conformation in the intact molecule, others could be cryptic and only exposed with proteolytic cleavage.

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Figure 8. Location of active sequences on laminin-1 identified by the series of the systematic peptide screening. The location of cell binding sites on the α1 chain reported in this study and on the G domain (20) are indicated by arrows. A-13, C-16, and AG-73 are found to have strong attachment activity in both the beads assay and plate assay. Many active sequences are clustered in globular domains.

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