Identification of Endothelial Cell Binding Sites on the Laminin γ1 Chain

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Abstract—The laminins belong to a family of trimeric basement membrane glycoproteins with multiple domains, structures, and functions. Endothelial cells bind laminin-1 and form capillary-like structures when plated on a laminin-1–rich basement membrane matrix, Matrigel. Laminin-1 is composed of 3 chains, α1, β1, and γ1. Because laminin-1 is known to contain multiple biologically active sites, we have screened 156 synthetic overlapping peptides spanning the entire laminin γ1 chain for potential angiogenic sequences. Only 7 of these peptides, designated as C16, C25, C30, C38, C64, C75, and C102, disrupted the formation of capillary-like structures by human umbilical vein endothelial cells on Matrigel. Dose-response experiments in the presence of 50 to 200 μg/mL showed that tube formation was prevented by most peptides at 150 and 200 μg/mL, except for C16, which showed strong activity at all concentrations. Active peptides promoted vessel sprouting from aorta rings and angiogenesis in the chick chorioallantoic membrane assay. In addition, the active peptides also promoted endothelial cell adhesion to dishes coated with 0.1 μg of peptide and inhibited attachment to laminin-1 but not to plastic or fibronectin. Four of the active peptides, C25, C38, C75, and C102, may have cell-type specificity with endothelial cells, since they did not promote PC12 neurite outgrowth or adhesion of B16-F10 melanoma and human submandibular gland cells. These results suggest that specific laminin γ1–chain peptides have angiogenic activity with potential therapeutic applications. **(*Circ Res*. 1999;84:688-694.)**

Key Words: angiogenesis ■ laminin ■ endothelium ■ extracellular matrix ■ adhesion

The laminins are large basement membrane matrix glycoproteins with multiple domains, structures, and functions.[1,2] Eleven different laminins have been characterized to date, and each isoform consists of 3 different chains (α, β, and γ), which form a cruciform-like structure.[3] Five α, 3 β, and 2 γ chains have been identified. Laminin-1, composed of α1, β1, and γ1 chains, was isolated from the Engelbreth-Holm-Swarm (EHS) tumor[4,5] and is the best characterized laminin.

In blood vessels, endothelial cells contact a basement membrane that contains laminin. Although the exact nature of the laminin isoforms present in this basement membrane has not yet been determined, polyclonal antibodies to laminin-1 bind to this matrix, suggesting that at least 1 of the α1, β1, or γ1 chains is present. In vitro, endothelial cells bind to laminin-1 and, when plated on Matrigel (a laminin-1-rich basement membrane matrix), differentiate and form capillary-like structures.[6] It has previously been shown that laminin-1 peptides containing the IKVAV (from the α1 chain) and YIGSR (from the β1 chain) sequences alter the formation of capillary-like structures on Matrigel.[7] In vivo angiogenesis assays in mice have demonstrated that the IKVAV peptide induces blood vessel formation, whereas the YIGSR peptide has an inhibitory effect.[8,9]

Recently the entire γ1 chain has been duplicated by mostly 12-mer overlapping synthetic peptides, and the biological activity of these peptides was tested for cell adhesion with 2 tumor cell lines.[10] Several cell attachment sites were identified for B16-F10 melanoma and HT-1080 fibrosarcoma cells. Because laminin-1 has multiple active sites with different cell types[11–13] and little is known about the effect of laminin peptides on endothelial cells, we have used an in vitro Matrigel tube forming assay[6] to identify sequences that affect in vitro morphological differentiation. In this study, we have screened 154 overlapping synthetic peptides that span the entire laminin γ1 chain, which is present in all of the laminins described to date except laminin-5. We have identified 7 peptides (C16, C25, C30, C38, C64, C75, and C102) that alter the formation of capillary-like structures by endothelial cells. Aortic explant studies showed that all 7 peptides can induce endothelial cell sprouting, and 4 were tested in the chick choriollantoic membrane assay and found to be highly angiogenic. Furthermore, we have shown that the 7 peptides promote endothelial cell attachment and compete for adhesion to laminin-1. Although 3 of these peptides, C16, C30, and C64, had been previously shown to promote tumor, neuronal, and submandibular gland cell adhesion in vitro,[10] peptides C25, C38, C75, and C102 showed cell-type speci-

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ficity with endothelial cells. These latter 4 peptides did not promote PC12 neurite outgrowth, adhesion of B16-F10 melanoma, and human submandibular gland (HSG) cells or inhibit HSG cell acinar formation. Our results demonstrate that specific laminin γ1 chain domains may be important in promoting the formation of blood vessels.

Materials and Methods

Matrigel and Peptides

Matrigel and laminin-1 were prepared from the EHS tumor as described.[5,14] One hundred and sixty-five overlapping peptides from the γ1 chain sequence[15] were manually synthesized as described by Nomizu et al.[10,16] Eleven of these peptides were insoluble and therefore could not be tested.[10]

Cells and Culture

Human umbilical vein endothelial cells (HUVECs) were obtained from freshly delivered umbilical cords by treatment with 0.1% collagenase.[17] Cells were grown in RPMI 1640 containing 20% defined and supplemented bovine calf serum (BCS) (HyClone Laboratories, Inc); 5 U/mL of heparin (Fisher Scientific); 100 U/mL penicillin; and (in μg/mL) endothelial cell growth supplement (ECGS) 200 (Collaborative Research), streptomycin 100, gentamycin 50, and amphotericin B 2.5 (Life Technologies). Only early cell passages[3–5] were used. Acinar formation by HSG cells[18] and neurite outgrowth by PC12 cells[10] were performed as previously described.

Tube Forming Assays

Screening of active peptides from the laminin γ1 chain was performed in triplicate on 96-well plates that had been coated with 106 μL of Matrigel per well. Confluent HUVECs were detached from the plates with 0.05% trypsin and 0.53 mmol/L EDTA in HBSS (Life Technologies). Cells were plated at a density of 13 000 cells per well in 100 μL of HUVEC medium containing a reduced amount of BCS (10%) and the test peptide at a concentration of 200 μg/mL. For dose-response studies, tube assays were done using 48-well plates containing 200 μL of Matrigel, 24 000 HUVECs, and 50 to 200 μg/mL of test peptides. As positive controls, cells were plated in the presence of 100 μg/mL of a peptide containing the IKVAV sequence or HUVEC medium with only 5% BCS. The plates were incubated overnight at 37°C with 95% air/5% CO_2. Cells were then fixed and stained with Diff-Quick fixative (methanol) and solution II (0.625 g/L each of azure A and methylene blue) (Baxter Scientific Products). Each peptide was tested in triplicate, and each assay was repeated at least 3 times. Tubes were scored by a blinded observer.

Aortic Ring Sprouting Assays

Aortas were harvested from 6-week-old Sprague-Dawley rats and cleaned of fatty tissue.[19] The aorta was cross-sectioned into thin rings with a scalpel. The rings were placed on 150 μL of gelled Matrigel on 48-well dishes; overlaid with 50 μL of Matrigel, which was allowed to gel for 30 minutes; and then incubated in the presence of 100, 200, or 400 μg/mL of test peptide in 200 μL of human endothelial serum-free medium (Life Technologies). On the 4th day, an additional 20 μg of peptide in 100 μL of fresh serum-free medium was added, and the assay was stopped on the 5th or 6th day after sprouts had developed. Assays were repeated 3 times in quadruplicate and scored by a blinded observer.

Cell Adhesion Assays

Adhesion of endothelial cells to laminin-1 and to synthetic laminin γ1-chain peptides was assayed on 96-well plates. Wells were coated overnight at room temperature with either 0.1 μg of peptide or 0.5 μg of laminin-1 in 100 μL of PBS. After decanting unbound material, wells were rinsed 3 times with PBS, blocked for 2 hours at room temperature with 0.2% BSA in PBS, and then rinsed 3 times with PBS. Uncoated wells blocked with BSA served as controls. Confluent cells were removed from dishes using Versene (0.2 g EDTA/L in PBS) (Life Technologies), and 35 000 endothelial cells were plated per well in 100 μL of RPMI 1640. After incubation for 1 hour at 37°C, plates were gently washed twice with PBS, fixed, and stained with 20% methanol containing 0.2% crystal violet. Wells were washed with distilled H_2O until the wash was dye-free. Bound dye was solubilized with 2% SDS and quantified in an ELISA Titertek microplate reader at 595 nm. For inhibition assays, the cells were allowed to attach to 96-well plates coated with either 0.5 μg of laminin-1, fibronectin, or collagen type I in the presence of 25 to 200 μg/mL of soluble laminin-1 peptides or as otherwise indicated. Under these conditions sufficient peptide and protein binds.[16] All assays were performed at least 3 times in triplicate.

Chick Chorioallantoic Membrane (CAM) Assay

Approximately 4 mL of ovalbumin were removed and windows were open from 3-day old embryonated eggs (Truslow Farms, Charlestown, Md). On day 10, 5 μL of distilled water containing various amounts of peptides, as specified, were dried on quartered 13-mm-diameter plastic coverslips (Thermanox, Nalge NUNC International) and placed on the CAM of the chick. The assay was scored and photographed on embryonal day 13. A positive score for angiogenesis was made when vessels appeared to radiate from the peptide source.

Results

Laminin γ1–Chain Peptides Alter Endothelial Cell Tube Formation

A total of 154 synthetic peptides were screened using the Matrigel tube forming assay to identify laminin γ1–chain sequences involved in angiogenesis. Only 7 of these peptides, C16, C25, C30, C38, C64, C75, and C102, altered tube formation at a concentration of 200 μg/mL (Table 1). Figure 1 shows microphotographs of tube-like structures formed by endothelial cells on Matrigel in the absence (Figure 1A) or presence (Figure 1B through 1F) of active laminin peptides. Different peptides had varying effects on tube morphology (Figure 1). C16 (Figure 1B) caused the cells to form clumps, whereas the remaining peptides produced varying degrees of incomplete tube formation. Although the reasons for the differences in tube morphology induced by the peptides are not known, the differences suggest that the peptides might be acting through different mechanisms, receptors, or both. Experiments in which the peptides were removed after 18 hours and the cells were washed and incubated with fresh medium alone suggested that the effect of the peptides on tube formation is irreversible, because complete tube structures were not formed even 48 hours after peptide removal (data not shown). Furthermore, peptide addition after tube formation had occurred (18 hours) did not cause any apparent morphological changes (data not shown), indicating that the peptides are not toxic and that their presence is required during cell differentiation to be effective. Cells remained viable after 48 hours of incubation in the presence of peptides as determined by trypan blue exclusion, further indicating that the effects were not due to toxicity. In an attempt to determine the minimum concentration at which each peptide affects tube formation, dose-response assays were performed. Most peptides were active at the 2 highest concentrations tested, 150 and 200 μg/mL; however, C16 showed strong activity even at 50 μg/mL (Table 1). It is important to note that the morpho-

TABLE 1. Effect of Laminin γ1–Chain Peptides on Endothelial Cell Tube Formation and Sprouting From Aorta Explants

		Tube Formation,* μg/mL of Peptide				Aorta Explants,† μg/mL of Peptide		
Peptide	Sequence	50	100	150	200	100	200	400
C16	KAFDITYVRLK	++	+++	+++	+++	+/−	+++	++
C25	AFSTLEGRPSAY	−	−	++	+++	+	++	+++
C30	NEPKVLKSYYYAI	−	−	++	+++	+/−	++	+++
C38	FDPELYRSTGHGGH	−	−	++	+++	++	+++	++
C64	SETTVKYIFRLHE	−	−	++	++	+/−	+++	++
C75	DPETGV	−	−	++	++	+++	++	++
C102	KEAEREVTDLLR	−	−	++	++	+/−	++	++

*Tube formation in the presence of peptides was compared with that in medium alone (control). − denotes tubes resemble control; ++, altered tube formation compared with control; and +++, complete tube alteration. An IKVAV-containing peptide was used as a positive control. Other γ1-chain peptides did not alter tube formation.

†Induction of endothelial cell sprouting by peptides was compared with that observed in the presence of medium alone (negative control) or ECGS (positive control). +++ denotes significant sprouting but lower than positive control; ++, significant sprouting above background levels; +, low sprouting levels; and +/−, some sprouting above background levels.

logical characteristics of the tubes formed at the highest doses were also observed at lower doses, suggesting that the different morphologies were not due to peptide concentration but rather to specific biological effects.

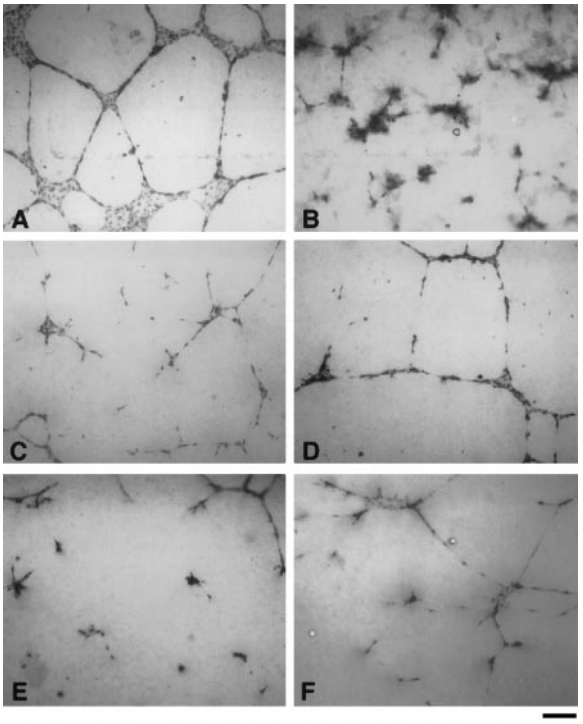

Figure 1. Endothelial cell tube formation in the presence of laminin γ1 chain peptides. Tube-forming assays were performed on 24-well dishes coated with 200 μL of Matrigel. HUVECs were allowed to form tubes in medium containing 10% BCS in the absence of peptides (A) or in the presence of 200 μg/mL of active laminin γ1 chain peptides (C16, C30, C38, C64, and C75; B through F, respectively). Of the 154 peptides screened, only 7 disrupted tube formation; of these, 5 are shown. Note that tube morphology varies in the presence of different peptides. Microphotographs were taken on a Zeiss Axiovert 25 microscope. Bar=100 μm.

Peptides Promote Endothelial Cell Sprouting in Aorta Rings

Because the Matrigel tube assay cannot distinguish between angiogenic and antiangiogenic stimuli, active peptides were tested using aorta explants. Figures 2C through 2J show microphotographs of aorta rings in the presence of 200 μg/mL of peptides C15, C16, C64S, C64, C25, C30, C38, and C75, respectively. All 7 active peptides (C16, C25, C30, C38, C75, and C102 [not shown]) promoted endothelial cell sprouting in a dose-dependent manner (except C16, which was active at all doses tested) (Table 1) above the negative controls, which contained either medium alone (Figure 2A) or peptides that did not disrupt tube formation. These latter peptides included C15 (Figure 2C); C3, C28, C57, and C63 (not shown); and a scrambled sequence of C64, designated C64S (Figure 2E). None of the 7 peptides was as active as the positive control, ECGS, which is a potent angiogenic mixture containing basic and acidic fibroblast growth factors (Figure 2B). Although some of the peptides (C25, C30, C64, and C75) appeared to promote more extensive endothelial cell sprouting than others (C16, C38, C102), it was interesting to note that the number and length of the sprouts also varied. For example, C75 (Figure 2J) did not promote a thick growth of cells, but the distance the cells migrated from the tissue exceeded that observed with the positive control. On the other hand, C16 and C25 (Figure 2D and 2G, respectively) induced strong sprouting but relatively little migration. We conclude that all 7 active peptides have the potential to be angiogenic in vivo because of their ability to promote microvascular growth and migration in 3-dimensional cultures of rat aorta explants.

Active Peptides Promote Cell Adhesion and Inhibit Laminin-1 Binding

Peptide-coated 96-well plates were used to determine whether peptides that altered endothelial cell tube formation could promote cell adhesion. Endothelial cells adhered only

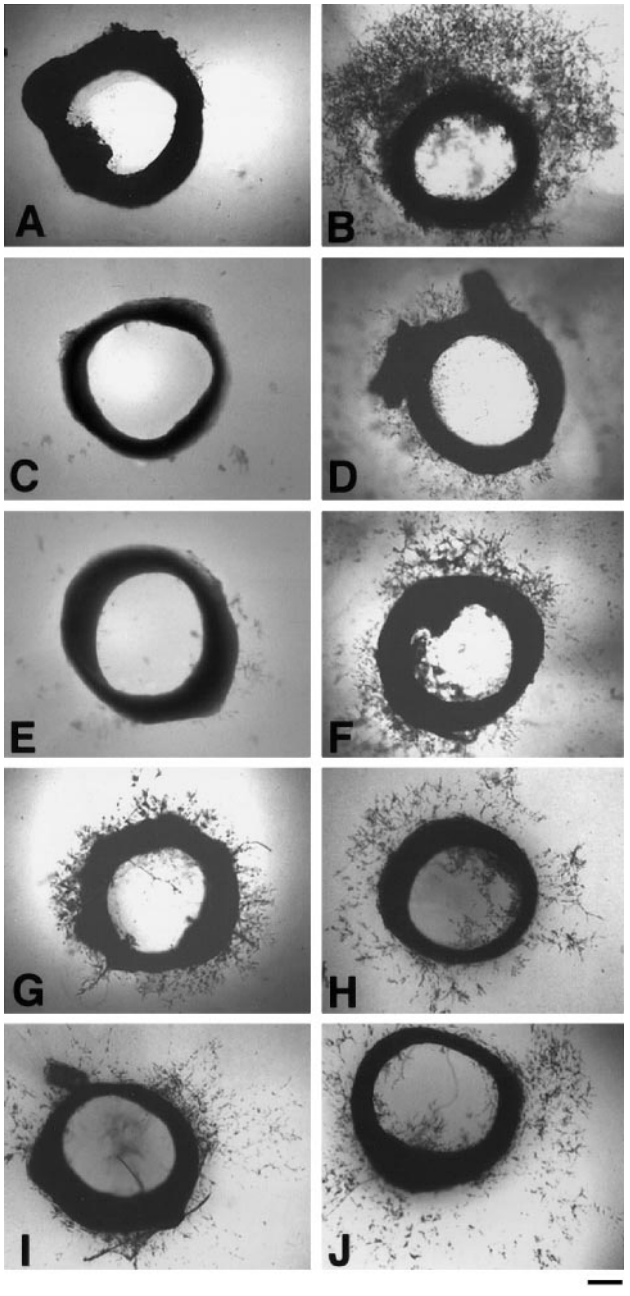

Figure 2. Effect of active laminin γ1 chain peptides on aortic ring sprouting. Aortas from 6-week-old rats were cross-sectioned and embedded in Matrigel on 48-well dishes. Rings were incubated with 200 μg/mL of active peptides C16 (D); C64 (F); and C25, C30, C38, and C75 (G through J, respectively) in serum-free endothelial cell medium. Negative controls included medium alone (A) or C15 (C) or C64S (YVKRTHLFTIESE) (E), a scrambled version of C64 (SETTVKYIFRLHE) (not shown). The positive control contained 200 μg/mL of ECGS (B). On day 4, the cultures were fed with either fresh medium alone or medium containing either peptides or ECGS. On culture day 5 or 6, the rings were fixed and stained with Diff-Quik fixative and solution II, respectively. Explants were photographed on a Zeiss STEMI V8 microscope. Bar=1 mm.

to those wells coated with 0.1 μg of the identified active peptides (Table 2) with the remaining peptides showing no activity. Inhibition experiments were performed to determine whether various concentrations of peptides in solution could compete for attachment to native laminin-1. The results

TABLE 2. Effect of Active Laminin γ1 Peptides on Different Cells

Peptide	Adhesion* HUVEC	HSG	B16-F10	Inhibition of Acinar Formation,† HSG	Neurite Outgrowth,‡ PC12
C16	++	++	+++	++	+
C25	++	−	−	−	−
C30	++	+	−	−	+
C38	++	−	−	−	−
C64	++	++	+	−	−
C75	++	−	−	−	−
C102	++	−	−	ND	−

*Adhesion of HUVECs, HSG cells, and B16-F10 cells to peptides was compared with that observed with laminin-1 (positive control) and to BSA (negative control). − denotes nonsignificant difference from negative control; +, adhesion above background levels; ++, significant adhesion but not as high as positive control; +++, adhesion comparable with that of laminin-1; and ND, not determined.

†Inhibition of salivary gland acinar formation was assayed using 100 μg/mL of peptide. ++ denotes strong activity; −, no effect.

‡Neurite outgrowth was assayed using 25 μg/mL of peptide. + denotes weak activity; −, no effect.

demonstrated that the 7 peptides (C25, C75, and C102; not shown) could prevent cell attachment to wells coated with 0.5 μg of laminin-1 in a dose-dependent manner (Figure 3). Once again, C16 showed the strongest activity, since it inhibited adhesion by 91% at 50 μg/mL, whereas the other peptides inhibited at 25% to 62%. C57, an inactive peptide in tube formation and aortic sprouting, did not significantly inhibit adhesion at any of the concentrations tested. These results suggest that all 7 peptides are active binding sites in the intact laminin-1 molecule. When similar inhibition experiments were performed on collagen type I, C16, C30, and C38 inhibited adhesion by 46% to 88% at 200 μg/mL. When plastic or fibronectin was used as a substrate, the peptides, with the exception of C16, did not inhibit binding, which suggests that at least some of the peptides are specific for laminin-1 (Table 3).

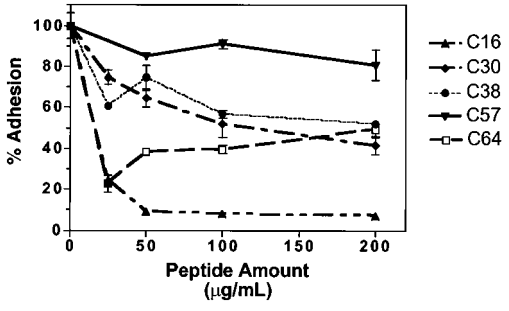

Figure 3. Effect of active laminin γ1 chain peptides on HUVEC adhesion to laminin-1. Ninety-six–well dishes were coated with 0.5 μg of laminin-1. HUVECs (35 000) were incubated for 1 hour at 37°C with 100 μL of RPMI 1640 with increasing doses of C16, C30, C38, C57 (control), and C64, ranging from 50 to 200 μg/mL. Results are expressed as percentage adhesion relative to respective laminin-1 control. Shown are data from representative experiment done in triplicate. $P \leq 0.025$ for all peptides at the highest dose compared with their C57 control.

TABLE 3. Effect of Peptides on Adhesion to Plastic, Fibronectin, and Collagen I

	Adhesion		
Peptide	Plastic	Fibronectin	Collagen I
None	100.0±2.7	100.0±3.4	100.0±3.5
C16	31.5±2.7	12.7±0.4	12.1±3.8
C25	93.2±1.0	85.7±3.8	70.6±11.2
C30	93.2±0.1	90.0±3.1	57.5±3.8
C38	104.0±1.0	94.5±0.7	54.0±6.1
C75	94.5±4.5	89.3±6.6	72.2±5.1

Peptide concentration, 0.2 mg/mL; fibronectin and collagen I coating, 0.5 μg/mL.

Peptides C25, C38, C75, and C102 Lack Activity With HSG, B16F10, and PC12 Cells

Because other laminin-1 peptides, such as IKVAV and YIGSR (from the α1 and β1 chains, respectively), have been shown to possess multiple biological activities with different cell types, we sought to determine whether any of the active γ1-chain peptides could promote the adhesion of nonendothelial cells, including B16-F10 melanoma, HSG cells, and PC12 neuronal cells, and the differentiation of the latter 2 cell lines. All 3 cell types attached to C16 and C64, whereas C30 promoted the attachment of HUVECs and HSG cells (Table 2). C16 inhibited HSG acinar differentiation and, along with C30, stimulated neurite outgrowth of PC12 cells (Table 2).[10] In contrast, we found that the B16-F10 melanoma, HSG, and PC12 cells did not attach to C25, C38, C75, and C102. In additional assays, these peptides did not promote PC12 neurite outgrowth. These results suggest that these 4 peptides may be endothelial cell specific.

Angiogenesis Is Induced In Vivo

Four peptides, C25, C30, C38, and C64, were further tested on the chick CAM assay to determine their ability to induce angiogenesis in vivo. As shown in Figure 4, all 4 peptides were active in inducing vessel formation in a typical radial formation as compared with the 3 types of controls used, including distilled water, C15 (which had been inactive in the tube, aortic sprouting, and adhesion assays), and C64S. Figure 4A shows the angiogenic effect of C25, C30, and C64 (panels 1 through 3) as compared with the C64S control (panel 4). The response of the 4 active peptides varied between 45% and 77%, with C25 being the weakest and C64 the strongest. The controls showed a response of 20% or less (Figure 4B). These results indicate that the laminin γ1 peptides that have been identified in this study are angiogenic in vivo.

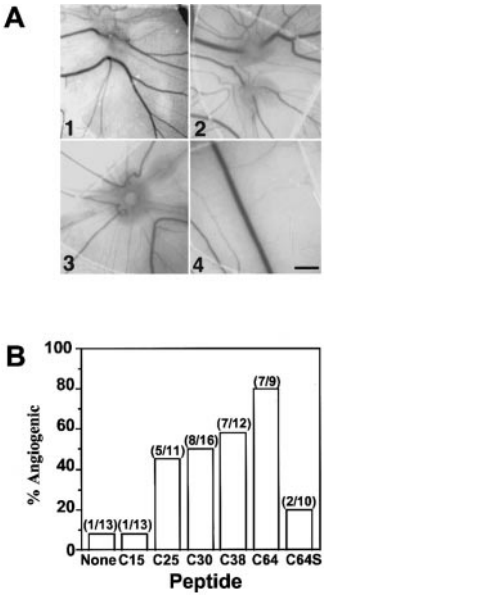

Figure 4. Laminin γ1–chain peptides promote angiogenesis in the chick CAM. A, One microgram each of peptides C25 (panel 1), C30 (panel 2), C38 (not shown), and C64 (panel 3) was dried on quartered 13-mm-diameter plastic coverslips and then placed on the CAM of 10-day-old embryos and scored on day 13. Negative control, C64S, is shown in panel 4. Bar=1 mm. B, Percentage of positive samples is shown for peptides C25, C30, C38, and C64. Controls included distilled water (solvent), C15, and C64S. A minimum of 8 different replicates was tested for each data point.

Discussion

The angiogenic process consists of several steps that include degradation of the basement membrane by endothelial cells, migration to the extravascular space, proliferation, and synthesis of a new basement membrane. Because the basement membrane of the endothelium contains laminin, among other extracellular matrix proteins, and certain laminin peptides derived from the α1 and β1 chain have been previously shown to play a role in angiogenesis,[8,20] it was important to determine whether other sequences derived from the γ1 chain affect this process. In the present study, we have systematically screened 154 soluble peptides spanning the entire laminin γ1 chain using an assay in which endothelial cells differentiate into organized capillary-like structures. Only 7 of these peptides disrupted tube formation at various concentrations. Each of the 7 peptides also promoted cell adhesion and competed for attachment to laminin-1. Furthermore, the peptides stimulated the sprouting of endothelial cells from aortic explants and angiogenesis in the chick CAM assay.

The peptides appear to play a role in the differentiation of endothelial cells into capillary-like structures. Removal

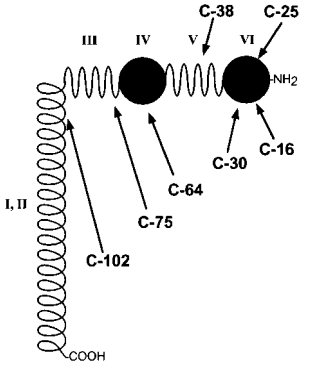

Figure 5. Schematic representation of the laminin γ1 chain and its peptides active with endothelial cells. γ1 chain domains are shown in Roman numerals.

TABLE 4. Sequence Comparison of Active Laminin γ1–Chain Peptides

	C16	C25	C30
Mouse	KAFDITYVRLK	AFSTLEGRPSAYN	NEPKVLKSYYYAI
Human	----------	----------	-D----------
	C38	C64	C75
Mouse	YFDPELYRSTGHGGH	SETTVKYIFRLHE	DPETGV
Human	----------	-------V-----	------
	C102		
Mouse	KEAEREVTDLLR		
Human	----------		

Dashed lines denote homologous amino acid sequences.

of the active soluble peptides from the disrupted tubes did not permit the formation of complete capillary-like structures, although we cannot rule out the possibility that some peptide remained deposited on the Matrigel substrate. Furthermore, cell attachment to Matrigel was not impaired, perhaps because the cells bound to other extracellular matrix components present in Matrigel such as proteoglycans and collagen type IV. These observations, together with the fact that the peptides did not have any effect on the morphology of tubes once they formed, suggest that the peptides play a role during the differentiation of the cells. Although it is not yet known how the peptides act, the morphological differences displayed by the tubes in the presence of each peptide indicate that they might be working through different mechanisms.

Of the 7 active peptides, the following 4 may be endothelial cell type-specific: C25, C38, C75, and C102. The remaining 3 peptides, C16, C30, and C64, promoted the adhesion of nonendothelial cells,[10] altered endothelial cell tube formation, and promoted the sprouting of vessels from aortic explants. C16 and C30 promoted neurite outgrowth by PC12 cells, whereas C16 was the only peptide that inhibited HSG acinar formation. The remaining 4 peptides identified in our screening were only active with endothelial cells, thus suggesting cell type specificity. Peptides C25, C38, C75, and C102 have not been previously reported to possess any type of biological activity when tested in full peptide screens for adhesion or differentiation of various nonendothelial cell lines.[10] These peptides, however, disrupted tube formation, promoted endothelial cell adhesion, and induced angiogenesis from vascular explants. Such cell-type specificity for endothelial cells is not observed with peptides from the α1 and β1 chains.[22] More than 679 peptides from all 3 laminin-1 chains have been screened now in our laboratory with endothelial cells as well as with other cell types, and only these 4 γ1 chain peptides are active with endothelial cells and not with either HSG, B16F10, or PC12 cells. Some cell-type specificity with α1 G-domain peptides has been previously observed with neuronal cells.[13]

It is not yet clear whether the 7 peptides active with endothelial cells are functional in the intact laminin-1 molecule in vivo. In vitro, the peptides block endothelial cell attachment to laminin-1, and 6 of them do not block binding to either plastic or fibronectin, although 3 of them partially inhibited binding to collagen type I. This suggests that most of the peptides promote cell adhesion to intact laminin-1 with high specificity and that they have the potential to function as attachment sites in vivo. The exact conformation of laminin-1 in vivo is not known because of multiple interactions with itself, collagen IV, perlecan, and other matrix components.[1] Recently, metalloproteinase 2 has been found to reveal active sites on the γ2 chain of laminin-5 that were not exhibited by the intact molecule.[21] Given that intact laminin-1 does not promote angiogenesis but the peptides do, a similar mechanism could occur with the γ1 chain in which the sequences described here could become active as angiogenic agents during development and/or tissue remodeling.

The active peptides are localized in different domains of the γ1 chain. Only 1 of the active peptides, C102, is located on the long arm of this chain (domain II), whereas the other 6 peptides are on the short arm (Figure 5). Of these, C16, C25, and C30 are in the first globular domain (domain VI), and C64 is in the second globular domain (domain IV), suggesting that the globular domains might be important functional sites. Peptides C38 and C75 are present in 2 of the EGF-like repeats of domains V and III, respectively. None of the active peptides are located in the region to which nidogen/entactin binds (peptides 82 to 85).[23,24] When a sequence comparison of all 7 peptides was performed, it was found that 5 (C16, C25, C38 C75, and C102) shared 100% homology with the human laminin γ1 sequence and that the other 2 (C30 and C64) only varied by 1 amino acid (Table 4). Interestingly, these sequences are exclusively found in the laminin γ1 chain and not in any other cloned proteins or laminin chains. The γ1 chain is present in all known laminins, except for laminin-5, which has a γ2 chain; this suggests that the γ1 chain and, therefore, its highly conserved active sites, are important.

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References

1. Timpl R, Brown JC. The laminins. *Matrix Biol*. 1994;14:275–281.
2. Mercurio AM. Laminin: multiple forms, multiple receptors. *Curr Opin Cell Biol*. 1990;2:845–849.
3. Burgeson RE, Chiquet M, Deutzmann R, Ekblom P, Engel J, Kleinman H, Martin GR, Meneguzzi G, Paulsson M, Sanes J, Timpl R, Tryggvason K, Yamada Y, Yurchenco PD. A new nomenclature for the laminins. *Matrix Biol*. 1994;14:209–211.

4. Orkin RW, Gehron P, McGoodwin EB, Martin GR, Valentine T, Swarm R. A murine tumor producing a matrix of basement membrane. *J Exp Med*. 1977;145:204–220.
5. Timpl R, Rhode H, Gehron Robey P, Rennard SI, Foidart JM, Martin GR. Laminin: a glycoprotein from basement membranes. *J Biol Chem*. 1979; 254:9933–9937.
6. Kubota Y, Kleinman HK, Martin GR, Lawley TJ. Role of laminin and basement membrane in the morphological differentiation of human endothelial cells into capillary-like structures. *J Cell Biol*. 1988;107:1589–1598.
7. Grant DS, Kinsella JL, Fridman R, Auerbach R, Piasecki BA, Yamada Y, Zain M, Kleinman HK. Interaction of endothelial cells with a laminin A chain peptide (SIKVAV) in vitro and induction of angiogenic behavior in vivo. *J Cell Physiol*. 1992;153:614–625.
8. Sakamoto N, Iwahana M, Tanaka NG, Osada T. Inhibition of angiogenesis and tumor growth by a synthetic laminin peptide, CDPGYIGSR-NH2. *Cancer Res*. 1991;51:903–906.
9. Kibbey MC, Grant DS, Kleinman HK. Role of the SIKVAV site of laminin in promotion of angiogenesis and tumor growth: an in vivo Matrigel model. *J Natl Cancer Inst*. 1992;84:1633–1638.
10. Nomizu M, Kuratomi Y, Song SY, Ponce ML, Hoffman MP, Powell SK, Miyoshi K, Otaka A, Kleinman HK, Yamada Y. Identification of cell binding sequences in mouse laminin γ1 chain by systematic peptide screening. *J Biol Chem*. 1997;272:32198–32205.
11. Kleinman HK, Weeks BS, Schnapper WH, Kibbey MC, Yamamura K, Grant DS. The laminins: a family of basement membrane glycoproteins important in cell differentiation and tumor metastases. *Vitam Horm*. 1993;47:161–186.
12. Beck K, Hunter I, Engel J. Structure and function of laminin: anatomy of a multidomain glycoprotein. *FASEB J*. 1990;4:148–160.
13. Richard BL, Nomizu M, Yamada Y, Kleinman HK. Identification of synthetic peptides derived from laminin α_1 and α_2 chains with cell type specificity for neurite outgrowth. *Exp Cell Res*. 1996;228:98–105.
14. Kleinman HK, McGarvey ML, Hasell JR, Star VL, Cannon FB, Laurie GW, Martin GR. Basement membrane complexes with biological activity. *Biochemistry*. 1986;25:312–318.
15. Sasaki M, Yamada Y. The laminin B2 chain has a multidomain structure homologous to the B1 chain. *J Biol Chem*. 1987;262:17111–17117.
16. Nomizu M, Kim WH, Yamamura K, Utani A, Song SY, Otaka A, Roller PP, Kleinman HK, Yamada Y. Identification of cell binding sites in the laminin α_1 chain carboxyl-terminal globular domain by systematic screening of synthetic peptides. *J Biol Chem*. 1995;270: 20583–20590.
17. Jaffe EA, Nachman RL, Becker CG, Minick CR. Culture of human endothelial cells derived from umbilical veins: identification by morphologic and immunologic criteria. *J Clin Invest*. 1973;52: 2745–2756.
18. Hoffman MP, Kibbey MC, Letterio JJ, Kleinman, HK. Role of laminin-1 and TGF-β_3 in acinar differentiation of a human submandibular gland cell line (HSG). *J Cell Sci*. 1996;109:2013–2021.
19. Nicosia RF, Ottinetti A. Growth of microvessels in serum-free matrix culture of rat aorta: a quantitative assay of angiogenesis in vitro. *Lab Invest*. 1990;63:115–122.
20. Grant DS, Tashiro K, Segui-Real B, Yamada Y, Martin, GR, Kleinman HK. Two different laminin domains mediate the differentiation of human endothelial cells into capillary-like structures in vitro. *Cell*. 1989;58: 933–943.
21. Giannelli G, Falk-Marzillier J, Chiraldi O, Stetler-Stevenson WG, Quaranta V. Induction of cell migration by matrix metalloprotease-2 cleavage of laminin-5. *Science*. 1997;277:225–228.
22. Malinda KM, Nomizu M, Chung M, Delgado M, Kuratomi Y, Yamada Y, Kleinman HK, Ponce ML. Identification of laminin α1 and β1 chain peptides active for endothelial cell adhesion, tube formation, and aortic sprouting. *FASEB J*. 1999;13:53–62.
23. Mayer U, Nischt R, Poschl E, Mann K, Fukuda K, Gerl M, Yamada Y, Timpl R. A single EGF-like motif of laminin is responsible for high affinity nidogen binding. *EMBO J*. 1993;12:1879–1885.
24. Poschl E, Fox JW, Block D, Mayer U, Timpl R. Two non-contiguous regions contribute to nidogen binding to a single EGF-like motif of the laminin γ1 chain. *EMBO J*. 1994;13:3741–3747.

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