RECOMBINANT HUMAN LAMININ-10 (α5β1γ1): Production, Purification and Migration Promoting Activity on Vascular Endothelial Cells

Masayuki Doi¹, Jill Thyboll¹, Jarkko Kortesmaa¹,², Katarina Jansson¹, Antti Iivanainen¹, Masomeh Parvardeh¹, Rupert Timpl³, Ulf Hedin⁴, Jesper Swedenborg⁴, and Karl Tryggvason¹

From the ¹Division of Matrix Biology, Department of Medical Biochemistry and Biophysics, Karolinska Institutet, 17177 Stockholm, ²Biostratum AB, 17177 Stockholm, ³Max-Planck-Institut für Biochemie, 82152 Martinsried, Germany, ⁴Division of Vascular Surgery, Department of Surgical Science, Karolinska Hospital, 17176 Stockholm

Correspondence should be addressed to:
Karl Tryggvason
Div. Matrix Biology
Dept. Medical Biochemistry and Biophysics
Karolinska Institute
S-17177 Stockholm, Sweden
Tel.:+46-8-728 7720; Fax: +46-8-316 165
E-mail: Karl.Tryggvason@mbb.ki.se

Running title: Recombinant Human laminin-10
Summary

The laminin (LN) family of large heterotrimeric extracellular matrix glycoproteins has multiple functions: LNs take part in the regulation of processes such as cell migration, differentiation, and proliferation, in addition to contributing to the structure of basement membranes. LN-10, composed of α5, β1, and γ1 chains, is widely distributed in most basement membranes of both epithelia and endothelia. We determined the complete human cDNA sequence for the LN α5 chain, and produced recombinant human LN-10 (rLN-10) in HEK293 cells by triple transfection of full-length cDNAs encoding the human LN α5, β1 and γ1 chains.

The rLN-10 was purified using affinity chromatography, and had an apparent molecular weight of approximately 800 kD in SDS-PAGE and a native domain structure in rotary shadowing electron microscopy. By using function-blocking monoclonal antibodies, integrin α3β1 was found to be a major mediator of adhesion of HT-1080 and human saphenous vein endothelial cells (HSVECs). HSVECs adhered to rLN-10 stronger than to LN-1 and LN–8, and showed better migration on rLN-10, compared to several other matrices. Considering the cell adhesive and migration-promoting properties that rLN-10 had on endothelial cells, this molecule could be useful in improving the biocompatibility and endothelialization of vascular grafts.
Introduction

The laminins (LNs) are a family of large extracellular matrix (ECM) proteins, composed of three disulfide-linked subunits, the α, β, and γ chains. To date, five α, three β, and three γ LN chains that may combine into at least 12 different isoforms have been identified (1,2). These LN isoforms are expressed in tissue-specific and developmentally regulated patterns and they play significant roles in adhesion, migration, proliferation and differentiation of many cell types (3-5).

In most tissues, mainly two isoforms, LN-8 (α4β1γ1) and LN-10 (α5β1γ1), are found in endothelial basement membranes (4,6-8). LN-8 seems to be an important component of the newly formed endothelial BMs, since mice lacking the α4 chain exhibit neonatal hemorrhages due to unstable microvessels (9). The LN α5 chain, a component of LN-10 and LN-11 (α5β2γ1), is expressed widely in adult tissues including placenta, heart, lung, blood vessels, skeletal muscle, kidney, and pancreas (4,7,8,10-14). Embryos lacking LN α5 exhibit several developmental abnormalities, such as exencephaly and syndactyly, as well as dysmorphogenesis of the placental labyrinth and die late in embryogenesis (15,16). LN α5 chain-containing isoforms may therefore be important in placental endothelial cell migration and blood vessel branching, and in formation of proper basal laminae.

The best-established type of LN receptors is the integrin family, which plays a central role in cell-matrix interaction. Integrin-mediated endothelial cell recognition of LN and other basement membrane (BM) molecules may determine cell-to-matrix adhesiveness and mediate cell behaviors, such as spreading, retraction, polarization, and migration that are essential for the maintenance and normal functioning of blood vessels (17-19).
Vascular endothelial cells undergo drastic morphological and functional changes during angiogenesis, and it is well established that the behavior of the cell is critically influenced by its interaction with components of the extracellular matrix. Because of this fact, endothelial cell attachment and migration on grafts used in vascular surgery might be improved if the surfaces of these non-biological materials would be pre-coated with ECM proteins, e.g. LNs. The ingrowth of endothelial cells on the surfaces of grafts, a process known as endothelialization, has been shown to be of critical importance for preventing thrombus formation on the graft material, and for reducing neointimal hyperplasia. Many adhesive substrate coatings to enhance endothelial cell attachment have been tested (20), but the long term patency of small-diameter vascular grafts is still disappointing, primarily due to stenosis and thrombus formation (21-23).

In this study, we report the full-length cDNA derived sequence of the human LN α5 chain, and the production of recombinant human LN-10. The transfected HEK293 clones produce large amounts of intact LN-10, which is otherwise difficult to obtain. We investigated the adhesive and migration-promoting activities and identified integrin-binding specificity of endothelial cells to LN-10 using the recombinant protein. LN-10 showed not only adhesive but also migration-promoting activities on endothelial cells, providing support for LN-10 as a good candidate for a coating substrate of vascular grafts.
Materials and methods

Cloning of the human laminin α5 cDNA

The previously published mouse sequence was used to search EST-databases. Based upon sequences of the identified ESTs, oligonucleotide primers were synthesized and used for PCR amplification of several human α5 specific probes with λgt11 cDNA library (Clontech) as template. These probes were used for screening of λgt11 cDNA libraries (Clontech) from human lung, fetal lung and fetal kidney. This resulted in the isolation of several clones and further screening was performed with PCR amplified selected regions of these clones. This walk generated clones covering 2134 base pair of coding sequence and 195 base pairs of 3’UTR in the C-terminal part, and 5354 base pairs in the N-terminal part, but lacking a translation initiation start site. The center part, comprising base pairs 5582-9316, was obtained by PCR amplification from a Human Lung Marathon Ready™ cDNA-mix (Clontech). The remaining 296 base pairs of coding sequence and a 67 base pair 5’UTR end was obtained with SMART™ RACE cDNA Amplification Kit (Clontech) using poly-A RNA purified with QuickPrep mRNA Purification Kit (Pharmacia Biotech) from HEK293 cell lysate. The reverse transcription was performed with the MMLV reverse transcriptase SuperscriptII (Life Technologies), and subsequent PCR amplification was performed with Advantage®-GC 2 PCR kit (Clontech). This 363 base pair N-terminal sequence was confirmed by sequencing genomic P1-clone (GenomeSystems) obtained by screening with a PCR generated probe from nucleotides 344-452. This generated a full-length sequence, but most of the sequence was only covered by a single λ-clone or PCR fragment. To further confirm the sequence, we used PCR amplification of SMART™RACE-generated cDNA-mixes from HEK293 cells, human placenta total RNA and from Human Lung Marathon
Ready™ cDNA-mix. This generated new clones so that all regions of the cDNA were covered by more than one clone from different sources. In the case of suspected polymorphisms, several clones from different sources were compared. Sequencing was performed on an ABI PRISM™ 310 Genetic Analyzer (Perkin Elmer) using ABI PRISM® BigDye™ Terminator Cycle Sequencing kit (PE Applied Biosystems). Sequence analysis was performed with AutoAssembler™ (PE Applied Biosystems) and sequence comparative analysis with the GCG-software (24).

**Expression constructs**

For expression of the human LN α5 chain containing a C-terminal FLAG epitope, the full-length cDNA was constructed as follows. To obtain overlapping cDNA clones, PCR amplification of SMART™ RACE-generated cDNA-mixes from HEK293 cells, human placenta total RNA and Human Lung Marathon Ready™ cDNA-mix was performed using Advantage®-GC 2 PCR kit and Pfu Turbo polymerase (Boeringer-Mannheim). All PCR derived cDNA fragments were cloned into the pCR2.1-TOPO vector (Invitrogen) and sequenced (AmpliTag FS on an ABI310 sequencer, Perkin-Elmer) to ensure that no mutations had occurred during amplification. All primers for PCR and pCR2.1-TOPO plasmids into which the PCR derived cDNA fragments were cloned are shown in Table I. To ensure efficient and correct translation initiation, the Kozak sequence (acccggcc, (25)) was edited to match the consensus. Primer KZK1 contained modified Kozak sequence and primer FLAG1 contained the FLAG sequence encoding the FLAG epitope (N-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-C).

The eight different cDNAs that overlap each other were ligated. The final expression construct named HLN5Full.pcDNA was made by inserting the 11.1-kilobase pair full-length cDNA into the EcoRI sites of pcDNA3.1/Zeo(-) mammalian expression vector (Invitrogen) in correct orientation. The construct used for expression of human LN β1 was constructed from
a baculovirus expression vector (26) by ligation of the insert into pIRES-vector (Clontech). The construct used for expression of LN γ1 (HG1) has been described previously (27).

**Antibodies and Control proteins**

Anti-LN α5 (15H5, (28)) monoclonal antibody (mAb) was kindly provided by Dr. K. Sekiguchi. Anti-LN β1 (DG10,(29)) mAb was kindly provided by Dr. I. Virtanen. Anti-LN γ1 (2E8, (30)) mAb was kindly provided by Dr. E. Engvall. Anti-FLAG M2 mAb, purified control mouse IgG, collagen type I from calfskin, collagen type IV (Col IV) from mouse-EHS-tumor and heparin (grade I-A) were purchased from Sigma. Anti-LN γ1 (clone 22) mAb was purchased from Transduction Laboratories. Mouse mAb against integrin α6 (BQ16) was purchased from Alexis Biochemicals. Mouse function blocking mAbs against integrin α1 (FB12), α2 (P1E6), α3 (P1B5), αv (AMF-7), αvβ3 (LM609), mouse mAb against integrin β4 (ASC-3) and control rat IgG2a were obtained from Chemicon. Rat function blocking mAbs against integrin α6 (GoH3), mouse function blocking mAbs against integrin β1 (4B4) and mouse mAbs against integrin α4 (HP2/1) and β3 (SZ-21) were obtained from Coulter. Secondary Ab conjugates anti-mouse IgG-horseradish peroxidase (HRP) and FITC-conjugated F(ab)2 fragments of rabbit anti-mouse immunoglobulin were purchased from Dako. RGDS-peptide, cyclic RGDS-peptide, control RAGS-peptide, mouse mAb against integrin α5 (P1D6) and human vitronectin from plasma were purchased from Life Technologies. Human fibronectin (FN) from plasma was obtained from Roche. EHS-derived LN-1/nidogen complex (LN-1/Nd) was kindly provided by Dr. J. Engel.

**Production and purification of recombinant laminins**

rLN-10 was produced in human embryonic kidney cells (HEK293, ATCC CRL-1573) essentially as described for the production of LN-8 (27). Wild-type cells were transfected
using the standard calcium-phosphate method with the HG1 construct and stable colonies were selected using 100 µg/ml hygromycin (Cayla). All further cell culture and clonal expansion was carried out in continuous presence of relevant selection antibiotics. A highly expressing clone was then transfected with the human LN β1 construct and stable clones were selected using 500 µg/ml G418 (Life Technologies). A clone highly expressing both LN γ1 and LN β1 was finally transfected with HLN5Full.pC DNA and stable colonies were selected using 200 µg/ml zeocin (Cayla). The clones showing the highest secretion were expanded further.

For production of rLN-10, confluent cells were cultured in DMEM supplemented with 1mM pyruvate and insulin-transferrin-selen supplement (Sigma) for up to five days. rLN-10 was affinity purified using anti-FLAG M2 matrix (Sigma). The collected medium was incubated in batch mode with the matrix overnight at 4°C with agitation. Bound rLN-10 was competitively eluted with 50 µg/ml FLAG peptide (Sigma) in TBS/E (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM EDTA) at room temperature. The eluate was concentrated and the buffer was replaced by PBS using 30 kD cut-off ultrafiltration (Millipore). Finally the concentrated solution was passed through 0.2 µm filter to remove self-aggregated polymers. Recombinant human laminin-8 (rLN-8) was produced in HEK293 cells and purified using anti-FLAG matrix and ion-exchange chromatography (JK, MD, KT: Manuscript in preparation).

**Characterization of recombinant laminin-10**

Secreted LN in medium and after purification was characterized using 5% SDS-PAGE and 4-15% gradient SDS-PAGE. Proteins were visualized using silver staining or transferred onto PVDF. The membranes were probed with mAbs described above. After washing, the membranes were incubated with HRP-conjugated goat anti-mouse antibody.
The immunoreactivity was detected by a chemiluminescent kit (Life Science Products) according to the manufacturer’s instructions.

Electron microscopy was performed by the rotary shadowing technique as described previously (31). Briefly, protein (25-50 µg/ml) in 0.2 M ammonium bicarbonate, pH 7.4 was mixed with an equal volume of glycerol and sprayed onto freshly cleaved mica discs. These were dried in high vacuum, shadowed with platinum/carbon at an angle of 9° and replicated.

**Cell culture and HSVEC isolation**

Human fibrosarcoma HT-1080 (CCL-121) cells were from ATCC. Immortomouse brain capillary endothelial (IBE, (32)) cells were kindly provided by Dr. L. Claesson-Welsh. All cells were cultured in humidified 5% CO₂ atmosphere. HT-1080 cells were cultured in DMEM, 10% FCS, pyruvate at 37°C. IBE cells were cultured in F-12, 10% FCS, 2 units/ml γ-interferon on gelatin-coated plastic at 33°C. Prior to assay, the IBE cells were cultured in serum-free F-12 at 37°C without γ-interferon for 24 hours.

As approved by the ethical committee at the Karolinska Hospital, Stockholm, Sweden, residual segments of the great saphenous vein were collected from patients undergoing coronary bypass surgery. HSVECs were isolated as previously described (33). Briefly, veins were rinsed with MEM (Life Technologies) and filled with 0.1% collagenase and 0.16% dispase (Boehringer Mannheim) in MEM for 20 min at 37°C in an 8% CO₂-humidified atmosphere. Cells were cultured in MEM containing 40% heat-inactivated pooled human serum (HS), 1 nmol/L choleratoxin (CT; Sigma), 33 µmol/L isobutylmethylxantine (IBMX; Sigma), and antibiotics. HSVECs were seeded on gelatin-coated plates and passaged (1:3). HSVECs were characterized with monoclonal anti-human von Willebrand factor-related antigen (Dako) and HSVECs between passages 4 and 7 were used in the experiments.
Cell adhesion assays

Adhesion assay was performed as described previously (27). Briefly, 96-well plates (Maxi-Sorp, Nunc) were coated with proteins overnight at 4°C. The remaining protein binding capacity was saturated by addition of 2% heat-inactivated BSA in PBS.

For the assay, cells were suspended in buffered serum-free medium at $3 \times 10^4$ cells/well. DMEM, 25mM Heps, pyruvate was used for HT-1080 cells, F-12, 25 mM Heps, 0.25% BSA for others. Antibodies or other test compounds were added to the cell suspension and the cells were allowed to recover at 37°C for 30 min. Integrin mAbs were used at 10 µg/ml, RGD-peptides at 0.25 mg/ml, heparin at 5 mg/ml, and EDTA at 5mM. The cells were then allowed to adhere for 60 min at 37°C. Bound cells were quantitated by crystal violet staining. None of the cell lines bound appreciably to BSA. When the quantitative results were calculated, binding to BSA was given a value of zero, while the relevant control was given the value 100. The mean and standard deviation (S.D.) were calculated from results obtained from parallel wells. Each assay was performed in three separate wells three times.

Immunofluorescence flow cytometry

Briefly, suspended HSVECs were incubated in PBS containing anti-integrin mAbs against $\alpha_1$-$6$, $\alpha_v$, $\beta_1$, $\beta_3$, and $\beta_4$ for 30 min at 4°C. Following washing, cells were incubated with FITC-conjugated F(ab)2 fragments of rabbit anti-mouse immunoglobulin for 30 min at 4°C. Cells were then analyzed in a FACScan flow cytometer (Becton Dickinson). Mouse IgG was used as a negative control.

Cell migration assays

Cell migration assay was performed as described previously (34). Flat-bottom 24-well culture plates (Corning) were coated with proteins overnight at 4°C. The remaining protein binding capacity was saturated by addition of 2% heat-inactivated BSA in PBS. Thereafter a
4×10 mm stainless steel-weight was put on the center of well, before seeding HSVECs at 2×10⁵ cells/well. After adhesion for 2 days in MEM with 30% HS, the steel-weight was removed. A gap devoid of HSVECs was thus created, with two broad (10 mm) EC-edges facing each other at a distance of 4 mm. During endothelialization, HSVECs were incubated in MEM with 40% HS, CT and IBMX for 2 days after removing the steel-weight. The cells were visualized by 0.1% crystal violet staining.

**Results**

**Sequence of human laminin α5 chain**

The full-length cDNA coding sequence consisting of 11,088 base pairs had an open reading frame encoding 3696 amino acids (GenBank™ accession number AF443072). Compared to the previously reported mouse sequence (GenBank™ accession number U37501 (10)), we obtained additional 79 amino acids in the N-terminal end, also identified later in mouse (GenBank™ accession number AJ293593). Comparison with the mouse laminin α5 showed an overall amino-acid identity of 79%. The two RGD-sequences were conserved in the human chain. The human sequence contained two extra cysteines (amino acid residues; 3173 and 3663) and a link region between LG3 and LG4-domains that is seven residues longer than the mouse sequence. In addition, there was a stretch of four extra amino acids in domain IV (amino acid residues; 1680-1683) in the human sequence. The mouse sequence also had an additional stretch of 25 amino acids in the C-terminus, compared to the human sequence. Alignment of mouse and human LN LG5-modules with other published sequences (not shown) revealed similar C-terminal length in all cases except for the mouse α5. A partial sequence covering 953 amino acids in the C-terminus of the human LN α5 has been reported earlier (12), and this contains no discrepancies compared to our sequence.
We generated cDNAs from four different sources (placenta, HEK293 cells, and lung-marathon-ready-cDNA from two sources), and when the same difference resulting in amino acid substitution was detected in two of them, we assumed these to be polymorphisms. Four of them were located in domain IIIa; 5698:A-G (1900: Met-Val), 5722: G-A (1908: Ala-Thr), 6158: G-A (2053: Arg-Thr), 6184: A-G (2062: Asn-Asp) and one in the G domain; 9235:T-C (3079: Trp-Arg). The first nucleotide of the predicted translation initiation site (ATG) was designated as nucleotide 1. The amino acids chosen for the rLN-10 construct at these possible polymorphic sites were the first of the two in each case. Four of the polymorphisms were located in domain IIIa that has laminin epidermal growth factor-like repeats (LE-modules). The two LE-modules (amino-acid residues; 1864-1912 and 2023-2069), containing these polymorphisms, have about 40% homology to LE-modules of LN γ1 (III4 and III5). Based on the crystal structures that were reported previously for these γ1 LE-modules (35,36), we modulated the tertiary structure of the LN α5 modules (not shown), and found that the observed polymorphisms would not seem to affect the structure of the LE-modules.

**Production and Characterization of Recombinant LN-10**

Conditioned medium from wild-type HEK293 cells did not react in western blotting with the anti-LN α5, anti-LN β1, anti-LN γ1, or anti-FLAG Abs, indicating that these cells express endogenous LNs at very low amounts if at all (data not shown). After triple transfection, the best cell clone produced 1-2 mg of rLN-10 per liter of medium.

Immuoaffinity purification with anti-FLAG M2 matrix followed by competitive elution with FLAG-peptide resulted in highly purified protein as seen in silver stained SDS-PAGE gels (Fig 1a). Under reducing conditions, two bands were seen, a 400 kD band corresponding to the laminin α5 chain and a 200 kD band corresponding to the laminin β1 and γ1 chains, which have similar molecular weights (Fig. 1a). In western blotting of the
conditioned medium, two bands of approximately 350 and 400 kD could be seen with the laminin α5 mAb (Fig. 1b). The anti-FLAG antibody reacted with a 400 kD and a 40 kD fragment (Fig 1b and not shown). Taken together, these data indicate that the 400 kD fragment is the intact laminin α5, the 350 kD is a N-terminal fragment and the 40 kD is a C-terminal fragment harboring the FLAG epitope. Under non-reducing conditions, most of the protein appeared at the top of the gel as a very high molecular weight band, which was immunoreactive with α5, FLAG, β1 and γ1 mAbs, showing that the rLN-10 was produced as disulfide-crosslinked heterotrimer (Fig. 1c). A minor band of approximately 400 kD was also seen in silver staining and in western blotting with α5, FLAG, β1 and γ1 mAbs. A band of similar size, likewise reactive with β1 and γ1 antibodies was also seen with rLN-8 (27), and thus we assumed that some of the rLN-10 molecules in the preparation consisted of a covalently cross-linked β1/γ1 dimer, which was non-covalently associated with the α5 chain. In case of rLN-8 the α4 chain is seen as a 200 kD band, but in case of rLN-10, the non-covalently associated α5 chain had an apparent molecular weight similar to the β1/γ1 dimer, which explains the immunoreactivity of the minor band with α5 and FLAG mAbs.

Rotary shadowing EM revealed the rLN-10 protein as having three short arms and one long arm in accordance with the expected structure. The lower panels in Fig. 2 show selected monomers, some of which have an elongated globular domain in one of the short arms, which could be the relatively large domain IVb. The upper panel of Fig. 2 shows representative oligomers, which dominate the preparation.

**Cell binding to rLN-10**

To investigate the biological activity of rLN-10, we assayed it for cell adhesion properties. As a general model, we used the HT-1080 fibrosarcoma cell line, which expresses a wide variety of integrin receptors such as α2, α3, α5, α6 and β1 (37). Different blocking
anti-integrin antibodies were used to identify the integrin receptors mediating cell binding to rLN-10. Two endothelial cell types were also studied; HSVECs were used as model for macrovascular endothelial cells and IBE cells for microvascular endothelial cells.

HT-1080 cells adhered to FN equally strongly as to rLN-10 (Fig. 3a) while LN-1 and rLN-8 were less effective in promoting cell adhesion (Fig. 3a). Similar results were obtained with IBE cells and HSVECs (Fig. 3b and c). Based on these results, further experiments with blocking integrin mAbs were performed using a coating concentration of 10 µg/ml.

Monoclonal Abs against either α3 or β1 inhibited HT-1080 cell binding to rLN-10 by approximately 80%, indicating that integrin α3β1 was a major mediator of adhesion to rLN-10 (Fig. 4). In addition, mAbs against integrin α2 had partial inhibitory effect on adhesion to rLN-10 (Fig. 4). Since some adhesion remained after blocking of the integrin β1, other receptor classes besides β1 integrins could be involved in the cell adhesion. Monoclonal Abs against integrins α6 and αv had no effects on the adhesion of HT-1080 cells to rLN-10 either alone (Fig. 4) or in various combinations (α3+α6, β1+α6, β1+αvβ3, β1+α6+αvβ3, β1+α6+αv; not shown). This indicates that α6 integrins (α6β1, α6β4) or αv integrins (αvβ1, αvβ3 or αvβ5) were not mediating the cell adhesion in a substantial manner.

HSVEC binding to the rLN-10 was also studied. To determine which integrins were present on the cell surface, we performed fluorescent cell sorting assay using mAbs against integrin subunits α1, α2, α3, α4, α5, α6, αv, β1, β3 and β4 (Fig.5). From these results it can be concluded that HSVEC express large amounts of α2β1, α5β1 and αvβ3, moderate amounts of α3β1, and small amounts of α1β1, α6β1 and α6β4, but integrin α4β1 was not detected. The HSVEC binding was most efficiently inhibited by mAbs against integrin α3 and β1, but mAb against α2 also had partial effect (Fig. 4), in a fashion similar to that
observed for HT-1080 cells. Integrin α6 was only weakly expressed on HSVECs and, consequently, mAbs against this integrin did not inhibit binding to rLN-10.

As expected, cell adhesion to rLN-10 was found to be dependent of divalent cations since it could be abolished by 5 mM EDTA in both HT-1080 cells and HSVECs (Fig. 4). Heparin, when used at 5 mg/ml, had no effect on the adhesion of either cell type (Fig. 4). Since the α5-chain has conserved RGD-sequences, we tested the effect of RGD peptides, which are reported to block the function of various RGD-dependent integrins (such as α5β1 and the αv family) (38). Neither linear nor cyclic RGD-peptides had any effect at 0.25 mg/ml concentration on adhesion of either HT-1080 cells or HSVECs to rLN-10 (Fig. 4). It was, furthermore, observed that the cell binding activity of rLN-10 was sensitive to air-drying, as we have previously reported for rLN-8 (27). When the coated protein was allowed to air dry for 20 min at room temperature before adding the cells, the cell binding activity of rLN-10 was completely lost (data not shown).

**HSVECs migration**

LN s have been shown to stimulate cell migration during development and in many pathological processes. We examined the ability to promote HSVEC migration on dishes coated with 10 µg/ml of rLN-10 or other adhesive proteins. The migration assay was repeated three times using HSVEC obtained from three different donors. Among the seven different adhesive proteins examined, rLN-10 was the most potent in promoting HSVEC migration *in vitro* (Fig. 6a and 6b). In addition to rLN-10, type IV collagen was also quite potent in promoting HSVEC migration. LN-1 and gelatin were of roughly equal potency but significantly lower than rLN-10, and rLN-8 was the least potent among the proteins examined.
Discussion

A major contribution of this work is that it provides a cell line source for human laminin-10 that is difficult to obtain in significant quantities from native tissues or cells. The quantity of rLN-10 produced by the HEK293 cells in monolayer cultures, was quite high considering the size and complexity of the protein. Rotary shadowing electron microscopy revealed the native structure of the rLN-10 molecules to be cross-shaped with three short arms and a long arm with a globular domain at the end (Fig. 2). Two forms of the LN α5 chain with molecular weights of approximately 350 and 400 kD were seen in western blotting of the conditioned cell medium. Other investigators have also observed LN α5 as two bands, and have suggested the difference to reflect different glycosylation forms (39). Based on western blotting, we conclude that the 350 kD fragment represents a proteolytically processed N-terminal fragment where the C-terminus, harboring the FLAG-epitope, was cleaved off. The 40 kD fragment detected with anti-FLAG mAb in western blotting is likely to be a cleaved C-terminal domain LG4-5 fragment and the 400 kD fragment represents the intact molecule. Such cleavage between the subdomains LG3 and LG4 has been reported for other LN isoforms (40). In SDS-PAGE under non-reducing conditions the majority of the rLN-10 appeared as disulfide cross-linked heterotrimer, while a minority consisted of the α5 chain non-covalently associated with a disulfide-linked β1/γ1 heterodimer.

Integrin-mediated recognition of ECM molecules results in intracellular signaling that affects a range of cell behaviors (41). In endothelial cells, these signals affect focal adhesions and cytoskeletal organization. Therefore, integrin-mediated endothelial cell recognition of LN and other BM molecules may determine cell-to-matrix adhesiveness and mediate signals that are essential for the maintenance and normal functioning of blood vessels (17-19). LN-8 and LN-10 are secreted by endothelial cells, and are major components of the subendothelial basement membrane (4,6-8,42,43). Several integrins have been
implicated as receptors for LN-10 or LN-10/11, including $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_1$, and $\alpha_6\beta_4$ (28,44-49), but no report exists concerning endothelial cells. In this study, rLN-10 was shown to promote cell adhesion and migration, and the main cellular receptors mediating adhesion in HSVECs and HT-1080 cells to be integrins $\alpha_2\beta_1$ and $\alpha_3\beta_1$. Although the $\alpha_6$ integrins have previously been implicated as receptors for LN-10 (44), our results do not support that notion. Cell-specific factors might contribute to the observed discrepancy.

HSVECs, as well as HT-1080 and IBE cells, attached to rLN-10 more strongly than to LN-1 and rLN-8. The poor adhesion of HSVECs to rLN-8 is not surprising considering the fluorescent cell sorting data (Fig. 5) showing that the cells have little $\alpha_6$ integrins, which have previously been shown to be receptors for rLN-8 (27). We can, therefore, conclude that the integrin-binding is distinctly different between the two main forms of endothelial LNs, as well as between different endothelial cell types, as we have previously shown that IBE and bovine capillary endothelial cell adhesion onto rLN-8 is mediated predominantly by $\alpha_6$ integrins (27).

Cell migration-promoting activities of different LNs appear to be dependent on cell specific factors. Human glioblastoma cell line T98G showed the best migration on LN-8 (50) compared to LN-2/4, LN-5, LN-10/11 and fibronectin, while LIM1215 carcinoma cells migrate more efficiently on LN-10 than on collagen type I, type IV or LN-1. (51). Here, we demonstrated that rLN-10 was the most potent matrix of the components tested in promoting endothelial cell (HSVEC) migration in vitro (Fig. 6). Interestingly, HSVEC adhesion to commercial LN-10/11 and to rLN-10 was equally strong, but the potency of LN-10/11 in promoting HSVEC migration was much lower than that of rLN-10. In agreement with these results, Sixt and co-workers (39) have reported that commercial LN-10/11 is a less potent migration substrate than intact native LN-10. Earlier studies on the function of LN-10 have frequently used commercial preparations, which are normally prepared using proteolytic
digestion and subsequent immunoaffinity chromatography resulting in a truncated mixture of $\alpha_5$ chain containing LN isoforms (39).

It has been shown that the domain IVa in the LN $\alpha_5$ chain mediate cell adhesion through RGD-dependent integrins (52). These integrins, such as $\alpha v\beta 3$ and $\alpha v\beta 5$, play an important role in cell migration, angiogenesis and neovascularization (53-55). Neither mAb against integrin $\alpha v$ nor RGD-peptides tested here inhibited cell adhesion to intact rLN-10. It seems likely that cell adhesion to LN-10 is mainly mediated through the C-terminal region, whereas the recognition of domain IVa by these integrins may mediate other activities such as cell migration.

Potential clinical uses of this laminin isoform includes pretreatment of vascular grafts used in the repair of vascular occlusive diseases, in order to improve endothelialization of the graft surface. Many substrate coatings, such as collagen, fibronectin, LN-1, and plasma have been tested in vitro (56-58). Preliminary data produced in our laboratory indicates that HSVECs migrate considerably better on rLN-10 coated graft materials than on BSA-coated ones.

The present work provided means of producing large amounts of high quality and biologically active LN-10, which is otherwise difficult to obtain. LN-10 showed not only strong adhesive activity but also migration-promoting activity, providing support for LN-10 as a good candidate for a coating substrate of vascular grafts.
Acknowledgments

We thank Maria Laisi, Mariette Lingquist and Hanna Wiedemann for expert technical assistance, Manuel Patarroyo for critical reading of the manuscript and gift of antibodies, Ekaterina Morgunova for assistance with structural analysis, Lena Claesson-Welsh for gift of cells, Kiyotoshi Sekiguchi, Ismo Virtanen and Eva Engvall for gift of an antibody, and Jürgen Engel for gift of purified proteins. This work was supported in part by grants from the Novo Nordisk Foundation, the Swedish Medical Research Council and EC contract QLK3-CT2000-00084.
References


Footnote

The nucleotide sequence reported in this paper has been submitted to the GenBank™ with accession number AF443072.

The abbreviations used are: LN, laminin; rLN, recombinant human laminin, HSVEC, human saphenous vein endothelial cell; IBE cell, immortomouse brain capillary endothelial cell; ECM, extracellular matrix; BM, basement membrane; LE, laminin epidermal growth factor-like repeats; LG, laminin G domain; DMEM, Dulbecco’s modified Eagle’s medium; MEM, minimal essential medium; FCS, fetal calf serum; BSA, bovine serum albumin, PVDF, polyvinylidene difluoride; PBS, phosphate-buffered saline; LN-1/Nd, laminin-1/nidogen complex from mouse-EHS-tumor; FN, fibronectin; Col IV, collagen type IV.
Figure legends

Fig. 1: **Characterization of rLN-10 using SDS-PAGE.** a, silver stain: Conditioned medium of triple-transfected HEK293 cells (CM) and recombinant purified laminin-10 (rLN-10), were analyzed by SDS-PAGE on 4-15% gradient gels under reducing conditions; b, Immunoblot of CM and rLN-10 under reducing conditions: Separated proteins on 5% gels were transferred onto PVDF membranes followed by staining with mAbs against laminin α5 (15H5), β1 (DG10), γ1 (clone 22) and FLAG-M2.; c, Silver stain and immunoblot of rLN-10 under non-reducing conditions: Separated proteins on 5% gels were visualized by silver staining or transferred onto PVDF membranes followed by staining with mAbs against laminin α5 (15H5), β1 (DG10), γ1 (2E8). The positions of molecular size markers are shown.

Fig. 2: **Rotary shadowing EM pictures of recombinant laminin-10 (rLN-10) molecules.** Top, low magnification field showing oligomeric association of rLN-10 molecules; Bottom, individual molecules. Each molecule has three short arms and one long arm.

Fig. 3: **Cell adhesion activity of recombinant laminin-10 (rLN-10) and other proteins.** a, HT-1080 cell adhesion to rLN-10, recombinant laminin-8 (rLN-8), laminin-1/nidogen complex (LN-1/Nd), and fibronectin (FN) coated at increasing concentrations.; b, IBE cell adhesion to rLN-10, rLN-8, LN-1/Nd, and FN coated at increasing concentrations.; c, HSVEC adhesion to rLN-10 and rLN-8 coated at 3 and 10 µg/ml, and LN-1/Nd, commercial laminin-10/11 (LN-10/11), collagen type IV (Col IV) and FN coated at 10 µg/ml. N.T. means not-tested. Bound cells were quantitated spectrophotometrically using adhesion to BSA as blank. Error bars indicate S.D.
Fig. 4: Inhibition of HT-1080 cell and HSVEC adhesion on rLN-10 coated at 10 µg/ml. Text under columns indicate the integrin subunit mAbs used or other added substances. Adhesion shown is relative to control, designated 100. Adhesion to BSA was designated zero. Error bars indicate S.D.

Fig. 5: Flow cytometry analysis of integrin receptor expression on HSVEC. Control is the binding of fluorescent labeled secondary antibody plus appropriate isotype control antibody. The vertical axis shows the cell number, and the horizontal axis shows the log fluorescence intensity. Integrin receptors investigated included α1, α2, α3, α4, α5, α6, αv, β1, β3 and β4.

Fig. 6: HSVEC migration (endothelialization) on plastic coated with laminin-10 and other proteins. Migration of HSVECs into the cell free area coated with BSA, laminin-1/nidogen complex (LN-1/Nd), recombinant laminin-8 (rLN-8), recombinant laminin-10 (rLN-10), fibronectin (FN), commercial laminin-10/11 (LN-10/11), gelatin, and collagen type IV (Col IV) was measured. a, photomicrograph of cells (donor 1) stained with crystal violet. Arrowheads indicate the 4 mm distance of the cell fronts at the time of seeding. b, The distance covered by cells from three different donors. N.T. means not-tested.
Table I: **Primers used in laminin α5 expression construct preparation.**

Upper row, forward primer; Lower row, reverse primer.

<table>
<thead>
<tr>
<th>plasmid</th>
<th>primer</th>
<th>primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>KBX3</td>
<td>KZK1</td>
<td>5’-gccaccatggcgaagcggctctg-3’</td>
</tr>
<tr>
<td></td>
<td>Ba3r</td>
<td>5’-aaggccaggatcactgggg-3’</td>
</tr>
<tr>
<td>BBL3</td>
<td>Bam4</td>
<td>5’-ctactgcaagctgctctt-3’</td>
</tr>
<tr>
<td></td>
<td>Bcl11r</td>
<td>5’-ccaggttgtctggtatc-3’</td>
</tr>
<tr>
<td>BNK2’</td>
<td>Bcl2</td>
<td>5’-gcgacaaatgctctctac-3’</td>
</tr>
<tr>
<td></td>
<td>Not4r</td>
<td>5’-agtgggttccaaagaatcc-3’</td>
</tr>
<tr>
<td>BNL12</td>
<td>Bpu1F</td>
<td>5’-ccctctgtgacagctcaag-3’</td>
</tr>
<tr>
<td></td>
<td>Not4r</td>
<td>5’-agtgggttccaaagaatcc-3’</td>
</tr>
<tr>
<td>D29D30l</td>
<td>D29</td>
<td>5’-gtatgtgcctctgctagcct-3’</td>
</tr>
<tr>
<td></td>
<td>D30l</td>
<td>5’-gtctgtgcagccctgtgagtt-3’</td>
</tr>
<tr>
<td>NSK5</td>
<td>Not3</td>
<td>5’-ctctcagttgctcactaacc-3’</td>
</tr>
<tr>
<td></td>
<td>Sal4r</td>
<td>5’-ctgactgtgcaagctgatg-3’</td>
</tr>
<tr>
<td>SFK2</td>
<td>Sal5</td>
<td>5’-ggagttggtcagctataca-3’</td>
</tr>
<tr>
<td></td>
<td>FLAG1</td>
<td>5’-taactgtgtcagctgtctctgtagcgcctggtaggc-3’</td>
</tr>
<tr>
<td>SFL13</td>
<td>Sal5</td>
<td>5’-ggagttggtcagctataca-3’</td>
</tr>
<tr>
<td></td>
<td>m19R</td>
<td>5’-aaggccagactcagg-3’</td>
</tr>
</tbody>
</table>
Recombinant human laminin-10(α5β1γ1): Production, purification and 
migration promoting activity on vascular endothelial cells
Masayuki Doi, Jill Thyboll, Jarkko Kortesmaa, Katarina Jansson, Antti Iivanainen, 
Masomeh Parvardeh, Rupert Timpl, Ulf Hedin, Jesper Swedenborg and Karl Tryggvason
J. Biol. Chem. published online January 30, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M111228200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/early/2002/01/30/jbc.M111228200.citation.full.html#ref-list-1