**Recombinant Human Laminin-10 (α5β1γ1)**

**PRODUCTION, PURIFICATION, AND MIGRATION-PROMOTING ACTIVITY ON VASCULAR ENDOTHELIAL CELLS**

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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 mass of ~800 kDa in SDS-PAGE and a native domain structure in rotary shadowing electron microscopy. By using function-blocking monoclonal antibodies, integrin αβ2 was found to be a major mediator of adhesion of HT-1080 and human saphenous vein endothelial cells. Human saphenous vein endothelial cells adhered more strongly to rLN-10 than to LN-1 and LN-8 and showed better migration on rLN-10, compared with several other matrices. Considering the cell adhesive and migration-promoting properties of rLN-10 on endothelial cells, this molecule could be useful in improving the biocompatibility and endothelialization of vascular grafts.

The laminins (LNs) are a family of large extracellular matrix 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 the adhesion, migration, proliferation, and differentiation of many cell types (3–5).

In most tissues, two main 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 basement membranes because 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 receptor is the integrin family, which plays a central role in cell-matrix interaction. Integrin-mediated endothelial cell recognition of LN and other basement membrane 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 nonbiological materials were precoated with extracellular matrix 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 the 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 Human Laminin α5 cDNA—The previously published mouse sequence was used to search expressed sequence tag data bases. Based upon sequences of the identified expressed sequence tags, oligonucleotide primers were synthesized and used for PCR amplification of several human α5-specific probes with αgt11 cDNA library (CLONTECH) as template. These probes were used for screening of αgt11 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 bp of coding sequence and 195 bp of 3′-untranslated region in the C-terminal part and 5354 bp in the N-terminal part but lacking a translation initiation start. The center part, comprising bp 5582–9316, was obtained by PCR amplification from a Human Lung Marathon Ready™ cDNA mix (CLONTECH). The remaining 296 bp of coding sequence and a 67-bp 5′-untranslated region end were obtained with SMART™ RACE cDNA amplicon libraries (CLONTECH) using poly(A) RNA purified with Quick-Prep mRNA purification kit (Amersham Biosciences) from HEK293 cell lysates. The reverse transcription was performed with the Moloney murine leukemia virus reverse transcriptase SuperscriptII (Invitrogen), and subsequent PCR amplification was performed with Advantage2–GC 2 PCR kit (CLONTECH). This 363-bp N-terminal sequence was confirmed by sequencing genomic P1 clone (Genome Systems) 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 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. Sequencing was performed on an ABI PRISM® 310 Genetic Analyzer (PerkinElmer Life Sciences) using the ABI PRISM® BigDye™ Terminator Cycle Sequencing Kit (PE Applied Biosystems). Sequence analysis was performed with AutoAssembler™ (PE Applied Biosystems), and sequence comparisons were performed with the GenBank database.

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 the Advantage2–GC 2 PCR kit and Phusion Turbo polymerase (Roche Molecular Biochemicals). PCR-generated cDNA fragments were cloned into the pcCR2.1-TOPO vector (Invitrogen) and sequenced (using AmpliTaq FS on an ABI 310 sequence; PerkinElmer Life Sciences) to ensure that no mutations had occurred during amplification. All primers for PCR and pcCR2.1-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 (acccg; Ref. 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-C).

The eight different cDNAs that overlap each other were ligated. The center part, comprising bp 5582–9316, was obtained with SMART™ RACE-generated cDNA mixes from HEK293 cells, human placenta total RNA, and 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. Sequencing was performed on an ABI PRISM® 310 Genetic Analyzer (PerkinElmer Life Sciences) using the ABI PRISM® BigDye™ Terminator Cycle Sequencing Kit (PE Applied Biosystems). Sequence analysis was performed with AutoAssembler™ (PE Applied Biosystems), and sequence comparisons were performed with the GenBank database.

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The eight different cDNAs that overlap each other were ligated. The final expression construct named HLN5Full.pcDNA was made by inserting the 11.1-kb full-length cDNA into the EcoRI sites of pcDNA3.1/Zeo(−) mammalian expression vector (Invitrogen) in the 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).

TABLE I

| Plasmid | Primer | Primer sequence |
|---------|--------|----------------|
| KBX3 | KZK1 | 5′-gcactactggaacaggtctg-3′ |
| | Blad1r | 5′-aagggagtgaactc-3′ |
| BBL3 | Bam4 | 5′-taagggagaggtctgtt-3′ |
| | Bclr1 | 5′-aagggagtgaactc-3′ |
| BNR2 | Bcl2 | 5′-gacaggctggattgat-3′ |
| | Not4r | 5′-agtggctcacaagaaa-3′ |
| BNL12 | Bpa1F | 5′-cctgtggaagctcag-3′ |
| | Not4r | 5′-agtggctcacaagaaa-3′ |
| D29D301 | D29 | 5′-gaaagtcggctgagtcgc-3′ |
| | D301 | 5′-ctggctggctgggtgag-3′ |
| NSK5 | Not3 | 5′-ctgtagctggactc-3′ |
| | Sal4r | 5′-tcagggctggagtgc-3′ |
| SKF2 | Sal5 | 5′-ggagggggcctaggc-3′ |
| | FLAG1 | 5′-taagggagaggtctgtt-3′ |
| SFL13 | Sal5 | 5′-ggagggggcctaggc-3′ |
| | m19R | 5′-aagggagtgaactc-3′ |

a Top row, forward primer; bottom row, reverse primer.

Production and Purification of Recombinant Laminins—LN-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 were carried out in the continuous presence of relevant selection antibiotics. A clone highly expressing LN γ1 was then transfected with the human LN β1 construct, and stable clones were selected using 500 μg/ml G418 (Invitrogen). A clone highly expressing both LN γ1 and LN β1 was finally transfected with HLN5Full.pcDNA, 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 Dulbecco’s modified Eagle’s medium supplemented with 1 mM pyruvate and insulin-transferrin-seleno-supplement (Sigma) for up to 5 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/8 (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, and 1 mM EDTA) at room temperature. The eluate was concentrated, and the buffer was replaced by phosphate-buffered saline using 30-kDa cut-off ultrafiltration (Millipore). Finally, the concentrated solution was passed through a 0.2-μm filter to remove self-aggregated polymers. rLN-8 was produced in HEK293 cells and purified using anti-FLAG matrix and ion-exchange chromatography.

Characterization of Recombinant Laminin-10—Secreted LN in medium was characterized after purification using 5% SDS-PAGE and 4–15% gradient SDS-PAGE. Proteins were visualized using silver staining or transfused onto polyvinylidene difluoride membranes. The membranes were probed with the mAbs described above. After washing, the membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse antibody. The immunoreactivity was detected by a chemiluminescence 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
polyvinylidene difluoride membranes, followed by staining with mAbs against laminin
immunoblot of rLN-10 under nonreducing conditions. Separated proteins on 5% gels were visualized by silver staining or transferred onto
37
0.16% dispase (Roche Molecular Biochemicals) in MEM for 20 min at
rinsed with MEM (Invitrogen) and filled with 0.1% collagenase and
VECs were isolated as described previously (33). Briefly, veins were
were collected from patients undergoing coronary bypass surgery. HS-

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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 endo-
thelial (IBE) (32) cells were kindly provided by Dr. L. Claesson-Welsh.
All cells were cultured in a humidified 5% CO2 atmosphere. HT-1080
cells were cultured in Dulbecco’s modified Eagle’s medium, 10% fetal
calf serum, and pyruvate were used for HT-1080 cells, and F-12, 25 mM Hepes,
cells were incubated in MEM containing 40% heat-inactivated pooled human serum, 1 nmol/
were cultured in Dulbecco’s modified Eagle’s medium, 10% fetal calf serum, and 2 units/ml interferon γ on gelatin-coated plastic at
33 °C. Before assay, the IBE cells were cultured in serum-free F-12 at
37 °C without interferon γ for 24 h.

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. HS-
VECs were isolated as described previously (33). Briefly, veins were
rinsed with MEM (Invitrogen) and filled with 0.1% collagenase and
0.16% dispase (Roche Molecular Biochemicals) 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, 1 mmol/
liter choleratoxin (Sigma), 33 μmol/liter isobutylmethylxantine (Sig-
ma), and antibiotics. HSVECs were seeded on gelatin-coated plates and
passaged (1:3). HSVECs were characterized with monoclonal anti-hu-
man von Willebrand factor-related antigen (Dako), and HSVECs be-
tween 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 the addition of 2% heat-inactivated BSA in phos-
phate-buffered saline.

For the assay, cells were suspended in buffered serum-free medium
at 3 × 10^5 cells/well. Dulbecco’s modified Eagle’s medium, 25 mm Hapes,
and pyruvate were used for HT-1080 cells, and F-12, 25 mm Hapes, and
0.25% BSA were used for other cells. Antibodies or other test com-
pounds 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 were used at 0.25 mg/ml, heparin was used at 5 mg/ml, and
EDTA was used at 5 mM. 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 0, whereas
the relevant control was given a value of 100. The mean and 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 phosphate-buffered saline containing anti-integrin
mAbs against α5, α6, α5, β1, β3, and β1, for 30 min at 4 °C. After washing,
cells were incubated with fluorescein isothiocyanate-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 (BC PharMin-
gen). Mouse IgG was used as a negative control.

Cell Migration Assays—The cell migration assay was performed as
described previously (34). Flat-bottomed 24-well culture plates (Cor-
ing) were coated with proteins overnight at 4 °C. The remaining protein
binding capacity was saturated by the addition of 2% heat-inactivated
BSA in phosphate-buffered saline. Thereafter, a 4 × 10-mm stainless
steel weight was put on the center of well before seeding HSVECs at 2 ×
10^5 cells/well. After adhesion for 2 days in MEM with 30% human
serum, the steel weight was removed. A gap devoid of HSVECs was
thus created, with two broad (10-mm) endothelial cell edges facing each
other at a distance of 4 mm. During endothelialization, HSVECs were
incubated in MEM with 40% human serum, choleratoxin, and isobutyl-
methylxanthine for 2 days after removing the steel weight. The cells were visualized by 0.1% crystal violet staining.

RESULTS

Sequence of Human Laminin a5 Chain—The full-length cDNA coding sequence consisting of 11,088 bp had an open reading frame encoding 3696 amino acids (GenBank® accession number AF443072). Compared with the previously reported mouse sequence (GenBank® accession number U37501 (10)), we obtained an additional 79 amino acids in the N-terminal end, also identified later in mouse (GenBank® accession number AJ293593). Comparison with the mouse laminin a5 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 the LG3 and LG4 domains that is 7 residues longer than the mouse sequence. In addition, there was a stretch of 4 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 with the human sequence. Alignment of mouse and human LN LG5 modules with other published sequences (data not shown) revealed similar C-terminal length in all cases except for mouse a5. A partial sequence covering 953 amino acids in the C terminus of human LN/H9251 has been reported previously (12), and this contains no discrepancies compared with 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 polymorphisms 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 was located 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/H9253 (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 a5 modules (data not shown), and we found that the observed polymorphisms did 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 a5, anti-LN β1, anti-LN γ1, or anti-FLAG antibodies, indicating that these cells...
express endogenous LNs at very low levels, if at all (data not shown). After triple transfection, the best cell clone produced 1–2 mg rLN-10/liter medium.

Immunoblotting 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-kDa band corresponding to the laminin α5 chain and a 200-kDa band corresponding to the laminin β1 and γ1 chains, which have similar molecular masses (Fig. 1c). In Western blotting of the conditioned medium, two bands of ~350 and 400 kDa could be seen with the laminin α5 mAb (Fig. 1b). The anti-FLAG antibody reacted with a 400-kDa and a 40-kDa fragment (Fig. 1b; data not shown). Taken together, these data indicate that the 400-kDa fragment is the intact laminin α5, the 350-kDa fragment is an N-terminal fragment, and the 40-kDa fragment is a C-terminal fragment harboring the FLAG epitope. Under nonreducing conditions, most of the protein appeared at the top of the gel as a very high molecular mass band, which was immunoreactive with α5, FLAG, β1, and γ1 mAbs, showing that the rLN-10 was produced as disulfide-cross-linked heterotrimer (Fig. 1c). A minor band of ~400 kDa 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 noncovalently associated with the α5 chain. In the case of rLN-8, the α4 chain is seen as a 200-kDa band, but in the case of rLN-10, the noncovalently associated α5 chain had an apparent molecular mass similar to that of the β1/γ1 dimer, which explains the immunoreactivity of the minor band with α5 and FLAG mAbs.

Rotary shadowing electron microscopy revealed that the rLN-10 protein had three short arms and one long arm, in accordance with the expected structure. The bottom 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 top 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 a model for macrovascular endothelial cells, and IBE cells were used as a model for microvascular endothelial cells.

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

Monoclonal antibodies against either α5 or β1 inhibited HT-1080 cell binding to rLN-10 by ~80%, indicating that integrin α5β1 was a major mediator of adhesion to rLN-10 (Fig. 4). In addition, mAbs against integrin α5 had a partial inhibitory effect on adhesion to rLN-10 (Fig. 4). Because some adhesion remained after the blocking of integrin β1, other receptor classes besides β1 integrins could be involved in the cell adhesion. Monoclonal antibodies against integrins α2, α6, and α7 had no effects on the adhesion of HT-1080 cells to rLN-10 either alone (Fig. 4) or in various combinations (α2 + α6, β1 + α6, β1 + α7, β2, β3, α4, α5, α6, α7, β1, β2, and β3). These results indicate that α6 integrins (α6β1 and α6β2) or α7 integrins (α7β1, α7β2, and α7β3) were not mediating the cell adhesion in a substantial manner.

HSVEC binding to rLN-10 was also studied. To determine which integrins were present on the cell surface, we performed a fluorescence cell-sorting assay using mAbs against integrin subunits α1, α2, α3, α4, α5, α6, α7, β1, β2, and β3 (Fig. 5). From these results, it can be concluded that HSVECs express large amounts of α1β1, α2β1, and α3β1; moderate amounts of α4β1, and small amounts of α5β1, α6β1, and α7β1, but integrin α5β1 was not detected. The HSVEC binding was most efficiently inhibited by mAbs against integrin α5 and β1, but mAb against α6 also had a partial effect (Fig. 4), in a fashion similar to that observed for HT-1080 cells. Integrin α4 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 on divalent cations because 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). Because the $\alpha_5$ 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 $\alpha_6\beta_1$ and the $\alpha_{IIb}\beta_3$ family) (38). Neither linear nor cyclic RGD peptides had any effect at 0.25 mg/ml on the adhesion of either HT-1080 cells or HSVECs to rLN-10 (Fig. 4). Furthermore, it was observed that the cell binding activity of rLN-10 was sensitive to air drying, as we have reported previously 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).

**HSVEC Migration**—LN-1ns 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 $\mu$g/ml rLN-10 or other adhesive proteins. The migration assay was repeated three times using HSVECs obtained from three different donors. Of the seven different adhesive proteins examined, rLN-10 was the most potent in promoting HSVEC migration in vitro (Fig. 6, a and b). 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 of the proteins examined.

**DISCUSSION**

A major contribution of this work is that it provides a cell line source for human laminin-10, which 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.

**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, LN-1/Nd, rLN-8, rLN-10, FN, commercial LN-10/11, gelatin, and collagen type 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., not tested.
at the end (Fig. 2). Two forms of the LN α5 chain with molecular masses of ~350 and 400 kDa were seen in Western blotting of the conditioned cell medium. Other investigators have also observed LN α5 as two bands and have suggested that the difference reflects different glycosylation forms (39). Based on Western blotting, we conclude that the 350-kDa fragment represents a proteolytically processed N-terminal fragment where the C terminus, harboring the FLAG epitope, was cleaved off. The 40-kDa fragment detected with anti-FLAG mAb in Western blotting is likely to be a cleaved C-terminal domain LG4–5 fragment, and the 400-kDa 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 nonreducing conditions, the majority of the rLN-10 appeared as disulfide-cross-linked heterotrimer, whereas a minority consisted of the α5 chain noncovalently associated with a disulfide-linked β1/γ1 heterodimer.

Integrin-mediated recognition of extracellular matrix 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 basement membrane 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 αβ1, αβ3, αβ5, and αβ6 (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 were shown to be integrins αβ1 and αβ6. Although the αβ3 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 fluorescence cell-sorting data (Fig. 5) showing that the cells have little α5 integrins, which have previously been shown to be receptors for rLN-8 (27). We can therefore conclude that integrin binding is distinctly different between the two main forms of endothelial LNs as well as between different endothelial cell types because we have previously shown that IBE and bovine capillary endothelial cell adhesion onto rLN-8 is mediated predominantly by α5 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) when compared with LN-24, LN-5, LN-10/11, and fibronectin, whereas LIM1215 carcinoma cells migrate more efficiently on LN-10 than on collagen type I, collagen 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, Six et al. (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 α5 chain-containing LN isoforms (39).

It has been shown that domain IVa in the LN α5 chain mediates cell adhesion through RGD-dependent integrins (52). These integrins, such as αβ3 and αβ6, play an important role in cell migration, angiogenesis, and neovascularization (53–55). Neither mAb against integrin α5 nor the RGD peptides tested here inhibited cell adhesion to intact rLN-10. It seems likely that cell adhesion to LN-10 is mediated mainly 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 include pre-treatment of vascular grafts used in the repair of vascular occlusive diseases 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 indicate that HSVECs migrate considerably better on rLN-10-coated graft materials than on BSA-coated ones.

The present work provides a 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.

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