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The role of a recombinant fragment of laminin-332 in integrin $\alpha 3 \beta 1$ -dependent cell binding, spreading and migration

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Abstract

The extracellular matrix (ECM) is thought to be an essential component of tissue scaffolding and engineering because it fulfills fundamental functions related to cell adhesion, migration, and three-dimensional organization. Natural ECM preparations, however, are challenging to work with because they are comprised of macromolecules that are large and insoluble in their functional state. Functional fragments of ECM macromolecules are a viable answer to this challenge, as demonstrated by the RGD-based engineered scaffolds, where the tri-peptide, Arg–Gly–Asp (RGD), represents the minimal functional unit of fibronectin and related ECM. Laminins (Ln) are main components of epithelial tissues, since they enter into the composition of basement membranes (BM). Application of Ln to epithelial tissue engineering would be desirable, since they could help mimic ideal functional conditions for both lining and glandular epithelial tissues. However, functional fragments of Ln that could be used in artificial settings have not been characterized in detail. In this paper, we describe the production and application of the recombinant LG4 (rLG4) fragment of laminin-332 (Ln-332), and show that it mimics three fundamental functional properties of Ln-332: integrin mediated cell adhesion, spreading, and migration. Adhesive structures formed by cells on rLG4 closely resemble those formed on Ln-332, as judged by microscopy-based analyses of their molecular composition. As on Ln-332, focal adhesion kinase (FAK) is phosphorylated in cells adhering to rLG4, and colocalized with other focal adhesion components. We conclude that rLG4 could be a useful substitute to recapitulate, *in vitro*, the tissue scaffolding properties of Ln-332.

Keywords

cell adhesion; laminin; integrins; extracellular matrix; biomimetic substrate

Introduction

Cellular interactions with the extracellular matrix (ECM) are necessary for proper development, organization, homeostasis, and repair of tissues *in vivo* [1]. ECM proteins, such

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as laminins (Ln), fibronectin (Fn), and vitronectin (Vn), have adhesive properties that direct cell binding and signaling events that are critical for cell survival, cell cycle progression, and determination of phenotype [2,3]. Therefore, incorporation of such ECM components into biotechnology and biomedical applications, such as biomaterials, development of artificial organs, and tissue engineering, is critical because they provide structural scaffolds and regulate cellular activities such as cell adhesion, spreading, migration, and infiltration [4].

Cell adhesion to ECM proteins is largely mediated by integrin receptors, a large family of $\alpha\beta$ heterodimeric proteins [5]. Upon cell binding, integrins cluster to form focal adhesion complexes that contain structural and signaling proteins [6], which have demonstrated control of cell adhesion, differentiation, proliferation, survival, and migration *in vitro* [7-10]. Integrins bind to specific amino acid sequences present on ECM, including the RGD motif present on ligands such as Fn and Vn [11]. Previous studies have shown that inclusion of short peptide sequences containing this motif enhance tissue healing responses *in vivo*, including bone formation, nerve regeneration, and corneal tissue repair [12-14]. Short peptides are particularly convenient for inclusion in artificial substrates. Unfortunately, the short-peptide surrogate paradigm does not extend well to Ln, key ECM macromolecules of epithelial tissues. Peptide sequences of Ln-1, including an Ile-Lys-Val-Ala-Val peptide (IKVAV) present on its $\alpha 1$ chain, have been shown to support adhesion, neurite outgrowth, and angiogenesis [15-17], but the molecular underpinnings of these activities remain unclear, since these Ln peptides do not bind integrins. Therefore, short sequence strategies appear to be limited for Ln. On the other hand, the identification of larger sequences within ECM structures that mediate integrin-dependent adhesion has proven useful in the development of biomaterials that serve as adhesive surfaces. For instance, it has previously been reported that recombinant fragments of Fn exhibit equivalent integrin-mediated adhesive activities to plasma Fn [20]. Similarly, recombinant Ln fragments could be a viable alternative to peptides for applications to epithelial tissue engineering, since they could help mimic functional conditions for both lining and glandular epithelial tissues.

Ln-332 (formerly termed laminin-5, epiligrin, kalinin, ladsin, and nicein) is a major basement membrane (BM) glycoprotein in epithelium tissue and consists of $\alpha 3$, $\beta 3$, and $\gamma 2$ chains. Similar to other laminin α chains, the $\alpha 3$ chain of Ln-332 possesses a large C-terminal G domain formed by five LG modules (LG1-5) that regulate cell behavior by interacting with various cell surface receptors, including integrins. Like other ECM components, Ln-332 affects cell behavior through integrins [21]. It is well established that the two main receptors for Ln-332 are integrins $\alpha 6\beta 4$ and $\alpha 3\beta 1$, which are expressed on the surface of many cells [22-24]. In resting epithelia, such as skin and colon, integrin $\alpha 6\beta 4$ is the main integrin in contact with Ln-332, where their interaction is crucial to the formation of hemidesmosomes [24]. These structures mediate the adhesion of epithelial cells to the underlying BM and connect the cytoskeleton to the ECM [25]. In contrast, integrin $\alpha 3\beta 1$ is mostly confined to lateral cell-cell adhesion sites in resting epithelia [23,24]. During wound-healing, integrin $\alpha 3\beta 1$ redistributes to the basal cell surface and mediates rapid adhesion, spreading, and migration of keratinocytes [25,26]. Cell motility activity of Ln-332 via integrins has been suggested to contribute to both wound healing and tumor invasion, making this particular Ln isotype an important protein for regulating cell behavior [21,27].

Native ECM preparations, such as Ln-332, are somewhat challenging to handle in the laboratory because they are comprised of macromolecules that are large (~450 kDa) and are often insoluble in their functional states. Therefore, it is of great advantage to develop smaller recombinant Ln-332 fragments with cell adhesive activities via integrin interactions. In a previous study, we developed small fragments derived from the Ln-332 G domain, and found that recombinant LG3 (rLG3) harbors cell adhesion and migration activity via integrin $\alpha 3\beta 1$ [28]. However, the cell binding activity of rLG3 was found to be lower than that of intact

Ln-332 molecule and required about 250-fold higher rLG3 concentration than Ln-332 to reach half-maximal adhesion [28]. This led us to hypothesize that other small fragments within Ln-332 could potentially fulfill functional activities of intact Ln-332. In this study, we developed another recombinant fragment of Ln-332, rLG4, and expressed it as a monomeric, soluble fusion protein using both bacterial and mammalian expression systems.

Materials and Methods

Cell Lines and Culture Conditions

Human embryonic kidney 293 EBNA (HEK-293) cells were purchased from Invitrogen (Carlsbad, CA). Suspension-adapted 293 EBNA (SA-293) cells were purchased from Biotechnology Research Institute, Canada (Montreal, Quebec). Human fibrosarcoma HT-1080 cells (CCL-121) and human epidermoid carcinoma A-431 cells (CRL-1555) were purchased from American Type Culture Collection (Manassas, VA). Wild-type kidney inner medullary collecting duct cells (IMCD-WT) and β 1 integrin null IMCD (IMCD- β 1) cell lines were a kind gift from Roy Zent (Vanderbilt University). Human epidermal keratinocytes were purchased from the Skin Diseases Research Core Center at Vanderbilt University. HEK-293, HT-1080, A-431, IMCD-WT and IMCD- β 1 null cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Inc., Rockville, MD) supplemented with 10 % fetal bovine serum (FBS; Gemini, Irvine, CA) and 1 % glutamine/penicillin/ streptomycin antibiotics (Life Technologies). Human keratinocytes were maintained in EpiLife calcium-free medium (Cambrex Bio Sciences Walkersville, Inc., Walkersville, MA). The SA-293 cell line was maintained between 0.2×10^6 and 1.2×10^6 cells/ml cell density by diluting every 2 days in 25 ml FreeStyle medium (Invitrogen) containing 0.1 % pluronic F68 (Invitrogen) and 25 μ g/ml G418 (AMRESCO, Solon, OH) in 125 ml Erlenmeyer flasks (Corning, Lowell, MA) and a shaker plate. All cells were maintained at 37°C in a humidified, 5 % CO₂, 95 % air atmosphere.

Antibodies and reagents

Linear polyethylenimine (PEI; 25 kDa) was obtained from Polysciences (Warrington, PA). Stock solutions of PEI (1 mg/ml) were prepared in hot water and neutralized with HCl and sterilized by filtration (0.22 μ m, Millipore), and the aliquots were stored at -80°C.

The following antibodies to specific integrin subunits were used: PIB5, an anti- α 3 integrin inhibitory monoclonal antibody (mAb) (Chemicon, Temecula, CA); 6S6, an anti- β 1 integrin inhibitory mAb (Chemicon); GoH3, an anti- α 6 integrin inhibitory mAb (Pharmingen, La Jolla, CA); TS2/16, an anti- β 1 activating mAb (a gift from Dr. M. E. Hemler, Harvard Medical School, Boston, MA); A3X8, an anti- α 3 integrin mAb (a gift from Dr. M.E. Hemler); PID6, an anti- α 5 integrin inhibitory antibody (a gift from Dr. A. M. Weaver, Vanderbilt University); AB1920, a polyclonal (pAb) anti- α 3 antibody (Chemicon); Cyt, an anti- α 5 integrin mAb (Chemicon); anti-paxillin mAb (Pharmingen); anti-FAK [pY³⁹⁷] phosphospecific pAb (Biosource); a pAb against total FAK (Abcam, Cambridge, MA); and anti-His mAb (Qiagen, Valencia, CA).

Construction of Bacterial and Mammalian Expression Vectors for Ln-332 LG4 Module

Rat Ln-332 cDNA was cloned from an 804G λ zap expression library (24). The cDNA clone pCR5C5 (base pairs 874-3180) was used as a template to amplify the sequence encoding the LG4 module. The LG4 construct was extended by 10 amino acids into the adjacent LG5 module and the N-terminal nonbonding cysteine residue (C₁₃₆₅) was mutated to serine. The recombinant molecule for bacterial LG4 (BacLG4) was expressed as a double-tagged fusion protein, containing an N-terminal GST tag and a C-terminal His₆ tag. The recombinant molecule for mammalian-derived LG4 (MamLG4) was expressed as a single-tagged fusion

protein, containing only a His₆ tag at the C-terminus of the protein. Polymerase chain reactions (PCR) were performed with pfu ULTRA High-fidelity DNA polymerase (Stratagene, La Jolla, CA) using the following primers: 5'-ACC TTC GGA TCC AGA GCC CTG CAG TTT GGA GAC AGC CCC ACC AGC CAC TTG CTA-3' (BacLG4; forward), 5'-CTG TCC GCG GCC GCT CAA TGA TGA TGA TGA TGG TGA TAA ATG CCT TTC TCT AAA G-3' (BacLG4; reverse), 5'-CTA CTA GCT AGC TAG AGC CCT GCA GTT TGG AGA CAG CCC CAC CAG CCA CTT GCT AC-3' (MamLG4; forward), and 5'-CTG TCC GCG GCC GCT CAA TGA TGA TGA TGA TGG TGA TAA ATG CCT TTC TCT AAA G-3' (MamLG4; reverse). These primers expressing LG4 contain restriction enzyme sites for pairs of either *Bam*HI and *Not*I or *Nhe*I and *Not*I, respectively. The resulting PCR fragments were digested with the appropriate restriction enzyme pair; BacLG4 was ligated into the pGEX4T-3 expression vector, and MamLG4 was ligated into the pCEP-Pu-Ac expression vector. GST and a His₆ tagged sialic acid synthases (SAS) were also cloned as negative controls for the bacterial and mammalian vectors. SAS expression vectors were a gift from M. Sundaramoorthy (Vanderbilt University).

Expression and Purification of Recombinant Proteins

BacLG4 was expressed in *Escherichia coli* (*E. coli*; BL21 strain), and cells were grown to mid-log phase in Luria-Bertani medium. Cells were induced with 1 mM isopropyl-D-thiogalactopyranoside (Roche Molecular Biochemicals, Indianapolis, IN), and the temperature was shifted to 25°C. After 3 hours of growth, cells were harvested by centrifugation at 6000 rpm for 10 min (Sorvall, GSA rotor). Pellets were washed with water and flash frozen at -80°C. To purify BacLG4, pellets were thawed and re-suspended in ice-cold lysis buffer (25 mM Tris-HCl, pH 8.0, 150 mM NaCl) containing a protease inhibitor cocktail (1000× stock; Roche Molecular Biochemicals). Cell lysis was carried out by sonication. The lysate was clarified by centrifugation at 12,000 rpm for 30 min, followed by further clarification through a 0.45 µm filter. Clarified cell lysates were incubated with Ni²⁺-nitrilotriacetic acid beads (Ni-NTA, Qiagen), equilibrated in lysis buffer, for 4 hrs at 4 °C. The beads were washed with wash buffer (25 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 20 mM imidazole). Protein elution was carried out with 300 mM imidazole in wash buffer. Elutions were pooled and dialyzed against lysis buffer overnight, with two changes of buffer. Protein concentration was determined by the Bradford assay [29].

In order to obtain a mammalian expression system with recombinant LG4 proteins took both stable and transient expression strategies. For establishment of stable HEK-293 cells expressing LG4, the pCEP-Pu α3 LG4 vector was transfected into cells using 50 µl of 1 mg/ml lipofectamine 2000 (Invitrogen) in 10 ml serum-free DMEM in each dish. The transfected cells were maintained for 24 hrs, split at 1:6 ratio and incubated with 5 µg/ml puromycin for 3 days for selection. The selected cells were maintained with DMEM containing 10 % FBS and 0.5 µg/ml puromycin. For transient expression of recombinant α3 LG4, SA-293 cells were prepared in 125 ml Erlenmeyer flasks with 25 ml of 293 FreeStyle medium at a cell density of 0.5 × 10⁶ cells/ml. After 24 hrs, cells were transfected with 2.5 ml of a mixture of pCEP-Pu α3 LG4: pEGFPc1 (total 25 µg, 9:1) and PEI (75 µg). After an additional 24 hrs, feeding medium tryptone N1 (ORGANOTECHNIE, La Courneuve, France) was added to the cell suspension at a final concentration of 0.5%. Transfected cells were maintained for 4 days as described above and collected to obtain proteins.

In-gel Protein Digestion and Mass Spectrometry of Peptides

After purification by Ni-NTA agarose, LG4 proteins were separated by SDS-PAGE under non-reducing conditions and visualized using Coomassie Brilliant Blue staining (Sigma-Aldrich). A standard marker (Precision Plus protein dual color standard; BioRad, Hercules, CA) was also run for comparison. The band of target was excised from the gel, cut into cubes,

equilibrated in 50 mM NH_4HCO_3 , reduced with dithiothreitol (3 mM in 50 mM NH_4HCO_3 , 37°C for 15 min), and alkylated with iodoacetamide (6 mM in 50 mM NH_4HCO_3 for 15 min). The gel cubes were then dehydrated with acetonitrile and rehydrated with 15 μl of 20 mM NH_4HCO_3 containing 0.01 $\mu\text{g}/\mu\text{l}$ modified trypsin (Promega, Madison, WI). Trypsin digestion was carried out for ~2 hrs at 37 °C. Peptides were extracted with 60 % acetonitrile and 0.1 % trifluoroacetic acid, dried by vacuum centrifugation, and reconstituted in 5 μl of 60 % acetonitrile with 0.1 % trifluoroacetic acid. A small aliquot (0.4 μl) was applied to a target plate and overlaid with 0.4 μl of a α -cyano-4-hydroxycinnamic acid matrix. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry and TOF/TOF tandem mass spectrometry was carried out using a Voyager 4700 mass spectrometer (Applied Biosystems, Foster City, CA) operated in reflectron mode. For intact peptide mass analysis, the mass spectra were calibrated to within 50 ppm using trypsin autolytic peptides present in the sample (m/z = 842.51 and 2211.09 daltons). Peptide ion masses ($M + H$) and MS/MS fragmentation data on specific ions were collectively used for data base interrogation against the NCBI database using GPS Explorer software (Applied Biosystems) running the MASCOT algorithm. Matched ions included m/z = 771.48, 904.43, 1093.67, 1174.53, 1274.71, 1350.66, 1364.69, 1400.7, 1451.73, 1626.87, 1674.83. Fragmentation spectra generated from the parent ions at above m/z validated the predicted amino acid sequences.

Microplate Adhesion Assays

Cell adhesion assays were performed in 96-well plates GSH-coated plates (Pierce, Rockford, IL) that were pretreated with SuperBlock™, a proprietary blocking reagent containing a mammalian derived protein (Pierce). Plates were coated with recombinant proteins (50 $\mu\text{g}/\text{ml}$ BacLG4; 10 $\mu\text{g}/\text{ml}$ MamLG4) or appropriate controls (1 $\mu\text{g}/\text{ml}$ Ln-332; 50 $\mu\text{g}/\text{ml}$ GST; 10 $\mu\text{g}/\text{ml}$ SAS) overnight at 4°C as previously described [28]. Using an enzyme-linked immunosorbent assay (ELISA) with a His₆-tagged antibody, we previously determined the appropriate concentrations of protein to be used by detecting the point when substrates approached saturation (data not shown). Unbound protein was removed with phosphate-buffered saline (PBS) washing. A-431 and HT-1080 cells were detached from tissue culture flasks with 0.025 % trypsin/0.53 mM EDTA (Life Technologies), washed twice with adhesion medium (serum-free DMEM, 25 mM HEPES), and resuspended in serum-free DMEM/HEPES. Human keratinocytes were trypsinized with 0.25 mg/ml trypsin/EDTA solution (Cambrex Bio Science Walkersville Inc.), neutralized with trypsin neutralizing solution (Cambrex Bio Science), and washed with EpiLife calcium-free medium. For cation studies, indicated cells were washed with $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free Hanks' balanced salt solution (HBSS; Life Technologies) containing 2 mM EDTA to deplete preexisting cations prior to trypsinization, washed twice with HBSS after trypsinization, and resuspended in HBSS. Where indicated, cells were pre-incubated with antibodies (10 $\mu\text{g}/\text{ml}$) or other reagents (5 mM EDTA; 0.01, 0.1, 1.0 mM Mn^{2+}) for 30 min at room temperature (RT) before plating. For each well, 100 μl of cell suspension (8.0×10^4 cells/well) was seeded and allowed to attach for 1 h at 37°C. Non-adherent cells were removed with a 30% Percoll wash (Amersham Pharmacia Biotech, Piscataway, NJ) and adherent cells were fixed with glutaraldehyde in PBS (3% v/v) for 15 min and stained with Crystal Violet (CV; 0.5% in 20% MeOH) for 30 min. Excess dye was washed off with water, new media added, and absorbance measured at 570 nm. Each experiment was done in triplicate and results are expressed as mean $\pm$ standard deviation (SD) of relative cell adhesion with control cells set as 100%.

Cell Migration Assays

Cell migration assays were carried out as previously described [30]. Briefly, polycarbonate Transwell filters (8 μm ; Corning Inc., Corning, NY) were coated on the lower side with purified rLGs (BacLG4 and MamLG4) at a concentration of 50 and 10 $\mu\text{g}/\text{ml}$, respectively. The lower side of the filter was then blocked with 5% milk in PBS for 2 h. Cells were prepared and

pretreated with antibodies/reagents in suspension as described in adhesion assays. 8.5×10^4 cells in 600 μ l of serum-free DMEM containing 0.1% bovine serum albumin (BSA) were added to the upper and lower chambers and allowed to migrate for 6 h at 37°C under tissue culture conditions. Cells that failed to migrate through the filter after incubation were scraped out using a sterile cotton swab. Cells that migrated to the bottom surface of the filter were fixed with methanol, stained with CV, and counted using light microscopy. Each substrate was repeated in duplicate wells, and within each well counting was done in four randomly selected microscopic fields (300 $\times$ magnification). Results are expressed as mean $\pm$ SD of relative cell migration with control cells set as 100%.

FAK Phosphorylation Assays

FAK phosphorylation assays were performed as previously described [28]. Briefly, A-431 cells or keratinocytes were trypsinized, washed, and kept in suspension in serum-free DMEM for 30 min at 37°C to reduce background FAK phosphorylation levels. Cells were plated on uncoated, SAS-coated, MamLG4-coated, or Ln-332-coated microplates and allowed to attach for 1 h. At the end of incubation, A-431 cells were lysed with lysis buffer. Isolated samples (from Ln-332 and MamLG4 containing wells) were analyzed by western blot with antibodies for Y397-pFAK and total FAK, and quantified using the Odyssey Infrared Imaging System (LI-COR Inc., Lincoln, NE).

Cell Spreading Assays

For IMCD-WT and IMCD- β 1 null cell spreading assays, 96-well plates were coated with Ln-332 and MamLG4 overnight at 4°C. Cells were added to wells (8×10^5 cells/ml) and incubated for 1 h at 37°C. After incubation, bound cells were stained with CV (0.5% in 20 % MeOH) for 30 min at RT. The number of spread cells was obtained by manually counting all cells present in three randomly selected microscopic fields (400 $\times$ magnification).

For human epidermal keratinocyte spreading assays, glass-bottomed microwell 35 mm dishes (MatTek Corporation, Ashland, MA) were coated with Ln-332 (1 mg/ml), MamLG4 (10 mg/ml), SAS (10 mg/ml), or PBS overnight at 4°C. Keratinocytes (less than passage 4) were trypsinized with 0.25 mg/ml trypsin/EDTA solution (Cambrex Bio Science Walkersville, Inc), neutralized with trypsin neutralizing solution (Cambrex Bio Science Walkersville, Inc.), and washed with EpiLife calcium-free medium. 500 μ l of cell suspension (2×10^5 cells) was added to each dish and incubated for 2 h at 37°C. After incubation, cells were fixed with paraformaldehyde for 10 min at 37°C and washed twice with PBS. After fixation, cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min at RT and were immediately blocked with PBS containing 10% goat serum and 1% BSA for 40 min at RT. Cells were incubated with Hoechst (5 mg/ml; blue) and anti-paxillin (5 mg/ml) antibody in 3% BSA in PBS for 2 h at RT. Bound primary antibody to focal adhesions or F-actin was detected using goat anti-mouse IgG Alexa Fluor 568 (red) and Alexa Fluor 488 phalloidin (green), respectively for 1 h at RT. After washing, cells were mounted and observed under an LSM510 confocal microscope (Carl Zeiss, Inc.). Cell spreading was quantified by manually counting cells in five randomly selected microscopic fields (630 $\times$ magnification).

Pull Down Assay of Integrin α 3 β 1

HT-1080 cells cultured on 150 mm diameter 4 dishes were detached with 1 mM EDTA, washed twice with PBS containing 1 mM CaCl_2 , 1 mM MgCl_2 and lysed at 4 °C with lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05 % Brij 35) containing EDTA-free complete protease inhibitor mixture (Roche Molecular Biochemicals) and phenylmethylsulfonyl fluoride (1 mM). 100 μ l of GSH-Sepharose (Amersham Pharmacia Biotech) were incubated for 1 h at 4°C with 500 μ g of GST or GST-fused LG4 (BacLG4), respectively. Cell lysates from 150 mm diameter 2 dishes were clarified (Sorvall SS34, 10,000 $\times$ g, 20 min) and mixed in batch with GST or

GST-fused LG4 (BacLG4)-bound Sepharose on a rotator for 2 h at 4 °C. After incubation, beads were centrifuged at 5,000 rpm for 5 min and washed 3 times with 5 bed volume of lysis buffer.

Bound material was mixed with non-reducing sample buffer for SDS-PAGE and boiled for 5 min at 100 °C. Western blot was carried out with antibodies against integrin $\alpha 3$ (pAb, AB1920, Chemicon, 1:500) or $\beta 1$ (mAb TS2/16, 1:200). Protein bands were visualized with the ECL+ plus system (Amersham Pharmacia Biotech).

Immunofluorescence Assay for Cell Culture

Glass coverslips were coated with various substrate at 4 °C overnight. After coating, coverslips were inserted into sterile 24-well plates. A-431 and HT-1080 cells were trypsinized and neutralized with DMEM containing 10% FBS and washed twice with serum-free DMEM. 500 μ l of cell suspension at 0.5×10^5 cells/ml was added to each well of 24-well plate and incubated for 1 hr at 37°C. Non-adherent cells were promptly washed from each cover slip after the incubation period, and remaining cells were fixed with 3.7% paraformaldehyde for 10 min at 37 °C and washed twice with PBS. After fixation, cells were permeabilized with 0.1 % Triton X-100 in PBS for 10 min at RT and then immediately blocked with PBS containing 10% goat serum and 1% BSA for 40 min at RT. Cells were incubated with Hoechst (5 μ g/ml; blue), anti-paxillin (5 μ g/ml) antibody in 3 % BSA in PBS, or anti-pFAK (5 μ g/ml) at RT for 2 h. Bound primary antibody to focal adhesions or F-actin was detected using goat anti-mouse IgG Alexa Fluor 568 (red) or mouse anti-rabbit IgG Alexa Fluor 568 (red) and Alexa Fluor 488 Phalloidin (green), respectively for 1 h at RT. For immunostaining of $\alpha 3$ integrin in IMCD cell lines, 35 mm glass-bottomed Microwell Dishes (MatTek Corporation) were coated with Ln-332 and MamLG4. WT and null $\beta 1$ -IMCD cells were added and incubated for 1 h at 37 °C. Anti- $\alpha 3$ integrin AB1920 (Chemicon) was used as a primary antibody. Bound primary antibody to focal adhesions or F-actin was detected using mouse anti-rabbit IgG Alexa Fluor 488 (green) and phalloidin (red), respectively for 1 hr at RT. After washing, cells were mounted and observed under a LSM510 Laser scanning confocal microscope (Carl Zeiss, Inc).

Bioinformatics

The Basic Local Assignment Search Tool for protein analysis (BLASTp) was used to investigate the homology of Ln-332 LG4 sequences (165 amino acids total) across rat (gi: 27545378), mouse (gi: 226423935), and human (gi: 38045908) species. The conservation line output represents that: an amino acid is fully conserved across species (denoted by *), a strong group is fully conserved (STA, NEQK, NHQK, NDEQ, QHRK, MILV, MILF, HY, FYW; denoted by :), a weaker group is fully conserved (CSA, ATV, SAG, STNK, STPA, SGND, SNDEQK, NDEQHK, NEQHRK, FVLIM, HFY; denoted by .), or not conserved across the species of interest (#).

Statistical Analysis

Student's two-sided t-tests were performed using SPSS version 17 (Chicago, IL) to detect significant differences ($P < 0.05$) across treatments of interest.

Results

Recombinant LG4 (rLG4) module production

Similar to other laminin α chains, the Ln-332 $\alpha 3$ chain possesses a large C-terminal G domain formed by five LG modules (LG1-5) that regulate cell behavior by interacting with various cell surface receptors. The five modules are linked together by short (~5-10 residues) intermodular sequences, except between the third and fourth module, where the linker sequence

is ~50 residues in length, essentially dividing the G domain into two separate structural components, the LG123 triplet, and the LG45 pair [31] (Fig. 1A).

To examine whether the LG4 module of the $\alpha 3$ chain of Ln-332 binds integrin $\alpha 3\beta 1$, we prepared purified rLG4 and expressed the monomer in both *E. coli* (BacLG4) and HEK-293 cells (MamLG4), as described in *Materials and Methods* (Fig. 1B). For BacLG4, we used a strategy similar to one we previously employed for production of rLG3 [28], whereby the rLG4 module was expressed in *E. coli* as a doubled-tagged fusion protein containing a GST moiety at the N-terminus and a His₆ tag at the C-terminus to provide convenient handles for protein purification and to increase solubilization. For MamLG4, rLG4 was instead expressed in HEK-293 cells as a single-tagged fusion protein containing a His₆ tag at the C-terminus. Additionally, GST alone was expressed and purified as a control for the GST tag of the bacterial vector, and SAS with a C-terminal His₆ tag was expressed in bacteria as a control for the His₆ tag present on both bacterial and mammalian vectors [32].

After production and purification, recombinant proteins were separated by SDS-PAGE in 4-12% polyacrylamide gel under reducing conditions and stained with Coomassie Brilliant Blue to confirm purity of samples. These results indicate that all recombinant proteins were purified to near homogeneity, since each resolved primarily as a single band (Fig. 1C). To confirm purified products, a western blot probed with anti-His₆ was performed against an identical gel. As expected, the anti-His₆ probe detected BacLG4, MamLG4, and SAS proteins, but not the GST protein due to its lack of His₆ (Fig. 1D). The other faint bands that resolved in both Fig. 1C and 1D (for BacLG4) are most likely degradation products.

Mass spectrometric analysis of rLG4

To further confirm our protein purification products, we also performed mass spectrometric analysis of both BacLG4 and MamLG4. In the BacLG4 sample, nine peptides sequences were identified (Fig. 2A), which aligned with cloned sequence from the LG4 region of rat Ln-332 $\alpha 3$ chain (Fig. 2B). Similarly, in the MamLG4 sample, nine peptides were also identified (Fig. 2C), which align with the cloned sequence, from the LG4 region of rat Ln-332 $\alpha 3$ chain (Fig. 2D).

Cell adhesion on rLG4

To examine the functions of the LG4 module of Ln-332, we first analyzed the cell adhesion activity of rLG4 proteins against human epidermoid carcinoma A-431 cells, human fibrosarcoma HT-1080 cells, and human epidermal keratinocytes, all of which express $\alpha 3\beta 1$ on their cell surfaces [33,34]. A-431 and HT-1080 cell lines readily adhered to BacLG4, MamLG4, and intact Ln-332 in a dose-dependent manner, whereas neither cell line adhered well to GST or SAS (Fig. 3A, B). Similarly, keratinocytes adhered to MamLG4 and Ln-332 in a dose-dependent manner, but not to SAS (Fig. 3C).

The integrin dependence of cell binding to rLG4 was further evaluated using adhesion assays in the presence of agents that modify integrin activity. Divalent cations, such as Ca²⁺, Mn²⁺, and Mg²⁺, are generally required for integrin-ligand recognition [35]. Therefore, any agent that strips cells of cations prior to introduction, such as the divalent cation chelator EDTA, is expected to decrease integrin-dependent cell adhesion. Both A-431 and HT-1080 cell lines adhered to BacLG4 and MamLG4 substrates similarly to intact Ln-332 (data not shown). Further, when 5 mM EDTA was added to cell suspensions prior to seeding, cell adhesion to BacLG4, MamLG4, and Ln-332 substrates were significantly reduced (Fig. 4A; N=3; P < 0.001 in all cases). These results suggest that rLG4 can recapitulate cell adhesion on Ln-332 and that integrin activity is necessary for recognition and subsequent adhesion of cell lines to rLG4.

The cation Mn^{2+} is known to enhance cell adhesion by integrin activation [36]. Therefore, to confirm that cell adhesion to rLG4 is integrin-mediated, we introduced Mn^{2+} at various concentrations to both HT-1080 and A-431 cells prior to adhesion assays. In both cell lines, Mn^{2+} significantly enhanced the number of attached cells that adhered to BacLG4, MamLG4, and intact Ln-332 (Fig. 4B, C; $P < 0.05$). In contrast, Mn^{2+} had almost no effect on background cell adhesion to GST or SAS proteins. Furthermore, the Mn^{2+} effect on rLG4 was comparable with that obtained for intact Ln-332, particularly at higher concentrations ($P \gg 0.05$ in all cases). These results are consistent with the idea that rLG4 harbors binding sites for integrin-type receptors.

The interaction between integrin $\alpha\beta1$ and Ln-332 initiates the formation of focal adhesions [37]. As focal adhesions become established, they connect with polymerized actin filaments [37]. In order to determine if cells interacting with rLG4 also form these structures, we examined the localization of a key focal adhesion component, paxillin, and associated organization of actin (using phalloidin, which stains F-actin) in A-431 cells. As shown in Fig. 5A, cells formed focal adhesions in a punctate-like pattern (red) containing paxillin and well-organized cortical actin (green) on BacLG4, MamLG4, and Ln-332 substrates. However, cells plated on SAS or GST protein alone exhibited little spreading or organization. Keratinocytes plated on MamLG4 exhibited cell spreading, actin organization, and focal adhesion formation similar to cells on Ln-332 substrate (Fig. 5B). Visual inspection of cell spreading revealed that nearly 100% of both cell types spread on Ln-332 and rLG4, compared to almost no spreading on negative controls (data not shown). Taken together, these results indicate that rLG4 is capable of initiating typical cell spreading and focal adhesion formation much like intact Ln-332, for both cancer and “normal” cells, presumably in an integrin-dependent manner.

FAK expression on rLG4

Integrins function not only as adhesive receptors, but also as signal transducers by activating associated protein kinases, such as FAK [38]. Of the two Ln-332 receptors, $\alpha\beta1$ is known to signal through this pathway, while $\alpha6\beta4$ does not. Therefore, FAK activation (phosphorylation) can be taken as an indication of $\alpha\beta1$ -dependent adhesion [37]. As shown in Fig. 5C, plating A-431 cells on either BacLG4 or MamLG4 induced spreading and phosphorylation of FAK (pFAK; red), similarly to cells on intact Ln-332. In contrast, plating cells on GST or SAS substrate did not induce spreading nor FAK activation. Similarly, keratinocytes spread and showed phosphorylated FAK on MamLG4 and Ln-332, but not on SAS or PBS (Fig. 5D). Furthermore, highly magnified regions of interest highlight the colocalization of paxillin and pFAK markers, which indicates that phosphorylation occurs at sites of focal adhesion. Western blotting analysis of pFAK confirmed that A-431 cells plated on rLG4 module had increased tyrosine phosphorylation of FAK similar to that of Ln-332, with expression levels for both substrates almost 2-fold greater than negative controls (Fig. 5E). Taken together, these results indicate that the Ln-332 rLG4 modules can induce cell spreading and focal adhesion formation via integrin-type receptors.

Role of integrins in cell adhesion to rLG4

It is well established that the two main receptors for Ln-332 are integrins $\alpha\beta1$ and $\alpha6\beta4$ [22, 23]. To identify the specific integrin involved in binding rLG4, we examined the inhibitory effects of integrin blocking antibodies on rLG4 supported cell adhesion. In these experiments, A-431 cells were used in combination with MamLG4 only (Fig. 6A, B) and HT-1080 cells were used with BacLG4 only (Fig. 6A, B). As shown in Fig. 6A, treatment of A-431 cells with anti- $\alpha3$ (P1B5) or anti- $\beta1$ (6S6) integrin antibodies significantly inhibited cell adhesion to MamLG4 ($P < 0.05$, in both cases). Similarly, these antibodies also significantly inhibited HT-1080 cells to BacLG4 ($P < 0.001$, in both cases). In contrast, incubation of either A-431 and/or HT-1080 cells with anti- $\alpha6$ integrin (GoH3) or anti- $\alpha5$ integrin (P1D6) antibodies had

almost no effect on adhesion to rLG4 ($P > 0.05$, in both cases). Furthermore, in line with earlier results, the addition of EDTA significantly inhibited adhesion of both cell lines compared to each corresponding control (no antibody present; $P < 0.01$, in both cases). Taken together, these results suggest that cell adhesion to rLG4 is dependent on $\alpha 3\beta 1$ integrin.

Cell migration on rLG4

To identify the specific integrin receptor(s) involved in migration on rLG4, we examined the effects of the same integrin blocking antibodies using Boyden chamber assays. As shown in Fig. 6B, treatment of A-431 cells with anti- $\alpha 3$ (P1B5) or anti- $\beta 1$ (6S6) integrin antibodies significantly inhibited haptotactic migration on MamLG4 ($P < 0.05$, in both cases). Similarly, the same antibodies significantly inhibited HT-1080 migration on MamLG4 ($P < 0.001$, in both cases). In contrast, incubation of either A-431 or HT-1080 with anti- $\alpha 6$ integrin antibody (GoH3) had almost no effect on cell adhesion to rLG4 ($P \gg 0.05$, in both cases). Furthermore, in line with earlier results, the addition of EDTA inhibited adhesion of both cell lines significantly ($P < 0.01$), compared to each control (no antibody present). Taken together, these results suggest that haptotactic migration on rLG4 is also dependent on $\alpha 3\beta 1$.

We also used IMCD- $\beta 1$ null cells to confirm the role of $\beta 1$ integrin in cellular functions on LG4 module. It has previously been reported that this cell line exhibits decreased cell spreading on $\beta 1$ -dependent ECM substrate [39]. As expected, our results demonstrated that these cells are unable to spread on LG4 module or Ln-332 (Fig. 7A, B). These results indicate that cell functions on rLG4 are mediated by $\alpha 3\beta 1$ integrin.

Role of integrin $\alpha 3\beta 1$ in rLG4 interactions

To obtain direct evidence for interaction of rLG4 with $\alpha 3\beta 1$ integrin, we performed GST pull-down assays using BacLG4 and HT-1080 cell lysates. GSH-sepharose bound GST or BacLG4 was incubated with HT-1080 cell lysates in the presence of Mn^{2+} ion. Pulled-down materials were extracted and analyzed by SDS-PAGE under non-reducing conditions, followed by western blotting with integrin antibodies (Fig. 8). Antibodies against $\alpha 3$ or $\beta 1$ recognized two bands with molecular masses of ~ 165 and ~ 110 kDa in BacLG4 (Fig. 8A). In contrast, no bands were detected for GST alone. These results directly demonstrate that the LG4 module directly binds $\alpha 3\beta 1$ integrin.

Discussion

In this study, we produced and applied a Ln-332-derived adhesive surface using recombinant LG4 module, a 25 kDa fragment within the G domain of the Ln-332 $\alpha 3$ chain. We used an approach similar in design to our previous work involving expression of rLG3 in bacteria [28]. However, in order to overcome any protein folding concerns with expression in a bacterial vector, we also expressed rLG4 within the mammalian HEK-293 cell line in these studies. In summary, our results show that cell functions on rLG4 expressed in either expression system recapitulated those on intact Ln-332 (although mammalian expression of rLG4 exhibited somewhat greater functionality in all assays performed).

Functionalized rLG4 surfaces induced integrin $\alpha 3\beta 1$ -mediated cell adhesion, migration, spreading, and assembly of paxillin-containing focal adhesions. The evidence supporting this conclusion is as follows: Using *in vitro* microplate-based adhesion assays, rLG4 supported cell adhesion similarly to intact Ln-332 for both cancer (A-431, HT-1080) and “normal” (human epidermal keratinocytes) cell lines (Fig. 3); Divalent cations (Mn^{2+}), known as modulators of integrin affinity states, up-regulated cell adhesion on rLG4 and Ln-332 (Fig. 4C), while EDTA inhibited adhesion on both substrates (Fig. 4A); 3) A431 cells and keratinocytes adherent to rLG4 exhibited typical morphological patterns including formation of focal adhesion

structures, actin organization, cell spreading, and FAK phosphorylation, similar to Ln-332 (Fig. 5); 4) Cell adhesion and haptotactic migration on both rLG4 and Ln-332 were blocked by anti- $\alpha 3$ and anti- $\beta 1$, but not anti- $\alpha 6$ integrin, antibodies (Fig. 6); 5) $\beta 1$ integrin null IMCD cells failed to spread on rLG4 or Ln-332 (Fig. 7); and 6) GST pull down assays showed specific binding of $\alpha 3\beta 1$ in cell lysates to rLG4 (Fig. 8). Taken collectively, these results clearly demonstrate that cell interactions with rLG4 occur in an integrin $\alpha 3\beta 1$ -dependent manner, recapitulating Ln-332 functions.

In these studies, we used rat Ln-332 as cDNA templates for recombinant LG4 fragments and human and mouse-derived cell lines. A search of the BLASTp database revealed that the Ln-332 LG4 sequences (165 amino acids total) of rat (gi: 27545378), mouse (gi: 226423935), and human (gi: 38045908) share high homology, with rat and human sequences sharing 87% of residues, and rat and mouse sequences sharing 92% of residues (Supp. Fig. 1). These data suggest that our enclosed experimental findings should translate to other species. It is well known that integrin binding to ECM is interchangeable in between species.

Other groups have previously reported that recombinant FN III₇₋₁₀ fragments fulfill the intact FN functional activities including cell adhesion, focal adhesion assembly, phosphorylation of FAK, and integrin $\alpha 5\beta 1$ binding activity [20]. Thus, the approach using recombinant protein fragments may provide a versatile means for increasing biofunctional activity and integrin specificity to ECM-like biomaterials. The use of functional fragments also offers several advantages over the entire molecule, including higher stability, reduced antigenicity, elimination of domains that may elicit adverse reactions, and enhanced cost efficiency [40].

Conclusions

A recombinant fragment of Ln-332, including the $\alpha 3$ G domain LG4 module (rLG4), expressed in either bacteria (BacLG4) or mammalian (MamLG4) systems, mimicked functional activities of intact Ln-332 *in vitro*. Our results showed that cells on rLG4 surfaces adhered, spread, exhibited FAK activation, and migrated similarly to those on Ln-332 substrate. Furthermore, we determined by several independent approaches that these activities were mediated by the integrin $\alpha 3\beta 1$. Since incorporation of native ECM proteins often proves difficult for reasons of size and solubility, this strategy represents a more robust alternative approach for incorporating functional activity and integrin specificity into the design and application of biomaterials. This is especially true for Ln, whose sizes approaches 500 kDa, and for which short peptide sequences appear not to be useful surrogates in terms of integrin binding.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations Used

The abbreviations used are:

BacLG4	bacterial recombinant LG4
BM	basal membrane
CV	crystal violet
DMEM	Dulbecco's modified Eagle's medium
ECM	extracellular matrix

FBS	fetal bovine serum
G domain	globular domain
GST	glutathione S-transferase
HBSS	Hank' balanced salt solution
Ln	laminin
LG	laminin G-like
MamLG4	mammalian recombinant LG4
mAb	monoclonal antibody
pAb	polyclonal antibody
p-FAK	phosphorylated FAK
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate- buffered saline
PCR	polymerase chain reaction
PEI	linear polyethylenimine
rLG	recombinant laminin G-like
RT	room temperature
SAS	sialic acid synthases
SA-293	suspension-adapted human embryonic kidney 193 cells
SCC	squamous cell carcinoma

Fig. 1. Ln-332 structure and recombinant $\alpha 3$ LG4 expression in bacteria and mammalian cells
 (A) Ln-332 is composed of $\alpha 3$, $\beta 3$, and $\gamma 2$ chains assembled into $\alpha 3\beta 3\gamma 2$ heterotrimer via a three-stranded α -helical coiled-coil domain. A globular domain (G domain) uniquely exists at the C-terminus of the $\alpha 3$ chain, which consists of 5 tandem laminin G-like (LG) repeats (LG1-LG5). (B) Schematic diagram of recombinant proteins. The recombinant $\alpha 3$ LG4 module was expressed using GST and His₆ tag fusion protein in bacteria (BacLG4), and a His₆ tagged protein in mammalian HEK-293 EBNA cells (MamLG4). GST alone and His₆ tagged recombinant SAS alone were also expressed for control. (C) After GST and SAS constructs were purified and rLG4 modules were expressed in *E. coli* and Hek-293 cells, eluted material was subjected to SDS-PAGE analysis (NuPAGE 4 - 12% gel) under reducing conditions and visualized by Coomassie staining, which indicated proteins were purified to near homogeneity since they show up as single bands. (D) Immunoblot analysis was carried out using an mAb against His₆ tag, which detected rLG4 proteins and SAS, but not GST due to its lack of this structure (D).

Fig. 2. Mass spectrometry analysis of bacterial and mammalian rLG4

After in-gel trypsin digestion of rLG4 modules, peptides were identified by MALDI-TOF mass spectrometry. (A) Mass spectrometry identified 9 different peptides in BacLG4 digestion. (B) Localization of peptides (*1-9) for BacLG4 detected by mass spectrometry was compared with the established rat Ln-332 α 3 chain sequence (1312-1552+His₆ tag). Labels A-M signify the folds in the β sheet structure. (C) Mass spectrometry identified 9 different peptides in MamLG4 digestion. (D) Localization of peptides (*1-9) for MamLG4 was compared with the established rat laminin-332 α 3 chain sequence (1312-1552+His₆ tag). Labels A-M signify the folds in the β sheet structure.

Fig. 3. HT-1080, A-431, and keratinocytes adhere to rLG4

96-well plates were coated at 4 °C overnight with BacLG4, GST protein, MamLG4, SAS, or Ln-332. A-431, HT-1080, or keratinocytes were seeded and incubated on substrates for 1-2 h at 37 °C. Non-adherent cells were then washed from wells and remaining cells were stained with CV, and absorbance was measured as indicated. Results are expressed as the mean absorbance $\pm$ standard deviation. (A, B) Both A-431 and HT-1080 lines adhered to BacLG4 and MamLG4, similarly to intact Ln-332 (N = 3; P = 0.016-0.91). In contrast, GST and SAS controls had almost no adhesion activity (N = 3; P < 0.001). (C) Similarly, human keratinocytes adhered to MamLG4 similarly to Ln-332 (N = 2; P < 0.001).

Fig. 4. Cell adhesion to rLG4 is integrin-dependent

(A) In adhesion experiments involving the use of EDTA, 96-well plates were coated at 4 °C overnight with BacLG4, MamLG4, or Ln-332 protein. Where indicated, A-431 and HT-1080 cells were treated with EDTA for 1 h prior to plating. When EDTA was added to either cell line prior to incubation, adhesion was significantly reduced on all substrates (N=3; $P < 0.001$). (B) In Mn^{2+} experiments, 96-well plates were coated at 4 °C overnight with BacLG4, MamLG4, Ln-332, GST, or SAS protein. Where indicated, cells were treated with Mn^{2+} at various concentrations for 1 h at 37 °C. Cells were seeded, and allowed to adhere to substrates for 1 h at 37 °C. Non-adherent cells were washed off, adherent cells were stained with CV, and absorbance was measured at 570 nm. Results are presented as the mean absorbance $\pm$ standard deviation (N=3). Stimulation of cells with Mn^{2+} significantly enhanced the number of cells that adhered to BacLG4, MamLG4, and Ln-332 ($P < 0.05$, in all cases), but not GST or SAS. Taken together, these results indicate that rLG4 stimulates binding activity in an integrin-dependent manner.

Fig. 5. rLG4 induces cell spreading and focal adhesion structures

(A) A431 cells were seeded on glass coverslips coated with BacLG4, MamLG4, Ln-332, SAS, or GST, and allowed to adhere for 1 h at 37°C. After a washing step, adherent cells were labeled with Hoechst to stain nuclei (blue), phalloidin to locate actin organization (green), and paxillin to target focal contacts (red). Cells plated on MamLG4 and BacLG4 exhibited cell spreading, actin organization, and focal adhesion formation similar to cells on Ln-332 substrate. (B) Human epidermal keratinocytes were also seeded on glass dishes coated with Ln-332, MamLG4, SAS, or PBS, and allowed to adhere for 2 h at 37°C. After washing, adherent cells were labeled with Hoechst (blue) and anti-paxillin (primary mAb), followed by secondary IgG Alexa Fluor 568 (red) and Alexa Fluor 488 (green) to detect focal adhesions and F-actin, respectively. Keratinocytes plated on MamLG4 exhibited cell spreading, actin organization, and focal adhesion formation similar to cells on Ln-332 substrate. Whereas cells plated on SAS or PBS exhibited little organization or spreading. (C) A-431 cells were handled similarly to part A, except cells were stained for p-FAK to identify tyrosine phosphorylation (red), in lieu of paxillin. This marker revealed that FAK is phosphorylated similarly in on both Ln-332 and rLG4. (D) Similarly, keratinocytes spread and showed phosphorylated FAK on MamLG4 and Ln-332, but not on SAS or PBS. Highly magnified regions of interest highlight the colocalization of paxillin and pFAK markers, which indicates that phosphorylation occurs at

sites of focal adhesion. (E) Western blotting analysis confirms that tyrosine phosphorylation of FAK was induced in A-431 cells attached to immobilized rLG4 (similarly to Ln-332), in contrast to cells on SAS or PBS.

Fig. 6. Integrin $\alpha 3 \beta 1$ mediates cell adhesion and migration on rLG4

Adhesion (A) and haptotactic migration (B) activity of HT-1080 and A-431 cell lines against rLG4 were measured using standard 96-well plate adhesion and migration assays. Where indicated, cells were pre-incubated with EDTA or the following inhibitory antibodies: P1B5 (anti- $\alpha 3$), 6S6 (anti- $\beta 1$), P1D6 (anti- $\alpha 5$), or GoH3 (anti- $\alpha 6$) for 30 min. Plots represent the mean $\pm$ standard deviation percentage of adhesion (N=3) or migration (N=2) relative to untreated cells set to 100% (on BacLG4 or MamLG4). Both cell adhesion and/or migration were significantly inhibited by anti- $\alpha 3$, anti- $\beta 1$, and EDTA ($P < 0.05$, in all cases), but not by anti- $\alpha 6$ or anti- $\alpha 5$ antibodies. These results indicate that cell adhesion and migration on rLG4 is dependent on $\alpha 3 \beta 1$ integrin.

Fig. 7. Integrin β 1-knockdown decreases cell spreading on rLG4

(A) IMCD-WT and IMCD- β 1 null cells were seeded and allowed to adhere for 1 h at 37 °C. After a wash step, bound cells were stained with CV, and microphotographs were taken at 400 $\times$ magnification. Values represent the mean $\pm$ standard deviation percentage of spread cells relative to total cell number set to 100% (N=3). (B) β 1-IMCD null and WT cells were plated on Ln-332 or MamLG4 and allowed to adhere for 1 h. After a wash step, adherent cells were labeled with Hoechst stain nuclei (blue), phalloidin to locate actin organization (red), and α 3 integrin pAb 1948 (green). Microphotographs were taken at 630 $\times$ magnification.

Fig. 8. Integrin $\alpha 3\beta 1$ specifically binds to rLG4 module

GST pull-down assays were performed to obtain direct evidence for interaction of rLG4 with $\alpha 3\beta 1$ integrin. Assays used BacLG4 and HT-1080 cell lysates. GSH-sepharose bound GST or BacLG4 was incubated with HT-1080 cell lysates in the presence of Mn^{2+} ion. After incubation, beads were centrifuged at 5,000 rpm for 5 min and washed 3 times with 5 bed volume of lysis buffer. (A) Bound material was mixed with non-reducing sample buffer for SDS-PAGE and boiled for 5 min at 100 °C. (B) Western blot was carried out with antibodies against integrin $\alpha 3$ or $\beta 1$. These results indicate $\alpha 3\beta 1$ binds specifically to rLG4.