The 67 kDa laminin receptor increases tumor aggressiveness by remodeling laminin-1

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Abstract

The association between expression of the 67 kDa laminin receptor (67LR) and tumor aggressiveness has been convincingly demonstrated although the exact function of this molecule in the metastatic process has remained unclear. In this study, we tested whether the laminin-1, upon interaction with 67LR, promotes tumor cell aggressiveness; the investigation was based on: (i) the previous demonstration that soluble 67LR, as well as a 20-amino-acid peptide corresponding to the 67LR laminin binding site, changes the conformation of laminin upon interaction with this adhesion molecule and (ii) the known relevance of microenvironment remodeling by the tumor, leading to structural modification of extracellular matrix components in tumor progression. MDAMB231 breast carcinoma cells plated on peptide G-treated laminin-1 exhibited a polygonal array of actin filament bundles compared with cells seeded on native laminin-1 which presented the actin bundles organized as multiple cables parallel to margins. Furthermore, in cells seeded on peptide G-treated laminin-1, 67LR was distinct from the α6 integrin subunit in filopodia protrusions in addition to colocalizing with this integrin in focal adhesion plaques as it occurs when cells are plated on native laminin-1. In addition to differences in tumor cell adhesion and migration found in cells exposed to peptide G-treated vs native laminin-1, breast carcinoma cells seeded on modified laminin-1 showed a 6-fold increase in invasion capability compared with cells seeded on unmodified laminin-1. Alterations in actin organization as well as adhesion, migration and especially invasion observed in MDAMB231 cells in the presence of peptide G-treated laminin-1 were even found in MDAMB231 cells that, after selection for 67LR high expression, were seeded on native laminin-1. As the 67LR shedding is proportional to its expression level, these findings indicate a role for 67LR in changing laminin structure.

Expression analysis of 97 genes encoding proteins that mediate cell matrix interactions, revealed significant differences between cells exposed to modified vs unmodified laminin-1 in 19 genes, 17 of which — including those encoding α3 integrin, extracellular matrix protein 1, proteolytic enzymes (such as MT1-MMP, stromelysin-3 and cathepsin L) and their inhibitors — were up-regulated in cells treated with modified laminin-1. Zymogram analysis clearly indicated a significant increase in the activity of the gelatinolytic enzyme MMP-2 in the culture supernatant from cells exposed to modified laminin-1, without an increase in mRNA abundance as observed in microarray analysis. Invasiveness of tumor cells conditioned by modified laminin-1, evaluated as the capability to cross Matrigel basement, was significantly more inhibited by MMP inhibitor TIMP-2 than invasiveness induced by native laminin-1. Taken together, our findings indicate that the role of 67LR in tumor aggressiveness rests in its ability to modify laminin-1 thereby activating proteolytic enzymes that promote tumor cell invasion through extracellular matrix degradation.

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Introduction

The recent view of tumors as functional tissue dynamically interconnected with the microenvironment suggests that the remodeling of the extracellular matrix (ECM) around the tumor is relevant in tumor invasion of surrounding tissues and dissemination to distant sites (Pupa et al. 2002). The key molecules in this process are adhesion molecules and their receptors, as well as proteolytic enzymes that degrade basement membrane components (Liotta & Kohn 2001). The high-affinity 67 kDa laminin receptor (67LR) expressed by tumor cells is known to be critically involved in the metastatic process (Castronovo et al. 1990, 1992, Ciocé et al. 1991, Martignone et al. 1993, Sobel 1993, Basolo et al. 1996, Sanjuán et al. 1996, Van den Brule et al. 1996, Fontanini et al. 1997, Waltregny et al. 1998, Molino et al. 2003), although its exact mechanism remains unclear. In vitro data indicate that the 67LR promotes adhesion, but this activity cannot account for its role in invasion since stabilization of the tumor cells at the primary site is implied.

Furthermore, after the initial description of 67LR, it became clear that integrins are the primary mediators of cell adhesion and signal transduction to the nucleus. The 67LR is an accessory molecule of the α6 integrin subunit (Ardini et al. 1997), and confocal microscope studies (Starkey et al. 1999) confirmed the presence of 67LR in the vicinity of focal adhesion plaques associated with α6β1 integrin. However, this molecule is also shed in soluble form by tumor cells in amounts proportional to its overexpression (Karpatová et al. 1996). The correlation between the magnitude of the shedding and the enhanced invasiveness in ‘in vitro’ experiments (Starkey et al. 1999) is consistent with the notion that shed 67LR promotes aggressive malignant behavior in solid tumors. Soluble 67LR, as well as a 20-amino-acid peptide (peptide G) corresponding to the 67LR laminin binding site, change the conformation of laminin upon interaction with this adhesion molecule (Magnifico et al. 1996). These 67LR-induced conformational changes result in increased proteolytic cleavage of laminin and consequent generation of proteolytic fragments that promote tumor cell migration (Ardini et al. 2002). Because receptor recognition domains of laminar are conformation dependent (Mercurio 1995), 67LR-induced remodeling of laminin and unmasking of cryptic sites might modulate the interaction between tumor cells and the ECM, with consequences for metastatic potential. To test this hypothesis we investigated the effect of peptide G-modified laminin-1 on cellular processes involved in metastasis such as adhesion, migration, invasion and activation of proteolytic enzymes.

Materials and methods

Cell lines and culture conditions

Human breast carcinoma cell line MDAMB231 (American Type Culture Collection Manassas, VA, USA) was maintained in RPMI 1640 (Sigma) supplemented with 10% fetal calf serum (FCS), 1% L-glutamine, and antibiotics (100 μg/ml) at 37°C in a humidified 5% CO2 atmosphere. Experiments to determine the effect of laminin-1 or peptide G-treated laminin-1 were carried out in serum-free medium. Separation of MDAMB231 cells according to 67LR expression levels was performed by incubating cells with anti-67LR MLuC5 monoclonal antibody (Mab) followed by anti-mouse immunoglobulin (Ig)-conjugated magnetic beads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) according to the manufacture’s procedure.

Reagents and antibodies

Peptide G (IPCNNKGALSVGLMWWMLAR), corresponding to amino acids 161–180 of the 37 kDa precursor protein of 67LR and scrambled peptide X (PMLRWGCHIAMVNKLSWGNA) were synthesized by Neosystem (Strasbourg, France). Murine laminin-1 and human purified TIMP-2 were obtained from Sigma and Chemicon International, Inc. (Temecula, CA, USA) respectively. MAb directed against α6-chain integrin (MAR6) (Bottini et al. 1993), 67LR (MLuC5) (Martignone et al. 1992), talin (8D4 clone) (Sigma) and laminin (LAM-89 clone) (Sigma) were used. Treatment of laminin-1 with peptide G or X was performed by incubating adhesion molecules with distinct peptides at a 1:1 (w/w) ratio for 1 h at 37°C.

Indirect immunofluorescence and confocal microscopy analyses

Glass coverslips were left uncoated or were coated with laminin-1 or laminin-1 pre-incubated with peptide G or peptide X overnight at 37°C. MDAMB231 cells serum-starved for 24 h were plated, allowed to grow for 30 min, fixed with 3.7% formaldehyde in the medium, saturated with 5% BSA and permeabilized with 0.1% Triton-X 100 in PBS. For phalloidin staining, cells were incubated with fluorescein isothiocyanate (FITC)-conjugated phalloidin (Molecular Probes Inc., Eugene, OR, USA) and observed with an inverted microscope connected to a Nikon DXM camera. Captured images were processed using the Image PC software package. For cell surface labeling, cells on coverslips were washed with PBS, incubated with MLuC5 (1:100), MAR6 (1:50) and 8D4 (1:50) primary antibody for 1 h at...
room temperature and incubated with specific secondary FITC (Alexa 488)- or TRITC (Alexa 546)- or Texas-red (Alexa 633)-conjugated goat anti-mouse antibody for 45 min (Molecular Probes Inc.). After three washes in PBS, coverslips were mounted on glass slides using Mowiol (Calbiochem, San Diego, CA, USA) and observed with a confocal microscope (Microradiance 2000, BioRad) equipped with Ar (488 nm) and HeNe (543 nm) lasers. Images were taken using a ×60 oil immersion lens (1024 × 1024 pixels). Images were analyzed using Lasersharp 2000 software. Control cells were exposed to relevant secondary antibodies alone and showed no significant degree of labeling.

**Cell adhesion assay**

Equal amounts (10 µg/ml) of intact or peptide G-treated laminin-1 were adsorbed on 96-well plates (Greiner Labortecnik, Frickenhausen, Germany) for 1 h at 37°C. MDAMB231 cells (1 × 10^4 cells per well) in serum-free culture medium were added and allowed to adhere at 37°C. After 1 h, plates were filled with PBS, inverted and shaken in a tank of PBS for 15 min. Regarding high or low 67LR-expressing cells, MDAMB231 cells separated as reported were plated on native laminin-1 for 6 h at 37°C. Afterwards they were filled with PBS, inverted and shaken in a tank of PBS for 15 min. Adherent cells were fixed in ice-cold 10% trichloroacetic acid, labeled with sulforhodamine B and evaluated based on optical density at 550 nm wavelength.

**ELISA assay**

Equal amounts (20 µg/ml) of native, peptide G- or peptide X-treated laminin-1 were adsorbed in 96-well plates (Greiner Labortecnik) for 3 h at 37°C. Anti-laminin MAB diluted 1:200 was incubated for 1 h at 37°C. After several washings with PBS, anti-mouse Ig horseradish peroxidase (HPR)-conjugated antibody was incubated for 1 h at 37°C. After additional washing, the immune complexes were detected by adding 3,3’5,5’-tetramethylbenzidine (TMB) (Sigma) as substrate for 30 min at room temperature in the dark. The enzymatic reaction was arrested by adding 1 M H_2SO_4. Plates were read at 450 nm using an automated ELISA reader.

**Cell migration assay**

The effects of peptide G-treated laminin-1 or laminin-1 on tumor cell migration were determined by a modified Boyden chamber assay using a 96-well cell migration kit (Chemicon International, Inc.), with each well chamber separated by an 8 µm pore membrane. Cell suspension containing 4 × 10^4 cells in RPMI medium without growth factors was transferred to the upper wells after addition of untreated (LN-1), peptide G (pG-LN-1)- or peptide X-treated (pX-LN-1) laminin-1, peptide G (pG) or peptide X (pX) alone to the lower chamber as attractants. Control chambers contained no added chemoattractants. After incubation at 37°C for 4 h, migratory cells were dissociated from the bottom of the membrane by incubation with cell detachment buffer. Regarding high or low 67LR-expressing MDAMB231 cells, they were allowed to migrate for 24 h using native laminin-1 as chemoattractant. Detached cells were lysed and stained for 15 min at room temperature with CyQuant GR dye (Chemicon International, Inc.), which exhibits strong fluorescence enhancement when bound to cellular nucleic acids. Fluorescence was measured using TECAN plate reader with a 480/520 nm filter set.

**Cell invasion assay**

Invasion assays were performed in a Matrigel invasion chamber (Becton Dickinson Labware, Bedford MA). For chemotaxis assay, 750 µl of serum-free culture medium and equal amounts (10 µg/ml) of native (LN-1), peptide G (pG-LN-1)- or peptide X-treated (pX-LN-1) laminin-1, peptide G (pG) or peptide X (pX) alone were added to the lower wells, whereas 500 µl MDAMB231 cells were transferred to the upper wells (4 × 10^4 cells per well). For experiments with MDAMB231 pre-incubated with treated or untreated laminin-1, lower chambers were filled with culture medium and cells previously seeded for 30 min on native or modified laminin-1 (10 µg/ml) at 37°C were transferred into the upper chambers as described for the chemotaxis analysis. After 6 h, cells on the upper side of the filters were mechanically removed. High or low 67LR-expressing MDAMB231 cells were allowed to migrate for 24 h using native laminin-1 as chemotactrant. Filters were fixed and stained with Diff-Quick solution (Dade Behring, Duedingen, Switzerland). The number of migrated cells underneath the membranes was counted in four high-power fields.

**Superarray**

The GEArray gene expression array system consisting of 97 cDNA fragments, printed in tetra-spot format, from genes associated with metastasis and with control sequences (PUC18 as negative control, β-actin and GAPDH for loading) was obtained from SuperArray (Bethesda, MD, USA). Total cellular RNA was isolated with Trizol reagent (Invitrogen) and RNA
integrity was assessed by visualization of ethidium bromide-stained gels. Microarray analysis was carried out according to the manufacturer’s instructions. Briefly cDNA was prepared from total RNA obtained from cells plated on native or peptide G-modified laminin-1 for 30 min, by reverse transcription with MMLV reverse transcriptase, radiolabeled using [\(\alpha\)-\(32\)P]dCTP (10\(\mu\)C/\(\mu\l, Amersham) and hybridized under specified conditions to a positively charged nylon membrane containing the array DNA. After washing, arrays were visualized by autoradiography and tetra-spot images were converted to numerical data using the free ScanAlyze software. GEArray Analyzer software was used to match raw data with the gene list for metastasis GEArray. Raw signal intensities were corrected for background by subtracting signal intensity of a negative control or blank, and normalized to signal intensity of a housekeeping gene using image-analysis software (AtlasImage version 2.0). For each gene, values corresponding to two different treatments were compared and a gene expression difference higher than that observed for housekeeping genes (1.6-fold) was considered indicative of change due to cell treatment. The array includes the genes described below.

**Cell adhesion molecules**

Integrins: ITGA1 (integrin \(\alpha_1\)), ITGA2 (integrin \(\alpha_2/\beta\)), ITGA2B (integrin \(\alpha_2/\beta\)), ITGA3 (integrin \(\alpha_3\)), ITGA4 (integrin \(\alpha_4/\beta\)), ITGA5 (integrin \(\alpha_5\)), ITGA6 (integrin \(\alpha_6\)), ITGA7 (integrin \(\alpha_7\)), ITGA8 (integrin \(\alpha_8\)), ITGA9 (integrin \(\alpha_9\)), ITGA10 (integrin \(\alpha_{10}\)), ITGA11 (integrin \(\alpha_{11}\)), ITGAL (integrin \(\alpha_l/\beta\)), ITGAM (integrin \(\alpha_M\)), ITGAV (integrin \(\alpha_V\)), ITGAX (integrin \(\alpha_X\)), ITGB1 (integrin \(\beta_1\)), ITGB2 (integrin \(\beta_2\)), ITGB3 (integrin \(\beta_3/\beta CD61\)), ITGB4 (integrin \(\beta_4\)), ITGB5 (integrin \(\beta_5\)), ITGB6 (integrin \(\beta_6\)), ITGB7 (integrin \(\beta_7\)), ITGB8 (integrin \(\beta_8\)). Immunoglobulin (Ig) Superfamily: CEACAM5 (CEA), DCC, ICAM1, MICA (MUC-18), NCAM1, NRCAM, PECAM1, VCAM1. Cadherin and catenins: CDH1 (E-cadherin), CTNNA1 (catenin a), CTNNA1L (catenin \(\alpha_L\)), CTNNB1 (catenin \(\beta\)), CTNND1 (catenin \(\delta_1\)), CTNND2 (catenin \(\delta_2\)). Selectins: SELE (ELAM-1/E-selectin), SELL (L-selectin), SELP (P-selectin). Other related genes: CD44, CNTN1.

**Extracellular matrix proteins**

CAV1 (caveolin-1), COL18A1 (LOC51695/endostatin), COL1A1, COL4A2, ECM1 (extracellular matrix 1), FGB (fibrinogen \(\beta\)), FN1 (fibronecin-1), LAMB1 (laminin B1), LAMC1 (laminin B2), SPARC, SPP1 (OPN, osteopontin), THBS1 (TSP-1), THBS2 (TSP-2), THBS3 (TSP-3), VTN (vitronectin).

**Proteases**

Matrix metalloproteinases: ADAMTS1 (Meth 1), ADAMTS8 (Meth 2), MMP1 (collagenase-1), MMP2 (gelatinase A), MMP3 (stromelysin-1), MMP7 (matrilysin), MMP8 (neutrophil collagenase), MMP9 (gelatinase B), MMP10 (stromelysin-2), MMP11 (stromelysin-3), MMP12 (macrophage elastase), MMP13 (collagenase-3), MMP14 (MT1-MMP), MMP15, MMP16, MMP17, MMP20 (elastase), MMP24, MMP26. Serine proteases: CTSG (cathepsin G), PLAT (tPA), PLAU (uPA), PLAUR (uPAR), TMPRSS4.

**Cysteine proteinases**

CASP8, CASP9, CST3 (cystatin C), CTSB (cathepsin B), CTSL (cathepsin L). Other related genes: CTSD (cathepsin D), HPSE (heparanase), MGEA5 (meningioma associated hyalurondiase). Protease inhibitors: SERPINB2 (PAI-2), SERPINB5 (maspin), SERPINE1 (PAI-1), TIMP-1, TIMP-2, TIMP-3.

**RT-PCR**

Reverse transcription was carried out at 42°C for 60 min using 2\(\mu\) total RNA, 75 pmol oligo(dT) primer, 0.5 mM of each dNTP and 50 U of MMLV reverse transcriptase (Invitrogen). RT product was amplified with primer pairs specific for the genes to be studied. Conditions and primer sequences are available on request. GAPDH cDNA was amplified in parallel to evaluate the efficiency of RNA extraction and cDNA synthesis. GAPDH primers were 5'-TCCAC-CACCTGTGGCTGTA-3' and primer 5'-ACCACA-GTCCATGCCTCAC-3', with an expected product of 452 bp. Each RT-PCR product was electrophoresed on a 1% agarose gel, visualized by ethidium bromide staining and the optical density (OD) of the bands was measured with Image Quant 5.2 Software (Molecular Diagnostics, San Francisco, CA, USA). The intensity of the bands was normalized for every sample, relative to the intensity of the respective GAPDH bands, according to the following formula: OD gene/OD GAPDH. For each gene, results are then expressed as the ratio pG-LN-1/LN-1.

**Detection of metalloproteinase activity**

Cells were grown for 30 min on plates precoated with laminin-1 or peptide G-treated laminin-1. Aliquots of cell supernatants were electrophoresed through a 10% SDS-PAGE gel polymerized with 0.1% gelatin (Sigma). Gels were washed three times for 20 min each in 2.5% Triton X-100 to remove SDS, and incubated overnight at 37°C in collagenase buffer containing 50 mM Tris–HCl (pH 7.5), 200 mM NaCl and 10 mM...
CaCl$_2$. Gelatinolytic activity was visualized as clear blue bands by staining with 0.5% Coomassie Blue and destaining with 10% methanol and 5% acetic acid. Previous data were confirmed using the MMP gelatinase activity assay kit (Chemicon International, Inc.) which is based on cleavage of biotinylated gelatinase substrate by active MMP-2 enzyme. The remaining biotinylated fragments were added to biotin-binding 96-well plates and detected with streptavidin enzyme complex. Addition of enzyme substrate results in a colored product, detectable based on optical density at 450 nm.

**Statistics**

Data are expressed as the means ± S.D. of three independent experiments. Statistical analysis was performed using an unpaired Student’s t-test. P values of <0.05 were considered statistically significant.

**Results**

**Effect of 67LR-treated laminin-1 on tumor cell morphology and aggressiveness**

MDAMB231 human breast carcinoma cells plated on laminin-1 untreated or treated with the 67LR mimic peptide G were analyzed for morphology after fixation and staining with FITC-phalloidin. Cells plated on native laminin-1 presented the actin bundles organized as multiple cables parallel to margins (Fig. 1B), whereas cells plated on peptide G-modified laminin-1 exhibited a polygonal array of filament bundles (Fig. 1C). Cells plated on peptide X-treated laminin-1 or uncoated substrate presented an elongated-shaped and round morphology respectively (Fig. 1D and A). To investigate whether 67LR shed from tumor cells had effects on the actin infrastructure similar to those induced by peptide G-modified laminin-1, MDAMB231cells — which are heterogeneous for the expression of monomeric laminin receptor — were separated according to 67LR expression. By cytofluorimetric analysis, two cell subpopulations were found to display a different expression of the 67LR with a mean fluorescence intensity of 328 ± 166 (high 67LR) and 67 ± 177 (low 67LR). After incubation on native laminin-1 for 6h, high 67LR-expressing cells presented the actin bundles organized as cells plated on peptide G-modified laminin-1 (Fig. 1E). On the other hand, cells presenting a low level of 67LR showed an elongated morphology similar to that of cells plated on native laminin-1 (Fig. 1F). Confocal microscopic analysis of cells plated on native laminin-1 revealed α6 integrin subunit, the canonical laminin-1 receptor, and 67LR concentrated at the tips of the cellular extensions co-localized in adhesion plaques as identified by talin labeling (Fig. 2A), whereas in cells seeded on peptide G-treated laminin-1, 67LR was distinct from the α6 subunit in filopodia, especially at the root of such protrusions, in addition to co-localizing with α6 integrin subunit in focal adhesion plaques (Fig. 2B and C). These results point to the different behavior of cells interacting with 67LR-modified laminin-1 as compared with cells interacting with the unmodified molecule.

To determine whether 67LR-modified laminin-1 affects activities related to tumor aggressiveness, in vitro cell adhesion and migration in the presence of peptide G-treated or untreated laminin-1 were analyzed. MDAMB231 cells seeded in wells containing native or peptide G-treated laminin-1 showed a significant increase in adhesion compared with cells seeded on uncoated matrix; a small but statistically significant increase in adhesion was observed on the modified vs native laminin-1 (P = 0.006). Cells incubated in the presence of peptide X-treated laminin-1 showed adhesion comparable with that of native laminin-1 while cells plated in the presence of peptide X or G displayed an adhesion superimposable on that observed for control (Fig. 3A). By ELISA analysis with anti-laminin-1 antibodies, results for binding of peptide G-treated laminin-1 to the plastic substrate were similar to those of untreated or peptide X-treated laminin-1 (Fig. 3B). When MDAMB231 cells separated according to 67LR expression were seeded for 6h on native laminin-1 a significant increase in adhesion (P < 0.0001) was found in high 67LR-expressing cells compared with cells presenting a low level of the laminin-1 receptor. Chemotaxis assay to quantitate the effect of 67LR-induced conformational change on laminin-1-stimulated cell motility revealed a 2.5-fold or 3-fold increase in MDAMB231 cell motility when native or peptide G-treated laminin-1, respectively, was used as chemoattractant (Fig. 3C). The difference in motility between unmodified and peptide G-modified laminin-1 was quite small but still statistically significant (P = 0.02). Cells in the presence of both peptides X and G alone or peptide X-treated laminin-1 showed migration comparable with that of cells without chemo-attractant or with native laminin-1. MDAMB231 cells separated according to 67LR expression and allowed to migrate for 24h using native laminin-1 as chemotactant, revealed that only those presenting high levels of 67LR increased their migration compared with cells expressing low levels, although this difference did not reach statistical significance (Fig. 3C). Because the invasive phenotype consists of coordinated attachment, detachment, migration and matrix
degradation, we analyzed the effect of 67LR-induced conformational change in laminin-1 on the ability of the tumor cells to penetrate the basement membrane. MDAMB231 cells seeded in invasion chambers containing a membrane coated with a uniform layer of basement membrane (Matrigel) migrated within 6 h when the peptide G-modified laminin-1 was present in the lower chamber \((P=0.035)\), whereas migration of these cells through the membrane in the presence of native laminin-1 was comparable with that in the absence of chemoattractant (Fig. 4A). However, extending incubation time with native laminin-1 was found to induce basement membrane penetration related to expression of 67LR (Fig. 4A) \((P=0.04\), high 67LR vs low 67LR). Furthermore, cells pretreated with modified laminin-1 for 30 min before invasion assay in the absence of chemoattractant revealed a 6-fold increase in invasion as compared with cells pretreated with

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**Figure 1** Morphology of breast carcinoma cells seeded on different laminin forms. MDAMB231 cells plated for 1 h on glass coverslips left uncoated (A) or coated with LN-1 (B), pG-LN-1 (C) or pX-LN-1 (D). MDAMB231 cells expressing high (E) or low (F) levels of 67LR seeded on LN-1 for 6 h. After fixation, cells were stained with FITC-phalloidin, and viewed using a fluorescence microscope. Photographs are representative of different actin stainings obtained in three separate experiments. The percentages of cells presenting the different morphologies were: 76 ± 1.4% (B), 67 ± 4.3% (C), 71 ± 3% (D), 65 ± 2% (E) and 77 ± 0.5% (F). Scale bars, 10 μm.
unmodified laminin-1 \( (P=0.005) \) (Fig. 4B) suggesting that laminin-1 modified by interaction with the 67LR provides a message that promotes invasion.

**Effect of 67LR-treated laminin-1 on gene expression in breast cancer cells**

To examine the signal derived from 67LR-modified laminin-1, MDAMB231 cells plated on native or peptide G-modified laminin-1 for 30 min were analyzed for gene expression by cDNA superarray, including genes for cell adhesion molecules, ECM proteins, proteases and their inhibitors. Arrays were hybridized with \( ^{32} \)P-labeled cDNA probes generated from RNA preparations of MDAMB231 cells seeded on untreated or peptide G-treated laminin-1. Spot intensities were normalized relative to expression levels of four housekeeping genes. For each gene, values corresponding to two different treatments were examined. Comparative analysis of values corresponding to each gene revealed differential expression for 19 genes (Table 1) presenting higher variation than that observed for housekeeping genes (1.6-fold). Seventeen of these genes were up-modulated in MDAMB231 cells seeded on peptide G-modified laminin-1 compared with cells treated with unmodified laminin-1, which presented up-modulation of only two ECM proteins: vitronectin and osteopontin. Cells plated on peptide G-treated laminin-1 showed overexpression of 12\% (7/58) of ECM proteins and adhesion molecules vs 26\% (10/38) of proteases and their regulators. In particular, all \( \alpha \)-subunits of integrins (1, 3 and 5) as well as catenin \( \delta 1 \), ECM1, fibronectin and laminin B1; and MMPs such as stromelysin-3, MMP-8, MMP-7 and MT1-MMP, as well as the cysteine proteases cathepsin L and cystatin C; and the serine protease tPA were dramatically overexpressed in modified laminin-1-seeded cells. Expression of molecules involved in regulation of proteolytic enzymes such as PAI-1, TIMP-1 and -2 was also augmented.

Microarray results were verified for some selected genes by RT-PCR analysis (Fig. 5A) with similar results even if the fold-change in mRNA levels that resulted was lower compared with array analysis (Fig. 5B). TIMP-2, cathepsin C, ECM1, stromelysin-3, \( \alpha1 \) and \( \alpha3 \) integrin subunits were more transcribed in cells plated on peptide G-modified laminin compared with cells plated on native laminin-1, whereas vitronectin had higher expression levels in cells plated on unmodified vs modified laminin-1. The MMP-2, whose expression was not found to change in microarray analyses, was equally transcribed in cells exposed to 67LR-modified or native laminin-1.

Because MT1-MMP and TIMP-2, the two molecules involved in MMP-2 activation, were both up-modulated in cells seeded on peptide G-treated laminin-1, the activity of this metalloproteinase was further investigated by zymographic analysis of the culture supernatant from these cells; the clear band corresponding to activated MMP-2 was more notable compared with cells seeded on native laminin-1, whereas levels of pro-enzyme did not change in any condition (Fig. 6A). Accordingly, using the MMP gelatinase activity assay kit, MDAMB231 cells showed...
a significant ($P=0.01$) increase in MMP-2 gelatinolytic activity when exposed to peptide G-modified vs native laminin-1 (Fig. 6B). To determine the relevance of increased MMP-2 activity in the invasiveness of cells pretreated with modified laminin-1, invasion of MDAMB231 cells was assayed in the presence or absence of the MMP inhibitor TIMP-2. Addition of such an inhibitor reduced the migration of these cells through the membrane by 33%, while the same treatment reduced the invasiveness of cells exposed to native laminin-1 by only 7% ($P=0.0003$). Moreover, TIMP-2-mediated inhibition of invasiveness was stronger in cells expressing high levels (23%) rather than low levels (13%) of 67LR ($P=0.014$) (Fig. 6C).

**Discussion**

The present study demonstrates that the interaction of 67LR-modified laminin-1 with tumor cells enhances their ability to invade and increases expression and activation of proteolytic enzymes and their regulators. As indicated by morphological analysis, MDAMB231 breast carcinoma cells line grown on peptide G-treated laminin-1 exhibited actin-containing structures different from those formed in the presence of native laminin-1. Specifically, the former cells developed filopodia, extensions by which cells establish dynamic adhesion and motility on the substratum where they are shaped (O’Connor *et al.*, 1990, Mitchison & Cramer 1996, Adams 2001). The functional involvement of 67LR in the dynamics of these actin-containing motility structures is also supported by the localization of this

adsorbed on a 96-well plate. After 6 h plates were filled with PBS, inverted and shaken in a tank of PBS for 15 min. Control represents adhesion of cells plated on uncoated substrate. (B) Adhesion of LN-1, pG-LN-1 or pX-LN-1 to culture substrates detected using anti-laminin MAb followed by anti-mouse Ig HPR-conjugated antibody together with TMB as substrate and H2SO4 for arresting the enzymatic reaction. The immune complexes were evaluated spectrophotometrically at 450 nm using an automated ELISA reader. Control represents uncoated wells. (C) Migration of MDAMB231 cells on LN-1, pG-LN-1, pX-LN-1, pG or pX. After 4 h migrating cells were evaluated using a CyQuant GR dye solution together with a lysis buffer. High or low 67LR-expressing MDAMB231 cells were allowed to migrate for 24 h using LN-1 as chemoattractant. Control represents migration of cells in absence of any chemoattractant. All experiments were repeated three times; error bars represent standard deviations of the means calculated in separate experiments; *, statistically significant ($P<0.05$) as determined using Student’s $t$-test.

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*Figure 3 Effect of peptide G-modified laminin-1 on tumor cell adhesion and migration. (A) Adhesion of MDAMB231 cells on LN-1, pG-LN-1, pX-LN-1, pG or pX adsorbed on a 96-well plate overnight at 37 °C. After 1 h plates were filled with PBS, inverted and shaken in a tank of PBS for 15 min. High or low 67LR-expressing MDAMB231 cells were seeded on LN-1*
receptor in filopodia distinct from that of the canonical laminin-1 receptor a6 integrin subunit with which the monomeric laminin-1 receptor reportedly colocalizes in focal adhesion plaques (Starkey et al. 1999). The small differences between unmodified and 67LR-modified laminin-1 in two steps of cell motility, i.e. adhesion and migration, do not explain the mechanism by which 67LR is associated with tumor invasiveness. By contrast, the enhanced ability, compared with the native adhesion molecule, of tumor cells induced by modified laminin-1 to traverse a Matrigel basement suggests that the invasive potential upon interaction with laminin-1 conformationally modified by 67LR underlies the high metastatic behaviour of tumors overexpressing the monomeric laminin-1 receptor.

The highly increased production of proteolytic enzymes by tumor cells treated with 67LR-modified laminin-1 compared with native laminin supports this hypothesis. Indeed, of the 24 integrin subunits and 19 non-integrin cell adhesion molecules investigated in microarray analysis, only 3 (12%) and 1 (5%), respectively, showed a change. By contrast, 10 of 38 (26%) proteases and their regulators were up-modulated by cell binding to 67LR-treated laminin-1. Specifically, expression was enhanced in 26% of MMPs, 20% of serine proteases, 40% of cysteine proteases and 50% of molecules involved in their regulation — such as TIMPs and PAI-1 — with consequent modulation of enzyme activation. Consistent with these findings, Givant-Horwitz et al. (2004) recently reported the down-modulation of MMP-2 collagenolytic activity in A375SM melanoma cells transfected with a monomeric laminin receptor precursor antisense construct.

The a3 integrin subunit was among the receptor molecules up-modulated in cells seeded on 67LR-treated laminin-1. This integrin usually does not participate significantly in laminin-1 binding to the cell surface, but does act in laminin-5 binding (Carter et al. 1990). Thus, up-modulation of the a3 subunit implies an increased probability of interaction with laminin-5, known to be implicated in tumor cell invasion (Shang et al. 2001, Wang et al. 2004). The similar degree of up-modulation for both a5 and its ligand fibronectin (Robinson et al. 2004) points to the ability of tumor cells interacting with 67LR-treated laminin-1 to modify the composition of the ECM as well as the ability to interact with it. A similar speculation can be made for the a1 and laminin B1 chain, both of which presented 4- to 5-fold expression up-modulation. Moreover, a1 is involved in protrusive cell-matrix contacts, such as filopodia, which are usually associated with rapid membrane remodelling, transient matrix adhesions and exploratory cell movements (Gardner et al. 1996, Hynes 2002). The 67LR-induced membrane protrusions with a matrix degradation activity suggest that expression of genes with cytoskeletal function might also be modulated by peptide G-modified

Figure 4 Invasion of MDA MB231 cells. Cell migration in vitro was measured using Matrigel as an ECM barrier in modified Boyden chambers with LN-1, pG-LN-1, pX-LN-1, pG or pX as chemoattractants (A) or as pretreatment agents (B). After 6h, filters were fixed and stained with crystal violet and viewed under light microscope with ×20 magnification. Cells migrating to the underside were counted in four microscopic fields. High or low 67LR-expressing MDA MB231 cells were allowed to migrate for 24h using LN-1 as chemoattractant. Migrating cells were fixed, stained and counted as described above. Controls represent migration of cells in the absence of any chemoattractant or following any pretreatment. All experiments were repeated three times; error bars represent standard deviations of the means calculated in separate experiments; *, statistically significant (P<0.05) as determined using Student’s t-Test.
laminin-1 even though RT-PCR analyses did not reveal differences in tropomyosin expression in cells stimulated with treated vs untreated laminin-1 (data not shown).

The supposition of induction by 67LR of a ‘different’ matrix is strengthened by the reduction of vitronectin and osteopontin, two cell adhesion molecules whose overexpression has been linked to differentiation and tumor progression respectively (Senger et al. 1983, Panda et al. 1997, Aaboe et al. 2003).

However, even cell–cell interaction was affected by 67LR-modified laminin-1, as indicated by the significantly increased expression of catenin δ1, a component of the adherens junction and with a role in cell motility. Indeed, ectopic expression of this molecule alters cell morphology, inducing the elaboration of lamellipodia and increasing the scattering response to hepatocyte growth factor treatment (Lu et al. 1999).

Consistent with the reported association between 67LR expression and tumor high neovascularization (Gasparini et al. 1995, Gebarowska et al. 2002), we found that laminin modified by interaction with the monomeric receptor strongly increases the expression of ECM1, a secreted glycoprotein implicated in cell proliferation, angiogenesis and differentiation. In primary breast carcinomas, ECM1 expression has been correlated with tumor metastatic properties (Wang et al. 2003). The most direct evidence for the role of ECM1 in angiogenesis is the finding that purified recombinant ECM1 stimulates the proliferation of cultured endothelial cells and promotes blood vessel formation in the chorioallantoic membrane of chicken embryos (Han et al. 2001). Because growth and metastasis are critically dependent on the formation of new vessels, and because 67LR is also overexpressed in endothelial cells during neoangiogenesis (Stitt et al. 1998, Tanaka et al. 2000, McKenna et al. 2001), laminin-1 modification induced by the monomeric laminin receptor might also support tumor progression through the induction of angiogenesis.

### Table 1 Peptide G-modified laminin-1-dependent genes differentially expressed by MBAMB231 cells

<table>
<thead>
<tr>
<th>Genebank</th>
<th>Description</th>
<th>Gene name</th>
<th>pG-LN-1/LN-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>M59911</td>
<td>Integrin, α3 (antigen CD49C, α3 subunit of VLA-3 receptor)</td>
<td>Integrin α3</td>
<td>14.11</td>
</tr>
<tr>
<td>X68742</td>
<td>Integrin, α1</td>
<td>Integrin α1</td>
<td>5.60</td>
</tr>
<tr>
<td>AF062343</td>
<td>Catenin (cadherin-associated protein), δ1</td>
<td>Catenin δ1</td>
<td>3.25</td>
</tr>
<tr>
<td>X06256</td>
<td>Integrin, α5 (fibronectin receptor, α polypeptide)</td>
<td>Integrin α5</td>
<td>3.19</td>
</tr>
<tr>
<td>NM_004425</td>
<td><em>Homo sapiens</em> extracellular matrix protein 1 (ECM1), transcript variant 1</td>
<td>ECM1</td>
<td>10.96</td>
</tr>
<tr>
<td>M61916</td>
<td>Laminin B1 chain</td>
<td>Laminin B1</td>
<td>4.06</td>
</tr>
<tr>
<td>X02761</td>
<td>Fibronectin 1</td>
<td>Fibronectin-1</td>
<td>2.03</td>
</tr>
<tr>
<td>X03168</td>
<td>Vitronectin (serum spreading factor, somatomedin B, complement S-protein)</td>
<td>Vitronectin</td>
<td>0.25</td>
</tr>
<tr>
<td>M83248</td>
<td><em>Homo sapiens</em> secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1) (SPP1),</td>
<td>Osteopontin</td>
<td>0.14</td>
</tr>
<tr>
<td>X57766</td>
<td>Human stromelysin-3</td>
<td>Stromelysin-3</td>
<td>5.50</td>
</tr>
<tr>
<td>NM_002424</td>
<td>Matrix metalloproteinase 8 (neutrophil collagenase)</td>
<td>Neutrophil collagenase</td>
<td>3.27</td>
</tr>
<tr>
<td>X07869</td>
<td>Matrix metalloproteinase 7 (matriksin, uterine)</td>
<td>Matriksin, uterine</td>
<td>2.70</td>
</tr>
<tr>
<td>D26512</td>
<td><em>Homo sapiens</em> mRNA for membrane-type matrix metalloproteinase 1</td>
<td>MT1-MMP</td>
<td>2.15</td>
</tr>
<tr>
<td>NM_000930</td>
<td><em>Homo sapiens</em> plasminogen activator, tissue (PLATa)</td>
<td>tPA</td>
<td>2.10</td>
</tr>
<tr>
<td>NM_000099</td>
<td><em>Homo sapiens</em> cystatin C (amyloid angiopathy and cerebral hemorrhage) (CST3)</td>
<td>Cystatin C</td>
<td>1.85</td>
</tr>
<tr>
<td>X12451</td>
<td>Cathepsin L</td>
<td>Cathepsin L</td>
<td>1.79</td>
</tr>
<tr>
<td>M16006</td>
<td>Plasminogen activator inhibitor, type 1 (PAI-1)</td>
<td>PAI-1</td>
<td>4.79</td>
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<td>NM_003255</td>
<td>Tissue inhibitor of metalloproteinase 2</td>
<td>TIMP-2</td>
<td>3.14</td>
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<tr>
<td>NM_003254</td>
<td>Tissue inhibitor of metalloproteinase 1 (erythroid potentiating activity, collagenase inhibitor)</td>
<td>TIMP-1</td>
<td>2.27</td>
</tr>
</tbody>
</table>
Most of the proteolytic enzymes up-regulated by modified laminin-1 interaction such as cystatin-C, cathepsin L and tPA might participate in ECM degradation (Birkedal-Hansen 1995, Fata et al. 2004), but the metalloproteinases appear to play the major role (Egeblad & Werb 2002). Enzymes such as MT1-MMP, MMP-7 and -8 which were considerably up-modulated have been widely described to cleave collagen, laminin and fibronectin respectively, and to be highly involved in tumor invasion mechanisms. The need for a delicate balance between proteases and their inhibitors in each phase of the migration process might provide a functional explanation for the up-regulation of TIMPs and PAI-1 in the same cells stimulated by modified laminin-1. The changes in expression of such MMP regulators explain the significant increase of MMP-2 activation upon treatment with 67LR-modified laminin-1 without mRNA modification and suggest that 67LR-enhanced invasiveness is strongly dependent on MMP-2 activation. The role of this protease is proved by TIMP-2 inhibition experiments. The synthesis and activation of different proteases induce physical alterations of ECM proteins, which expose cryptic domains (Ardini et al. 2002, Khan et al. 2002, Palmieri et al. 2003), and facilitate the activation of different signal transduction molecules that regulate cytoskeleton arrangement and cellular response in pathological conditions.

Figure 5 (A) Validation of microarray results for selected genes by RT-PCR. Comparison of expression analyses in cells seeded on LN-1 or pG-LN-1 of transcripts for α3 and α1 integrin subunits, ECM1, vitronectin, stromelysin 3, cystatin C, TIMP-2, MMP-2. GAPDH cDNA was amplified in parallel to evaluate the efficiency of RNA extraction and cDNA synthesis. (B) Comparative expression levels of a panel of eight genes assayed by RT-PCR and microarray.
superarray data were confirmed by RT-PCR. The lower ratio of PCR assay compared with array results can be explained by different saturation levels of the two methodologies.

Together, our results on the interaction of tumor cells with 67LR-modified laminin-1 to enhance tumor cell invasion, point to a mechanism by which the role of 67LR in tumor cell dissemination stems from its ability to modify laminin-1. This conclusion is strengthened by data obtained investigating the effects induced by native laminin-1 in tumor cells expressing high or low levels of 67LR. Indeed, alterations in actin organization as well as adhesion, migration and especially invasion were found in 67LR highly positive cells but not in low-positive cells when these cells were seeded for extensive periods on unmodified laminin-1. As the 67LR shedding is proportional to its expression level (Karpatová et al. 1996, Starkey et al. 1999), these findings support a role for 67LR in changing laminin-1 structure and consequently in promoting the metastatic phenotype.

Therefore, this laminin-1 receptor appears to play a prominent role in the continually evolving interactions between neoplastic cells and the ECM that are essential for tumor invasion and metastasis.

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Figure 6 Analysis of MMP-2 activity and its involvement in cell invasion. (A) Conditioned medium of MDA MB231 cells seeded on plates left uncoated (control), or coated with LN-1 or pG-LN-1 for 30 min, was assessed for gelatinase activity by zymography. (B) MMP-2 activity was also tested with a gelatinase activity assay kit that utilizes a biotinylated gelatinase substrate, which is cleaved by active MMP-2 enzyme. The remaining biotinylated fragments are then added to biotin-binding 96-well plates and detected with streptavidin-enzyme complex. Addition of enzyme substrate results in a colored product, detected based on optical density at 450 nm. Control represents supernatant of cells plated on uncoated substrate. Error bars represent standard deviations of the means calculated in separate experiments; *, statistically significant (P<0.05) as determined using Student’s t-test. (C) Inhibition of invasion by TIMP-2 in MDA MB231 cells pretreated with LN-1 or pG-LN-1 and in MDA MB231 cells selected for high or low level of 67LR expression.
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