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Peptide G, Containing the Binding Site of the 67-kDa Laminin Receptor, Increases and Stabilizes Laminin Binding to Cancer Cells*

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We investigated the effect of peptide G, a synthetic peptide derived from the sequence of the 37-kDa laminin receptor precursor, on the interaction of laminin in two tumor cell lines one of which produces laminin and one of which does not. Addition of peptide G to the culture medium induced a significant increase in the amount of endogenous laminin detectable on the cell membrane of both cell lines. Moreover, pretreatment of exogenous laminin with peptide G dramatically increased laminin binding on both cell lines. Kinetics analysis of membrane-bound labeled laminin revealed a 3-fold decrease in the k_d of peptide G-treated laminin compared with untreated or unrelated or scrambled peptide-treated laminin. Moreover, the affinity constant of peptide G-treated laminin increased 2-fold, with a doubling of the number of laminin binding sites, as determined by Scatchard analysis. Expression of the VLA6 integrin receptor on the cell membrane increased after incubation with peptide G-treated laminin. However, the lower binding inhibition of peptide G-treated laminin after anti-VLA6 antibody or cation chelation treatment indicates that membrane molecules in addition to integrin receptors are involved in the recognition of peptide G-modified laminin. These "new" laminin-binding proteins also mediated cell adhesion to laminin, the first step in tumor invasion. Together, the data suggest that peptide G increases and stabilizes laminin binding on tumor cells, involving surface receptors that normally do not take part in this interaction. This might explain the abundant clinical and experimental data suggesting a key role for the 67-kDa laminin receptor in the interaction between cancer cells and the basement membrane glycoprotein laminin during tumor invasion and metastasis.

The complex process of tumor invasion and metastasis is a cascade of sequential steps in which interactions between cancer cells and laminin, the major glycoprotein of the basal membrane, play a critical role (1–3). These interactions are mediated by different cell surface molecules, including heterodimeric integrins (4–8) and various monomeric receptors, such as the 67-kDa laminin receptor (67LR)[1] (9–12). Several studies to elucidate this receptor's role in tumor progression have clearly demonstrated an increase in 67LR expression in tumors compared with normal tissues (13, 14) and a correlation between 67LR expression, invasive phenotype of the tumor (15), and poor prognosis (16, 17).

cDNA clones of the 67LR have been isolated from various vertebrate sources, including human, mouse, hamster, bovine, and rat (18–22). The full-length clones encode a 295-amino acid polypeptide with a molecular mass of 32 kDa (18, 19). The nascent translation product (37LRP) of hybridized human laminin receptor mRNA has an apparent molecular mass of 37 kDa on SDS-polyacrylamide gel electrophoresis, and pulse-chase experiments have revealed that the 37-kDa polypeptide is a precursor of the mature 67LR (23). A 20-amino acid synthetic peptide derived from the 37LRP sequence, designated peptide G, was found to bind to laminin (9). *In vitro*, this peptide eluted the 67LR from a laminin affinity column (9), and its interaction with laminin was found to be heparin-dependent (25). *In vivo*, peptide G significantly increased the metastatic potential of melanoma cells (26). These findings, together with the observations of high metastatic potential of laminin-coated tumor cells (27, 28), suggest the relevance of interactions between the 67LR expressed on the tumor cell surface, integrin receptors, and laminin in the metastatic process. Recently, coexpression of the 67LR and the integrin laminin receptor VLA6 on small cell lung cancer cells was demonstrated (29), as well as their colocalization in the same cytoplasmic compartment and on the cell membrane as determined by immunoelectronmicroscopy (30). After exposure of the cells to laminin, a number of these cytoplasmic molecules translocated from the cytoplasm to the plasma membrane (30).

In light of these data, we investigated the role of the 67LR in the interaction of laminin with the tumor cell surface using peptide G. We found that peptide G stabilized laminin binding to tumor cells by increasing the number of laminin binding receptors and by decreasing the k_d of laminin from the cell surface.

MATERIALS AND METHODS

Reagents—Peptide G (IPCNNKGAHSVGLMWWMLAR), corresponding to the 161–180 region of the 37LRP; scrambled peptide GX (PMLRWGCHIAMVNKLSWGNA); and both hydrophobic and neutral peptide X (CEQKEENQGKNESNIKPVQTV) were obtained from Neosystem (Strasbourg, France) in N-terminal biotinylated form. High pressure liquid chromatography analysis showed 95% purity of pep-

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[1] The abbreviations used are: 67LR, 67-kDa laminin receptor; VLA6, very late antigen-6; 37LRP, 37-kDa laminin receptor precursor; BSA, bovine serum albumin; PBS, phosphate-buffered saline; HLA, human leukocyte antigen; mAb, monoclonal antibody.

tides G and X and 85% purity of peptide GX. Peptides were dissolved in distilled water and centrifuged, and the concentration was evaluated by densitometry. Whereas peptide G and X were easily dissolved, peptide GX was rather insoluble and 60% was lost after centrifugation. Peptides were stored at −20 °C at 1 mg/ml concentration. Laminin purified from the mouse Engelbreth-Holm-Swarm tumor was kindly provided by Dr. G. Taraboletti (Mario Negri Institute for Pharmacological Research, Bergamo, Italy). After iodination to a specific activity of 6 μCi/μg using the lactoperoxidase method (31), laminin (50 μg) was treated with peptide G, X, or GX (50 μg each) in a final volume of 1 ml for 30 min at 37 °C. Human placenta collagen was obtained from Sigma.

Cell Lines—Vulvar epidermoid carcinoma A431 and small cell lung carcinoma N592 human cell lines were provided by American Type Culture Collection (Rockville, MD). Cell lines were maintained in RPMI 1640 (Sigma,) supplemented with 10% fetal calf serum (Sigma), L-glutamine, and antibiotics.

Solid-phase Binding Assay—Microwells of a 96-well polyvinyl chloride plate (Becton Dickinson Labware, Oxnard, CA) were coated with 100 μl of laminin, collagen, or ovalbumin (10 μg/ml) in PBS and 0.01% of BSA for 2 h at 37 °C. After three washes with PBS, nonspecific binding sites were blocked with 1% BSA in PBS for 30 min at 37 °C. Biotinylated peptide G, X, or GX was added at 1 μg/ml in 100 μl PBS supplemented with 0.05% Tween 20 and 1% BSA (binding buffer), and wells were incubated for 2 h at 4 °C with gentle rocking, washed four times with binding buffer, and incubated further with ^{125}I-avidin (1×10^5 cpm/well; Amersham Corp.) for 1 h at room temperature. After being washed, wells were treated with 100 μl of 2 N NaOH for 20 min at room temperature, and the supernatant was assessed for radioactivity in a gamma counter. All experiments were performed in triplicate. Specific cpm was calculated as the difference between cpm bound to laminin or collagen and cpm bound to ovalbumin.

Flow Cytometric Analysis—Indirect immunofluorescence was used to analyze the binding of biotinylated peptides, the presence of endogenous or exogenous laminin, and expression of the α6 integrin subunit and human leukocyte antigen (HLA). Live cells (3×10^5), treated with murine laminin (50 μg/ml) or untreated, were incubated with biotinylated peptides at 37 °C for 30 min. After three washings, cells were assayed for bound peptide G, GX, or X with fluorescein-labeled streptavidin (5 μg/ml; Amersham Corp.). To analyze endogenous laminin production on the cell surface, A431 cells (3×10^5) treated for 30 min with peptides or untreated were incubated with a rabbit polyclonal serum directed against human laminin (1:100) (Telios Pharmaceuticals, San Diego, CA), washed three times, incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit Ig (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) for 30 min at 0 °C, washed three times, and analyzed. Murine laminin, treated with peptides (50, 25, 12.5, 6, 3, and 1.5 μg/ml) or untreated, was incubated with cells for 30 min at 37 °C and detected using a rabbit polyclonal serum directed against murine laminin (1:200; Telios) and fluorescein isothiocyanate-conjugated goat anti-rabbit Ig.

Expression of the α6 integrin subunit and HLA under various conditions was studied by incubation with purified mAbs, MAR6 (32) and W6/32, directed against the α6 subunit and against a monomorphic epitope on the 45-kDa polypeptide products of the HLA A, B, and C loci (Coulter Immunology, Hialeah, FL), respectively (10 μg/ml mAbs), followed by fluorescein isothiocyanate-conjugated goat anti-mouse Ig. Labeled cells were analyzed using a FACScan flow cytometer with LYSYS II software (Becton Dickinson, Mountain View, CA). Each experiment was repeated at least three times, with highly reproducible results. Staining of cells with fluorescein isothiocyanate-conjugated goat anti-rabbit Ig, fluorescein isothiocyanate goat anti-mouse Ig, or fluorescein-labeled streptavidin was performed to determine background values.

Dissociation of ^{125}I-Laminin from the Cell Surface—A431 and N592 cells (2×10^5 cells/sample) were incubated in complete culture medium for 30 min at 37 °C with ^{125}I-laminin (10^6 cpm/sample) alone or complexed with peptide G or peptide X, washed five times, incubated at 37 °C, and tested at different times (from 0 to 720 min) for residual radioactivity. Cell viability after a 720-min incubation was more than 90% as determined by trypan blue exclusion staining. Bound laminin was calculated as a percentage of the initial binding. Results are given as the average of triplicate determinations; counts in each experiment differed by <10%.

The k_d was calculated as follows: $\log_e(X_0/X_1) = k_d \times t_1$, where X_0 = bound radioactivity at 0 min and X_1 = bound radioactivity at 720 min (t_1).

Scatchard Analysis—N592 cells (2×10^5 cells/sample) were incubated for 180 min at 0 °C with 100 μl of ^{125}I-laminin treated with peptide G or X or untreated, at different serial dilutions in the presence or absence of a 100-fold excess of unlabeled laminin. After four washings, cells were assessed for bound radioactivity in a γ counter. The number of binding sites per cell was extrapolated from the Scatchard plot, and the affinity constant (K) was evaluated as the slope of the regression curve (33).

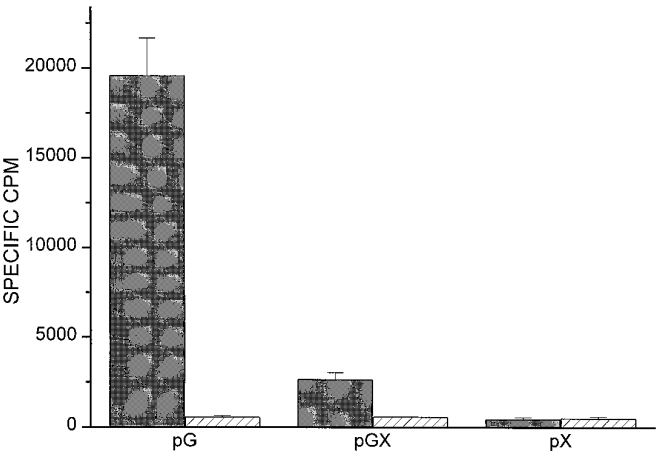

FIG. 1. **Synthetic peptide binding to immobilized laminin.** Microwells of a 96-well polyvinyl chloride plate were coated with 100 μl of laminin, collagen, or ovalbumin (10 μg/ml) in PBS and 0.01% of BSA for 2 h at 37 °C. After washing and blocking of nonspecific binding sites in wells with PBS and BSA (1%), N-terminal-biotinylated peptide G, GX, or X was added for 2 h at 4 °C at 1 μg/ml in 100 μl of PBS supplemented with 0.05% Tween 20 and 1% BSA. Peptide binding was monitored with ^{125}I-avidin (1×10^5 cpm/well). Specific cpm was calculated as the difference between mean cpm bound to laminin (*filled column*) or collagen (*hatched column*) and mean cpm bound to ovalbumin. *Bars* represent S.D.

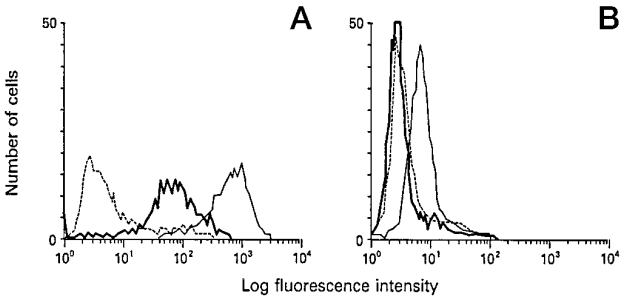

FIG. 2. **Peptide G binding to tumor cell lines.** A431 (*A*) and N592 cells (*B*), 3×10^5/sample, treated with murine laminin (50 μg/ml) (*light line*) or untreated (*bold line*) were incubated with peptide G (50 μg/ml) for 30 min at 37 °C. Bound peptide was monitored with fluorescein-labeled streptavidin (5 μg/ml). *Dotted lines* show background values.

Binding Assays of ^{125}I-Laminin—N592 cells (2×10^5 cells/sample) were incubated with 50 μl of iodinated laminin (10^6 cpm/sample) treated with peptide G and X or untreated. In one experiment, N592 cells were saturated with 50 μl of GOH3 mAb (200 μg/ml; Dako, Amsterdam, the Netherlands) directed against the laminin binding site of α6 integrin subunit, and were incubated for 30 min at 37 °C with labeled laminin treated with the peptides or untreated. After being washed, cells were assessed for bound radioactivity and the percent binding inhibition calculated as follows: 100 − (bound cpm to treated cells/bound cpm to control cells × 100). In a second experiment, N592 cells (2×10^5 cells/sample) were equilibrated with 80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, 10 μM EDTA, pH 7.4 (EDTA buffer) to deplete extracellular levels of divalent cations and incubated for 2 h at 37 °C with ^{125}I-laminin dissolved in complete PBS or EDTA buffer and treated with peptide G or X or untreated. After three washings, cells were assayed for bound radioactivity and percent binding inhibition was calculated as above.

Cell Adhesion—N592 cells, coated with 200 μg/ml of GOH3 mAb (Dako) or uncoated, were plated in 96-well plates at a density of 2×10^4 cells/well, alone or with 10 μg of laminin previously treated with peptide G or X (50 μg/ml) or untreated. After a 90-min incubation as described (29), followed by three washings, adherent cells were incubated for 4 h at 37 °C with 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl

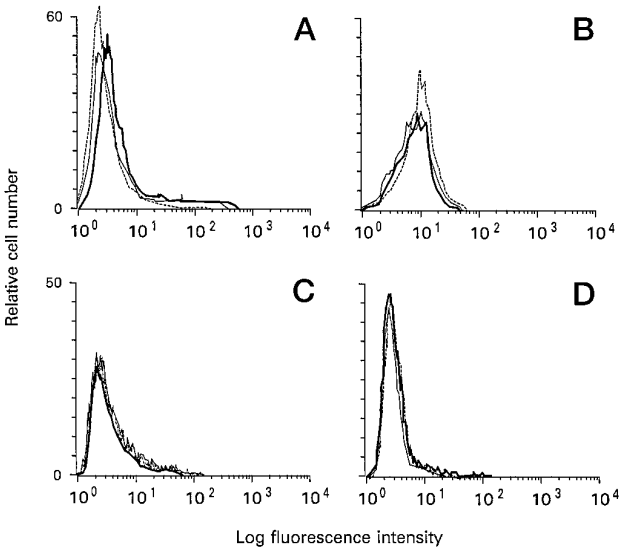

FIG. 3. **Peptide X and GX binding to tumor cell lines.** A431 (*A*) and N592 (*B*) cells, treated with murine laminin (*light line*) or untreated (*bold line*), were incubated with peptide X (*panels A* and *B*) or GX (*panels C* and *D*) in the conditions described in the legend to Fig. 2. *Dotted lines* show background values.

tetrazolium bromide (Sigma) dissolved in PBS at 5 μg/ml. Acid isopropyl alcohol (100 μl of 0.04 N HCl in isopropyl alcohol) was added to the wells and mixed thoroughly to dissolve the dark blue crystals for 5 min at room temperature. Plates were read on a Titertek Multiskan spectrophotometer (Flow Laboratories, Inc., McLean, VA) at a wavelength of 550 nm. Specific A_{550} was calculated as follows: A_{550} of treated sample $- A_{550}$ of control. Untreated N592 cells served as a control.

RESULTS

Binding of Synthetic Peptides to Laminin—The binding of N-terminal biotinylated peptides G, GX, and X to laminin was investigated in a solid-phase binding assay. Peptide G bound specifically to laminin but not to collagen, whereas neither peptide X nor peptide GX bound to either molecule (Fig. 1).

Cytofluorimetric analysis of peptide G binding to A431 tumor cells, which express laminin on the cell surface, and to N592 cells, which do not produce laminin, revealed positive and negative binding, respectively; treatment with exogenous laminin strongly increased the binding to A431 cells (Fig. 2*A*) and rendered N592 cells positive for peptide G binding (Fig. 2*B*). Peptide GX and peptide X binding were not detectable on either cell line even after laminin treatment (Fig. 3). Binding of peptide G, preincubated with laminin for 30 min at 37 °C before addition to tumor cells, was 10-fold higher in A431 cells and 100-fold higher in N592 cells (Fig. 4*A*) compared to peptide G binding on the respective cells coated with exogenous laminin (Fig. 4*B*).

Peptide G-induced Increase in Laminin Binding to Cells—The effect of peptide G on the binding of endogenous or exogenous laminin to the cell surface was investigated. A431 cells were treated for 24 h with 50 μg/ml peptide G, GX, or X and analyzed by cytofluorimetry for the amount of laminin present on the cell surface. As shown in Fig. 5, the amount of endogenous laminin detected on the cell membrane was 10-fold higher in peptide G-treated cells than in untreated cells. Peptide G treatment of N592 cells under the same conditions did not induce laminin detection (data not shown). Both A431 and N592 cells were able to bind exogenous laminin, but laminin pretreatment with peptide G increased this binding by 30- and 100-fold, respectively, whereas peptides X and GX had no such effect (Fig. 6). The peptide G-induced increase in binding of murine laminin was concentration-dependent; titration of peptide G from 50 to 1 μg/ml indicated that 3 μg/ml, corresponding to a 30-fold excess of peptide, doubled the levels of laminin bound to A431 cells.

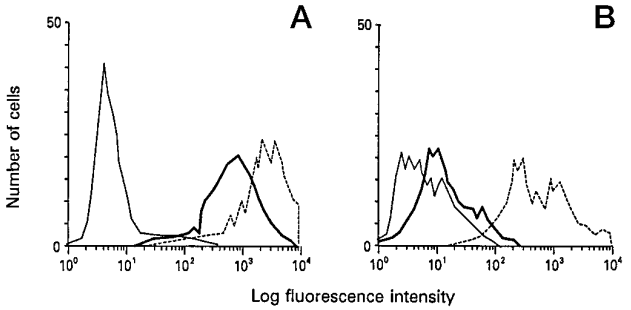

FIG. 4. **Binding of peptide G, alone or complexed with laminin, to tumor cell lines.** Peptide G (50 μg/ml) was added to A431 (*A*) and N592 (*B*) cells (3×10^5 cells/sample) for 30 min at 37 °C in the presence of exogenous laminin in suspension (*dotted line*) or in the presence of exogenous laminin coated onto the cell surface (*bold line*). Bound peptide was monitored with fluorescein-labeled streptavidin (5 μg/ml). *Light lines* show background values.

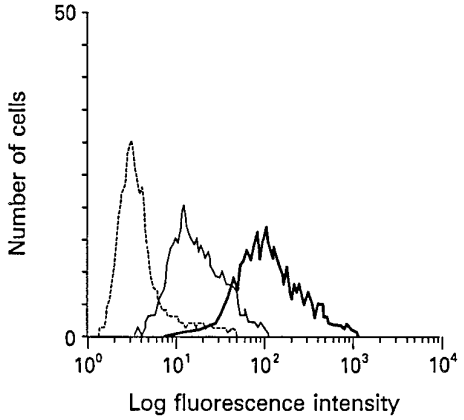

FIG. 5. **Increased endogenous laminin expression by tumor cells after treatment with peptide G.** A431 cells were treated with peptide G (50 μg/ml) for 24 h at 37 °C (*bold line*) or untreated (*light line*). Endogenous laminin on the cell surface was measured by indirect immunofluorescence using a rabbit polyclonal serum directed against human laminin (1:100) and fluorescein isothiocyanate-conjugated goat anti-rabbit Ig. *Dotted lines* show background values.

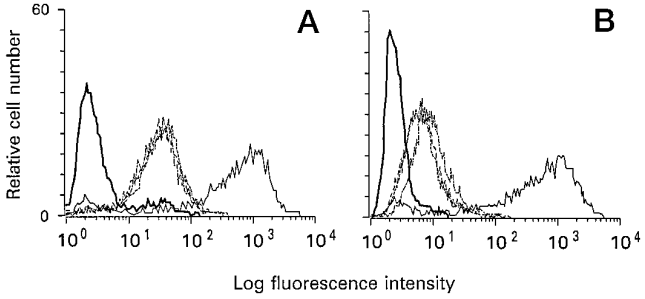

FIG. 6. **Increased cell surface binding of exogenous laminin in the presence of peptide G.** A431 (*A*) and N592 (*B*) cells were treated for 30 min at 37 °C with murine laminin, alone (*dashed line*) or preincubated with peptide G (*light line*), peptide X (*dotted line*), or peptide GX (*dashed-dotted line*) at 50 μg/ml. Exogenous laminin was detected by indirect immunofluorescence using a rabbit polyclonal serum directed against murine laminin (1:200) and fluorescein isothiocyanate goat anti-rabbit Ig. *Bold lines* show background values.

Binding Affinity of Membrane-bound Laminin—To investigate the effect of peptide G treatment on laminin binding affinity, the time course of dissociation of membrane-bound peptide-treated ^{125}I-laminin was analyzed in N592 and A431 cells. After 12 h, A431 cells released 50% of labeled laminin treated with peptide X ($k_d = 13.8 \times 10^{-6}$ s^{-1}) or untreated

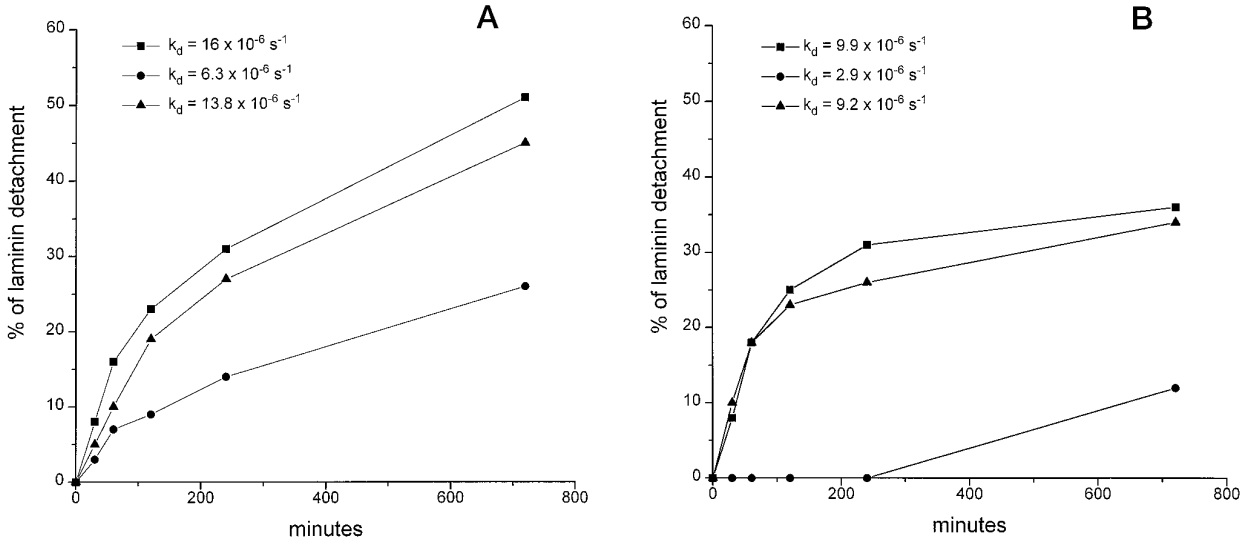

FIG. 7. **Stabilization of ^{125}I-laminin binding to tumor cells by peptide G treatment.** A431 (*A*) and N592 (*B*) cells, 2×10^5/sample, were incubated for 30 min at 37 °C with ^{125}I-laminin (10^6 cpm/sample), untreated (■) or treated with peptide G (●) or peptide X (▲). After five washings, cells were assessed for bound radioactivity in a γ counter at different times. Bound laminin is reported as a percentage of the initial binding. k_d values were calculated as follows: $k_d = \log_e(X_0/X_1)\cdot 1/t_1$ where X_0 and X_1 are bound radioactivity at 0 and 720 min, respectively.

laminin ($k_d = 16 \times 10^{-6}$ s^{-1}) into the supernatant, whereas peptide G-treated laminin dissociation was only 25% ($k_d = 6.3 \times 10^{-6}$ s^{-1}) (Fig. 7*A*). In N592 cells, the peptide-induced effects were even more pronounced, with only 12% of peptide G-treated laminin dissociation ($k_d = 2.9 \times 10^{-6}$ s^{-1}) *versus* 35% of peptide X-treated ($k_d = 9.2 \times 10^{-6}$ s^{-1}) or untreated ($k_d = 9.9 \times 10^{-6}$ s^{-1}) laminin dissociation (Fig. 7*B*).

Different amounts of labeled laminin, treated with peptide G or X or untreated, were tested for binding. The ratio between bound and free ligand in relation to the bound ligand was calculated by Scatchard analysis. The intercept on the *x* axis indicated that the number of binding sites recognizing the peptide G-treated laminin was double the number of binding sites recognizing untreated or peptide X-treated laminin, whereas the slope of the rate was slightly increased, corresponding to *K* values of 3×10^9, 1.6×10^9, and 1.8×10^9 M^{-1} for peptide G-treated, untreated, and peptide X-treated laminin, respectively (Fig. 8).

Expression of VLA6 on N592 Cells—The effect of peptide G-treated laminin on expression of the α6 integrin subunit, which is part of the VLA6 coexpressed with 67LR on N592 cells, was analyzed by immunofluorescence assay. The cells expressed a small amount of the α6 subunit, which increased slightly after incubation with peptide G-treated laminin but not after incubation with laminin alone or with peptide X-treated laminin (Fig. 9*A*). HLA expression, evaluated as a control, showed no changes (Fig. 9*B*).

Inhibition of Laminin Binding in N592 Cells by Anti-α6 mAb or EDTA Treatment—To identify the membrane receptors involved in the binding of peptide G-treated laminin, N592 cells previously saturated with a high concentration of an anti-α6 mAb (GOH3), directed against the laminin binding site of the α6 integrin subunit, were analyzed for their residual ability to bind labeled laminin pretreated with the peptides (G or X) or untreated. As shown in Fig. 10, the antibody treatment inhibited ^{125}I-laminin binding on the cell surface by 40% when the adhesion molecule was untreated or treated with peptide X and by only 20% when labeled laminin was incubated with peptide G.

Because divalent cations are required for integrin activity, the binding of labeled laminin, untreated or treated with the two peptides, was assayed in EDTA buffer. Binding of untreated laminin or peptide X-treated laminin was inhibited by 50%; in comparison, peptide G-treated laminin was inhibited by 30% (Fig. 10).

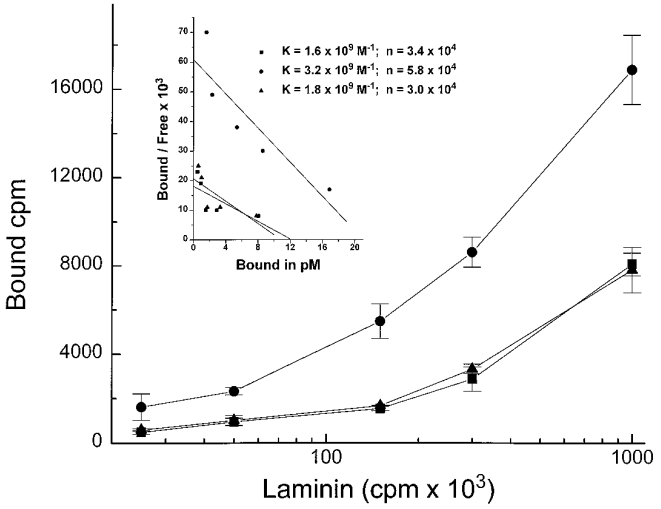

FIG. 8. **Scatchard analysis of membrane-bound laminin untreated or treated with peptide G.** N592 cells were incubated for 180 min at 0 °C with 100 μl of ^{125}I-laminin untreated (■) or treated with peptide G (●) or peptide X (▲) at different serial dilutions. After four washings, cells were evaluated for bound radioactivity in a γ counter. Specific binding was calculated as the difference between binding of ^{125}I-laminin in the presence and absence of a 100-fold excess of unlabeled laminin. The *inset* shows the Scatchard plot of the binding data.

Adhesion of N592 Cells to Laminin—An adhesion assay in which N592 cells in suspension were seeded in medium containing untreated or peptide-treated laminin was used to investigate the effect of peptide G treatment of soluble laminin on cell adhesiveness. Laminin alone or treated with peptide X induced 30% cell adhesion, whereas peptide G-treated laminin induced nearly 90% adhesion. Treatment of these cells with mAb GOH3 decreased adhesion by 50% in all samples (Fig. 11).

DISCUSSION

In the present study, peptide G, corresponding to the 67LR laminin binding domain, increased and stabilized laminin binding on tumor cells. This effect is specific because two other peptides of the same length, one hydrophobic and poorly soluble and one neutral, did not induce these effects. The stabili-

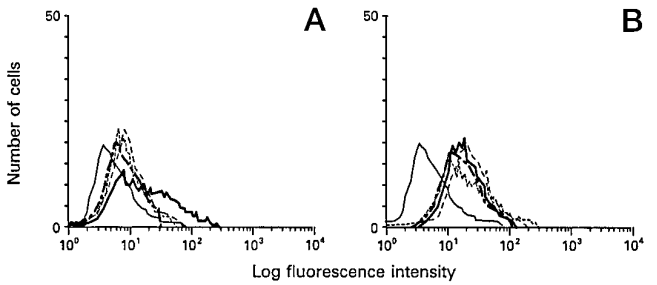

FIG. 9. **Effect of peptide G-treated laminin on expression of the α6 integrin subunit in N592 cells.** Cells (3×10^5 cells/sample) were treated for 30 min at 37 °C with exogenous laminin (50 μg/ml) alone (*dashed line*) or previously incubated with peptide G (*bold line*) or peptide X at 50 μg/ml (*dotted line*). Expression of the α6 integrin subunit (*A*) and HLA (*B*) was analyzed by indirect immunofluorescence using purified mAbs MAR6 and W6/32, respectively, at 10 μg/ml, and fluorescein isothiocyanate-conjugated goat anti-rabbit Ig (1:100). *Light lines* and *dotted-dashed lines* show the values of background and basal α6 expression, respectively.

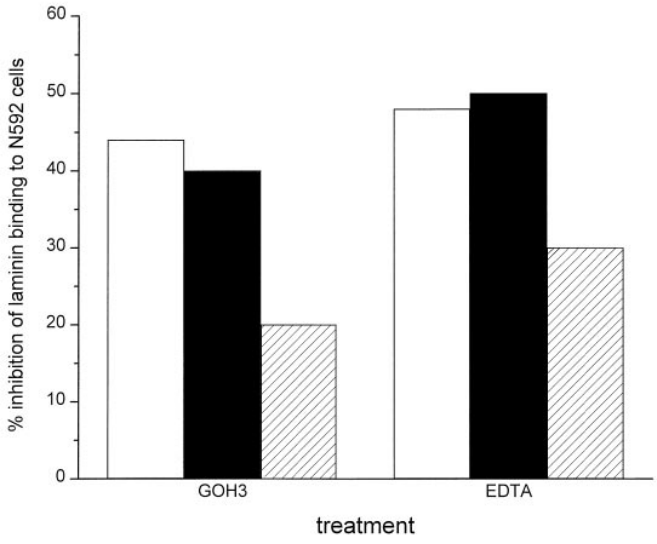

FIG. 10. **Effect of peptide G on ^{125}I-laminin binding to N592 cells in the presence of anti-α6 mAb or EDTA.** N592 cells (2×10^5/sample) were saturated with anti-α6 mAb GOH3 at 200 μg/ml in a 50-μl volume for 1 h at 37 °C or equilibrated with PBS without divalent cations. The same cells were incubated with 50 μl of ^{125}I-laminin (10^6 cpm) alone (*open bar*), with peptide G (*hatched bar*), or with peptide X (*filled bar*) in PBS or EDTA buffer for 2 h at 37 °C. After being washed, cells were evaluated for bound radioactivity, and the percentage of binding inhibition was calculated as follows: 100 − (mean cpm bound to treated cells/mean cpm bound to control cells × 100).

zation, evaluated as a decrease in the k_d of membrane-bound laminin, is due principally to an increase in the number of laminin binding sites on the cell surface and a slight increase in affinity. Scatchard analysis indicated a 2-fold increase in the affinity constant of peptide G-treated laminin and a 3-fold decrease in its k_d. Assuming that the mass equilibrium equation used in the Scatchard plot analysis is adequate for this system, the differences in K and k_d correspond to a 1.5-fold decrease in the association constant. This means that peptide G-treated laminin binds to cells with a similar or slightly lower association rate than untreated laminin, but once bound, it remains associated with the membrane far longer than does untreated laminin. The higher k_d in A431 cells can be attributed to production of endogenous laminin, which competes with the labeled laminin for cell binding. Thus, findings on N592 cells were considered more reliable, and this cell line was chosen for Scatchard analysis.

Peptide G has been shown to bind heparin, and its role in

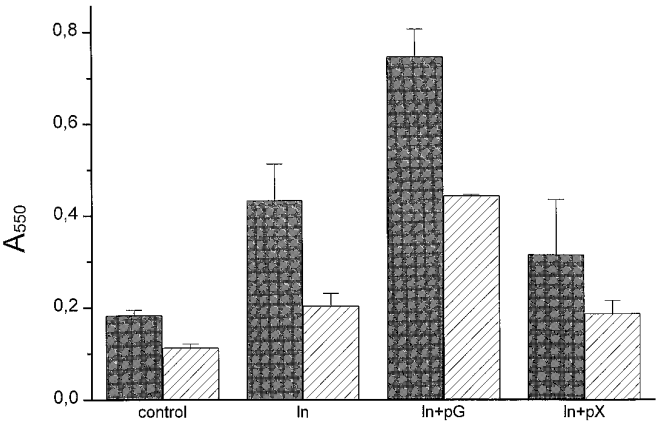

FIG. 11. **Effect of peptide G-treated laminin on N592 cell adhesiveness in the presence of anti-α6 mAb.** N592 cells, uncoated (*filled bar*) or coated with 200 μg/ml of anti-α6 mAb (*hatched bar*), were seeded in 96-well plates at a density of 2×10^4 cells/well alone or in the presence of 10 μg of murine laminin previously treated with peptide G or X or untreated. After 90 min at 37 °C, adherent cells were detected in a colorimetric assay by 4 h of incubation at 37 °C with 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide dissolved in PBS (5 μg/ml). Acid isopropyl alcohol (100 μl of 0.04 N HCl in isopropyl alcohol) was added to wells for 5 min at room temperature to dissolve the dark blue crystals. Plates were read on a Titertek Multiskan spectrophotometer at 550 nm. Specific A_{550} was evaluated as follows: (A_{550} of treated sample − A_{550} of control).

mediating the interaction with laminin through heparin has been suggested (25). Because heparan sulfate is likely present in all laminin preparations, as well as in culture medium, the binding of peptide G to purified laminin or to endogenously produced laminin is not indicative of a direct peptide G-laminin interaction but rather might reflect an indirect effect mediated through heparin. In either case, the final effect at the cell membrane level is a stabilization of laminin binding.

The increase in α6 expression on the membrane upon treatment with peptide G-modified laminin strongly suggests that the VLA6 integrin, the only α6-containing integrin expressed on N592 cells (29), participates in the binding increase. Considering that laminin might sterically interfere with the detection of its receptor, α6 expression could well be underestimated. Indeed, treatment of N592 cells with laminin had been shown to induce translocation of both 67LR and α6 from the cytoplasm to the cell's outer surface (30).

The decreased inhibition of binding of peptide G-treated laminin observed after mAb GOH3 or cation chelation treatment (34, 35), together with the finding that laminin did not dissociate from suspended cells at a time in which integrins might be inactivated, suggests that the laminin binding molecules involved in recognition of the peptide G-modified laminin are not restricted to the integrin family. Because the 67LR binds to laminin with different binding sites (36), the possibility that membrane 67LR itself plays a role in the recognition of peptide G-treated laminin cannot be excluded.

One possible explanation for the peptide G-induced increase in laminin binding ability is that the peptide changes the conformation of laminin. We found a greater increase in binding when laminin was treated in solution with peptide G than when it was first allowed to react with cells and then treated with the peptide. Thus, soluble laminin might be more sensitive to a conformational change than bound laminin. Because receptor recognition domains of laminin appear to be conformation-dependent (8), this modified laminin may interact better with integrins or other surface molecules that normally do not participate with significant affinity in laminin binding to the cell surface (37–39). The involvement of different laminin binding molecules, recognizing different sites, increases the

probability of multivalent linkages that stabilize the binding. Integrins and other laminin-binding proteins may cooperate to provide specific recognition of the laminin modified by peptide G; "new" laminin-binding proteins might receive functional signals from the multiple integrins involved, and this information might increase their binding specificity. Such new laminin-binding proteins might also mediate N592 cell adhesion to laminin, as indicated by the increased cell attachment to peptide G-treated laminin compared with the untreated adhesion molecule. Indeed, mAb GOH3, which has been reported to partially inhibit N592 cell adhesion (29), reduced cell attachment to the same extent in the presence of untreated or peptide-treated laminin.

Peptide G also increases the binding of endogenous laminin on the cell surface: in fact, addition of the peptide to the culture medium for 24 h increased the expression of membrane laminin in laminin-producing cells.

We recently showed that tumor cells release 67LR into the medium and that the soluble form retains its ability to bind laminin (40). This soluble receptor might induce the same conformational change in laminin as that induced by peptide G, leading to increased laminin binding to the cell surface. This mechanism, which provides the cells with a greater number of binding sites, might modulate the interaction between tumor cells and laminin, with consequences for their metastatic potential. Consistent with this suggestion, 67LR expression has been associated with an unfavorable prognosis in breast carcinomas, but only in those producing laminin (24).

In conclusion, the role of the 67LR in tumor progression appears to be related to its ability to increase and stabilize laminin binding on tumor cells that, when coated with laminin, show increased metastatic potential (26).

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