Co-regulation and Physical Association of the 67-kDa Monomeric Laminin Receptor and the α6β4 Integrin

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The interactions between tumor cells and laminin or other components of the extracellular matrix have been shown to play an important role in tumor invasion and metastasis. However, the role of the monomeric 67-kDa laminin receptor (67LR) remains unclear. We analyzed the regulation of 67LR expression under different culture conditions with respect to the expression of other well characterized laminin receptors. In A431 cells treated with laminin for different time periods, the regulation of 67LR expression correlated with expression of the α6 integrin subunit but not with the expression of other laminin receptors. Moreover, cytokine treatment resulted in down-modulated expression of the α6 integrin subunit and the 67LR. Co-regulation of the expression of the two receptors was further suggested by the observation that specific down-modulation of the α6-chain by antisense oligonucleotides was accompanied by a proportional decrease in the cell surface expression of 67LR. Biochemical analyses indicated co-immunoprecipitation of 67LR and the α6 subunit with an anti-α6 but not an anti-β1 monoclonal antibody. Co-regulation of 67LR and α6 subunit expression, together with the physical association between the two receptors, supports the hypothesis that 67LR is an auxiliary molecule involved in regulating or stabilizing the interaction of laminin with the α6β4 integrin.

The interaction of tumor cells with laminin, the major basement membrane glycoprotein, is considered a critical determinant of metastatic dissemination. Cells can bind laminin through different specific receptors, including the integrins, a large family of heterodimeric transmembrane molecules consisting of non-covalently associated α and β subunits (1–3).

13 years ago, three independent laboratories isolated a non-integrin protein of 67 kDa, designated 67-kDa laminin receptor (67LR),1 by affinity chromatography on laminin-Sepharose (4–6). To date, the structure of this molecule has not yet been elucidated, and only the cDNA encoding a cytoplasmic precursor of 37 kDa (3’7LRP) has been identified (7). Although pulse-chase experiments performed on melanoma cells demonstrated that this 37-kDa polypeptide is the precursor of the 67-kDa form, the post-translational mechanism by which the 67LR is synthesized from the precursor is still unknown (8).

Cell surface expression of the 67LR has been shown to correlate with metastatic potential of solid tumors. Indeed, increased expression of the 67LR was found in different neoplasias, where it is associated with poor prognosis (9–11). In a study aimed at elucidating the role of different laminin receptors in the metastatic process, we found a strong correlation between the expression of 67LR and the α6β1 integrin on small cell lung carcinoma cells (12). Consistent with these results, immunoelectron microscopy indicated that the 67LR localized in the same cytoplasmic structures positive for the α6 and β1 integrin subunits (13). After a brief exposure to laminin, these cytoplasmic complexes were exported to the cell surface by a mechanism which is still unclear.

In the present study, we analyzed whether membrane expression of the 67LR and of integrins involved in laminin recognition is co-regulated and whether the different molecules are physically associated.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture Conditions—Human vulvar epidermoid carcinoma cell line A431 was provided by ATCC. Cells were maintained in RPMI 1640 medium (Sigma) supplemented with 10% fetal calf serum (FCS), penicillin (100 mg/ml), and streptomycin (100 µg/ml). Experiments involving treatment with laminin were performed using murine laminin purified from the mouse Engelbreth-Holm-Swarm tumor (Sigma).

Monoclonal and Polyclonal Antibodies—The following monoclonal antibodies were used as purified Ig: MLuC5, directed against the 67-kDa laminin receptor (14); GoH3, directed against α6-chain integrin (ImmunoTech, Marseille, France); P1E6, directed against α2-chain integrin (Telios, San Diego, CA); P1B5, directed against α3-chain integrin (Telios); 3E1, directed against β4-chain integrin (Telios); MAR4, directed against β1-chain integrin (15); MGR1, directed against the EGF receptor (12); MPLR2, directed against the 37-kDa laminin receptor precursor and 67LR mature form of the receptor;2 and W6/32, directed against a monoclonic epitope on the 45-kDa polypeptide product of the human leukocyte antigen (HLA) A, B, C loci (Coulter Immunology, Hialeah, FL).

Fluorescein isothiocyanate (FITC)-conjugated goat anti-rat IgG and anti-mouse IgG or IgM (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD) were used as second-step reagents.

Oligonucleotide Synthesis—Unmodified DNA oligonucleotides corresponding to integrin subunit α6 nucleotides 142–160 (18-mer) were synthesized on an automated synthesizer (Cyclone Plus DNA Synthesizer, Millipore Corp.). The sense and antisense α6-chain sequences were 5’-GGCATGGCCGGGCCGCGG-3’ and 5’-CGGCACCCGCGCTGATG-3’, respectively. After cleavage from a controlled pore glass column and deblocking in concentrated ammonium hydroxide at 55 °C for 18 h, the oligonucleotides were purified by ethanol precipitation from a 100 mM

2 S. Butò, E. Tagliabue, E. Ardini, A. Magnifico, V. Castronovo, M. I. Colnaghi, M. Sobel, and S. Menard, manuscript in preparation.
A431 cells, treated with exogenous soluble laminin for indicated times, were analyzed by indirect immunofluorescence for the expression of different laminin receptors.

| Time of treatment | α2 | α3 | α6 | β1 | β4 | 67LR |
|------------------|----|----|----|----|----|------|
| 0 min            | 31 ± 5⁺ | 97 ± 15 | 68 ± 7 | 241 ± 20 | 73 ± 9 | 975 ± 47 |
| 30 min           | 27 ± 9 | 91 ± 10 | 102 ± 12 | 250 ± 23 | 97 ± 10 | 1150 ± 50 |
| 5 days           | 35 ± 5 | 102 ± 15 | 125 ± 14 | 273 ± 25 | 152 ± 20 | 1412 ± 73 |
| 30 days          | 32 ± 7 | 103 ± 10 | 130 ± 15 | 268 ± 20 | 160 ± 15 | 1430 ± 68 |

⁺ Mean fluorescence intensity ± S.D. obtained from three experiments by immunofluorescence assay and FACScan analysis.

**RESULTS**

**Effect of Laminin Treatment on Laminin Receptor Expression**—Modulation of 67LR and the other well characterized laminin receptors following laminin treatment was investigated using A431 cells, which express high levels of both 67LR and the integrin laminin receptors. A431 cells were treated with exogenous laminin at a concentration of 50 μg/ml for different periods of time and analyzed for cell surface laminin receptor expression by FACScan analysis. After 30 min of laminin treatment, membrane expression of the α6 and β4 integrin subunits and 67LR was significantly increased, whereas the levels of other laminin receptors such as α2 and α3 integrin subunits remained unchanged. The up-modulation was even more evident after 5 days of exposure to laminin, but no further increase was observed after longer treatment (Table I). Expression of the β1-chain was only slightly increased after long-term treatment.

**Down-modulation of α6 Integrin Subunit and 67-kDa Laminin Receptor after Cytokine Treatment**—Treatment of different tumor cell lines with cytokines has been reported to down-modulate the expression of integrin subunit α6 (16, 17). We therefore investigated whether cytokines might also modulate 67LR expression. A431 cells were treated with a combination of 20 ng/ml TNF-α and 20 ng/ml IFN-γ for 48 h and analyzed by FACScan for the expression of integrins and 67LR. As shown in Fig. 1, down-modulation of α6 integrin subunit expression was accompanied by a decrease in the level of 67LR on the cell membrane. Time-course experiments showed that cytokine-mediated down-regulation of the protein expression was detectable 24 h after addition of TNF-α and IFN-γ to the cells and maximal by 48 h (data not shown). No effect on class I HLA expression was observed (Fig. 1).

**Effect of Laminin Treatment on Tumor Cells**—In addition to the above described experiments, we also analyzed the effect of laminin treatment on tumor cell growth and survival using FACScan. As shown in Fig. 2, laminin treatment had no significant effect on cell proliferation, whereas it significantly increased the number of apoptotic cells (Fig. 2). These results suggest that the binding of laminin to its receptors may modulate the survival of tumor cells in a manner similar to that observed for other types of cells.
ment with an antisense oligonucleotide targeted to α6 integrin subunit mRNA; cationic liposome-mediated transfection of A431 cells with the specific antisense led to a marked decrease in α6 cell surface expression, which was detectable after 24 h of treatment (Fig. 2A). Antisense-induced α6 down-modulation was accompanied by a proportional decrease in 67LR cell membrane expression (Fig. 2C). No change in α6 and 67LR expression was observed after transfection with the sense oligonucleotide used as negative control (Fig. 2, panels B and D), as well as in the amount of EGF receptor, used as unrelated control, with either antisense or sense oligonucleotides (Fig. 2, panels E and F). As expected, Western blot analysis of total cell lysates obtained from antisense-treated A431 cells revealed a decreased level of the α6 protein (Fig. 3A). By contrast, no difference in the amount of the 67LR and of the 37-kDa precursor was observed (Fig. 3B).

Co-purification of the 67LR with α6 Integrin Subunit—Immunoprecipitation of A431 soluble extracts was performed with mAbs to α6 and β1 integrin subunits and EGF receptor. Western blot analysis of the immunoprecipitated proteins using a mAb that recognizes the 67LR revealed 67LR in the material immunoprecipitated with the anti-α6 integrin antibody, whereas no co-immunoprecipitation of the 67LR was found with antibodies directed to the β1-chain or the EGF receptor (Fig. 4).

DISCUSSION

The present study demonstrates that the 67LR and the α6 integrin subunit are physically associated and their expression on the membrane is co-regulated. Indeed, up- or down-modulation of α6 induced the same change in 67LR expression.

Previous studies with small cell lung cancer cell lines demonstrated the co-expression of 67LR and α6β1 integrin and a correlation between the level of both receptors and cell adhesion to laminin (12). Moreover, on a human melanoma cell line treated with laminin, a co-translocation of these two molecules to the cell membrane was detected (13).

Our experiments demonstrate on a human epidermoid carcinoma cell line that the α6 integrin subunit and 67LR are not only co-expressed but also co-regulated. In A431 cells, despite the presence of the β1 subunit, α6 is found exclusively associated with β4 integrin (18). Therefore, we can speculate that in this cell line, 67LR is specifically associated with β4α6, in keeping with the observation that the α6 subunit can bind either the β1 or β4 subunit, but when given a choice it preferentially associates with β4 (19, 20).

Together, these data suggest that the linkage of 67LR is actually through the α6 subunit independently of the β-chain to which this α-chain is associated, i.e. β1 in the case of small cell lung cancer cells and β4 in the case of A431 cells.

TNF-α and IFN-γ lead to a decrease in the expression of the
α6 integrin subunit (16, 17). Our data show that decreased expression of the α6 chain after cell treatment with a combination of two cytokines is accompanied by 67LR down-modulation. Even though this does not indisputably demonstrate the association of α6 and 67LR, since cytokine treatment can profoundly perturb the expression of many different molecules (21–23), the data are in keeping with the results of the antisense treatment. Indeed, the inhibition of α6 mRNA translation followed by inhibition of α6 membrane expression, which also led to inhibition of 67LR membrane expression, strongly indicates the interaction between the two molecules. The absence of a decrease in the total amount of the mature form of 67LR as well as in the total level of the 37-kDa precursor indicates that the antisense treatment does not influence the 67LR expression. Indeed, the interaction of 67LR and α6 integrin subunit seems to occur at the cytoplasmic level, when the two molecules co-translocate from the cytoplasm to the cell surface. After anti-α6 antisense treatment the lower availability of α6 protein might be responsible for the relevant decrease of the α6–67LR complexes exported to the cell surface.

Consistently, up-modulation of the membrane expression of both α6 and 67LR after exposure to laminin, which is detectable already after 30 min of treatment, suggests an increased delivery of the two associated receptors from the cytoplasm to the cell surface, as previously reported (13, 24).

The close association between the two molecules raises the possibility that the two receptors are specifically involved in the same process. This hypothesis is supported by the co-immunoprecipitation experiment demonstrating that the two receptors are not only co-expressed and co-regulated but are also physically associated on the cell membrane.

The 67LR, even though it binds directly to laminin, might be an accessory molecule for α6β1 and α6β4 integrins, acting in the regulation of integrin binding to laminin. The interaction of laminin with 67LR might induce some conformational changes that render the adhesion molecule more accessible for integrin binding. The finding that treatment of cells with the peptide YIGSR blocks cell adhesion to laminin is consistent with this hypothesis (25). Indeed, this five-amino acid peptide corresponding to the site on the short arm of the laminin 1 β1-chain recognized by 67LR can prevent laminin interaction with the monomeric receptor, thus inhibiting integrin-mediated adhesion as well.

The possible requirement for binding of laminin by both the integrin and 67LR to obtain sufficient affinity is consistent with a recent study demonstrating that affinity-purified 67LR reapplied to a laminin-Sepharose column in the same conditions used for initial purification was recovered mostly in the unbound fraction and that the ability of the purified protein to bind laminin-Sepharose was restored only by addition of two fractions of the low affinity eluate (26). According to our hypothesis, these fractions might contain the α6β4 or α6β1 integrin receptor, and the combination of purified 67LR with this material allows reconstitution of the complex able to bind laminin with high affinity. In this light the evaluation of the affinity of integrins and of the 67LR must take into account the association of the two receptors, which participate together in the recognition of laminin.

In conclusion, our data suggest that 67LR is an auxiliary molecule of the α6 integrin, forming a complex with this molecule that can interact with laminin with high affinity.

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