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Shedding of the 67-kD Laminin Receptor by Human Cancer Cells

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Abstract The 67-kD laminin receptor (67LR) is a cell membrane-associated molecule exhibiting high affinity for the basement membrane glycoprotein, laminin. While export of the 67LR toward the extracellular matrix has been recently suggested by electron microscopy studies, there is to date no evidence of shedding of the 67LR from cells. Using two monoclonal antibodies directed against the 67LR, we developed a double-determinant radioimmunoassay that demonstrates that the 67LR is released from cancer cells into the culture medium. The shed molecule exhibited the same apparent molecular weight as that of the membrane-associated 67LR, suggesting that no proteolytic cleavage is involved in the process. Furthermore, we demonstrate that the 67LR is not anchored to the membrane through a glycolsyl–phosphatidylinositol bridge. However, the observation that lactose increased the release of 67LR suggests that a lectin-type interaction is involved in the cell membrane association of this laminin binding protein and the cell surface. Interestingly, the released 67LR recovered after HPLC gel filtration was found free as well as associated to high molecular weight complexes. The free 67LR retained its ability to bind to the cell surface. Our study is the first demonstration that the 67LR is effectively shed by cancer cells. The released free 67LR could play an important role in modulating interactions between cancer cells and laminin during tumor invasion and metastasis.

Key words: Monomeric laminin receptor, shedding, metastasis, double determinant assay, adhesion, prognostic factor

Among several cell surface molecules able to interact with laminin, the 67LR laminin receptor (67LR) is the most studied in cancer progression. While its association with increased invasiveness and metastatic competence has been abundantly documented in a variety of human cancers [Wewer et al., 1986; Yow et al., 1988; Castronovo et al., 1990; Cioce et al., 1991; Martignone et al., 1992], its exact structure and biosynthesis have not been yet fully elucidated. A 37-kD polypeptide (37LRP) was cloned from a λgt11 library using an anti-67LR monoclonal antibody (mAb) [Wewer et al., 1986]. This 37 LRP was found in pulse-chase experiments to be the precursor of the 67LR. The mechanism responsible for the molecular-weight increase of the 37LRP up to 67 kD is unknown, although dimerization, heavy glycosylation, phosphorylation, and acylation have been ruled out as potential post-translational events that would account for the increase. A model of the 67LR in which the 37LRP is associated with another polypeptide has been proposed [Castronovo, 1993] but remains to be demonstrated.

An interesting aspect of the 37LRP polypeptide is the possibility that it could be multifunctional. It has been proposed that the 37LRP is part of the translational machinery and that it is associated in the cytoplasm with polysomes [Auth et al., 1992]. The 37LRP bears within its amino acid sequence a functional domain related to laminin interactions [Taraboletti et al., 1993]. A 20-amino acid synthetic peptide derived from the 37LRP cDNA sequence binds laminin with an affinity comparable to that calculated for 67LR. Because the 37LRP contains neither a classical transmembrane domain nor a signal peptide [Castronovo et al., 1991b], the

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mechanisms by which it is exported to the cell membrane are unclear. Yet epitopes corresponding to the carboxy terminal end of the 37LRP are detected on the surface of cancer cells [Wewer et al., 1987].

Recently, we studied the cell distribution of the 67LR and the 37LRP by electron microscopy using two monoclonal antibodies directed against the 67LR (MLuC5 and MLuC6) as well as anti-37LRP synthetic peptide polyclonal antibodies [Romanov et al., 1994]. As expected, we found that both 37LRP and 67LR epitopes colocalized in electron-dense structures in the cytoplasm as well as on the cell surface. Interestingly, after exposure of the cell to laminin, the number of such cytoplasmic structures increased, especially in the vicinity of the plasma membrane, and were exported to the cell surface. Those data suggested that the 67LR might be released into the extracellular matrix medium. To investigate this possibility, we developed a double-determinant radioimmunoassay (DDIRMA) using the two anti-67LR mAbs, MLuC5 and MLuC6. Using this assay, we demonstrate that the 67LR is released into the medium. In this soluble form, it retains its ability to bind laminin and was indeed found in high-molecular-weight complexes.

MATERIALS AND METHODS

Cell Lines

Human cell lines vulvar epidermoid carcinoma A431, lung adenocarcinoma CaLu3, small cell lung carcinoma N592, and breast carcinomas SKBr3 and MCF-7 were all provided by ATCC (Rockville, MD). Ovary carcinoma OVCa432 and melanoma cell line MeWo were kindly provided by Dr. W. Knapp (Dana Farber, Boston, MA) and the late Dr. J. Fogh (Sloan Kettering Cancer Center, New York, NY), respectively.

Cell lines were all maintained in RPMI-1640 medium (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum (FCS) (Sigma), L-glutamine, and antibiotics. Culture medium from human cell lines grown for 3 days in medium without FCS was concentrated by filtration on a Centriprep-30 concentrator (Amicon, Beverly, MA). Co-cultivation experiments were performed in Transwell culture plates containing 3-μm pore polycarbonate membranes (Costar, Cambridge, MA).

Antibodies

The following mAbs were used: MLuC5 and MLuC6, which recognize different epitopes on the 67 kD laminin receptor [Martignone et al., 1992], MGR1 against EGF receptor [Pellegrini et al., 1991], and W6/32 [Parham et al., 1979] reacting with a monomorphic determinant of human HLA-A,B,C molecules obtained from Dakopatts (Glostrup, Denmark). Rabbit antiserum to human laminin was from Telios Pharmaceutical Inc. (San Diego, CA).

Production of Cellular Conditioned Media, Double Determinant Radioimmunoassay, and Binding Assay

Cells (10^7) were washed, resuspended in 0.5 ml phosphate-buffered saline (PBS), and incubated with rotation for 2 h at 37°C. In some experiments, cells were treated with one of the following: 0.1 M lactose, 0.25 U/ml phospholipase C isolated from *Bacillus thuringiensis* (Oxford Glycosystem, Abingdon Oxon, UK), 1–10 μg/ml brefeldin A (Boehringer-Mannheim, Germany), 1–20 μM monensin (Sigma), 50 μg/ml mouse laminin (Sigma), or 50 μg/ml human fibronectin (Boehringer). The conditioned medium was separated from the cells by centrifugation and tested in double-determinant radioimmunoassay (DDIRMA) as described previously [Diotti et al., 1987] for the presence of the 67-kD laminin receptor. Briefly, supernatants were incubated overnight with polystyrene beads adsorbed with 5 μg/bead of purified mAb MLuC5. The beads were washed in PBS, incubated with ^{125}I-MLuC6 (5×10^5 cpm/well) for 2 h, extensively washed and assayed for bound radioactivity in a gamma counter.

Indirect binding test was performed using 10 μg/ml MLuC5 or W6/32 as first antibody and ^{125}I-labeled goat antimouse Ig (Amersham, Little Chalfont, UK) as second antibody on triplicate samples consisting of 10^7 CaLu3 cells pretreated or not with conditioned medium for 1 h at 37°C. After 1-h incubation with the labeled antibody, cells were extensively washed and assayed for bound radioactivity.

Flow Cytometric Analysis

Indirect immunofluorescence (IF) was used to analyze cell surface antigens. Approximately 10^5 cells were incubated with antibodies (10 μg/ml) for 30 min at 4°C. After three washings, cells were incubated with fluorescein isothiocyanate-

conjugated goat antimouse Ig (Kirkegaard and Perry Laboratories, Gaithersburg, MD) for 30 min at 4°C, washed, and analyzed using a FACScan flow cytometer with LYSYSII software (Becton Dickinson, Mountain View, CA).

Immunoprecipitation and SDS–PAGE

A431 cells (3×10^6) were metabolically labeled overnight with 50 μCi ^{35}S-methionine/ml (Amersham) in methionine-free medium containing 5% FCS. After several washings, labeled cells were incubated in PBS for 2 h at 37°C. Both cells and conditioned medium obtained after centrifugation were used in immunoprecipitation experiments with mAb MLuC5 linked to protein A–Sepharose CL-4B (Pharmacia, Uppsala, Sweden) according to described procedures [Tagliabue et al., 1991]. Immunoprecipitates were analyzed by SDS–PAGE on a 10% polyacrylamide gel under reducing conditions [Laemmli, 1970].

Western Blotting

Conditioned medium obtained from A431 cells and CaLu3 cells were separated on a 10% polyacrylamide gel and electrophoretically transferred to a Hybond-C nitrocellulose membrane (Amersham) as described [Towbin et al., 1979]. The membrane was first blocked overnight in PBS containing 5% nonfat milk and 0.1% Tween 20, and then incubated with purified MLuC5 mAb (50 μg/ml) for 1 h, washed, and further incubated with biotin-conjugated sheep antimouse antibody (Amersham) diluted 1:200 for 1 h. The membrane was washed and finally incubated for 1 h with streptavidin-biotinylated horseradish peroxidase (HPO) complex (Amersham) diluted 1:100. Immunodetection was performed with ECL reagents according to the manufacturer's instructions (Amersham). Nitrocellulose membrane treated only with biotinylated antibodies served as negative control.

HPLC Gel Filtration

Proteins from A431 cell conditioned medium were separated by HPLC (BioRad, Richmond, CA) on a TSK G4000 SWG column (21.5 × 600 mm) LKB (Bromma, Sweden) at a flow rate of 2 ml/min or on a TSK G3000 SW column (7.5 × 600 mm) at a flow rate of 0.5 ml/min. Both columns were equilibrated with 0.1 M sodium phosphate buffer, pH 7.35, and eluted with the same buffer solution. Before column separation, the conditioned medium was filtrated through a 0.45-μm Millipore filter (Nihon, Yonezawa, Japan). Elution volumes of known molecular-weight proteins were used to generate a standard curve.

RESULTS

Release of the 67-kD Laminin Receptor by Tumor Cells

A DDIRMA based on two mAbs directed against different epitopes on 67LR was set up, with MLuC5 mAb as carrier and labeled MLuC6 mAb as tracer. As shown in Figure 1, high DDIRMA reactivity was found in the concentrated culture medium from A431, a tumor cell line overexpressing the 67LR, whereas the medium from MeWo cells, which do not express detectable levels of this receptor, was negative.

Analysis of different tumor cell lines for 67LR surface expression by cytofluorometry and for 67LR release by a 2-h release assay, respectively, revealed high levels of released receptor in cells with high expression of membrane 67LR (A431, MCF-7 and N592) and low or no detectable levels of released 67LR in cells that were negative or weakly positive at the membrane level, although 67LR release was not directly proportional to membrane expression (Table I). When A431 cells were incubated for 2 h at 4°C instead of 37°C, only a low level of soluble receptor was detected in the conditioned medium (300 ± 30 cpm at 4°C vs 3100 ± 300 cpm at 37°C).

Mechanism of Release of the 67LR

To determine whether the 67LR is bound to the membrane via glycosyl–phosphatidylinositol and therefore can be removed from the cell

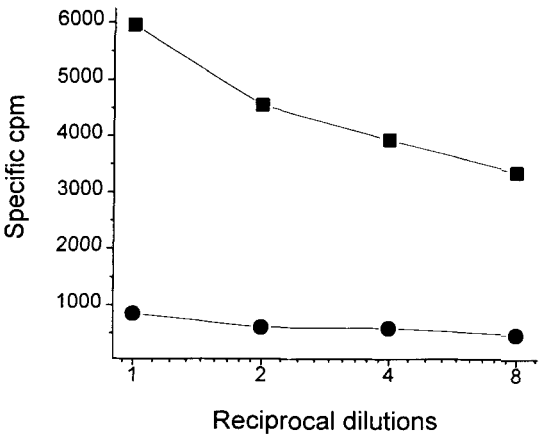

Fig. 1. DDIRMA analysis of released 67LR in concentrated culture medium of A431 (-■-) and MeWo (-●-) cells.

TABLE I. Flow Cytometric Analysis of 67LR Surface Expression and DDIRMA Analysis of the Released 67LR from Different Cell Lines

Cell line	IF intensity mean	IF intensity median	Specific cpm in DDIRMA[a]
N592	1,030	865	3,400
MCF-7	920	621	3,300
A431	1,136	1,084	2,700
OVCA432	37	31	200
SKBr3	192	135	50
CaLu3	29	4	12

[a]A 2-h release assay was set up in which 10^7 cells were incubated in 0.5 ml PBS with rotation at 37°C. Unconcentrated media were analyzed. Cell viability was >90% as determined by trypan blue exclusion.

by phospholipase C, A431 cells were treated with the enzyme and tested for membrane expression and the presence of the receptor in the conditioned medium. Flow cytometric analysis using MLuC5 or W6/32 mAb revealed no difference between 67LR cell surface expression in treated versus untreated cells, and DDIRMA analysis indicated no increase in the release of the 67LR in the conditioned medium (data not shown). Under the same conditions, ovarian carcinoma cells were found to release the folate binding protein associated with the membrane by a glycosyl–phosphatidylinositol linkage [Alberti, 1992].

To evaluate the molecular weight of the released molecule, metabolically labeled A431 cells were incubated for 2 h in PBS, and the soluble extract from these cells and the corresponding conditioned medium separated by centrifugation were analyzed in SDS–PAGE after immunoprecipitation with MLuC5 mAb. As shown in Figure 2A, in addition to minor bands, a 67-kD doublet band was found in both the conditioned medium and the cell extract. A major band at 55 kD was also detected in the cell extract but appeared only as a faint band in the conditioned medium. Immunoblotting analysis of conditioned medium from A431 cells and CaLu3 cells using MLuC5 mAb (Fig. 2B) revealed the 67-kD molecule in the A431 conditioned medium (lane 1) but not in the CaLu3 conditioned medium (lane 2).

Treatment of A431 cells with different concentrations of brefeldin A or monensin had no detectable effect on the quantity of 67LR expressed on the cell surface, as analyzed by flow cytometry using MLuC5 and MGR1 mAbs, or on the amounts released into the conditioned medium, as detected by DDIRMA (data not shown),

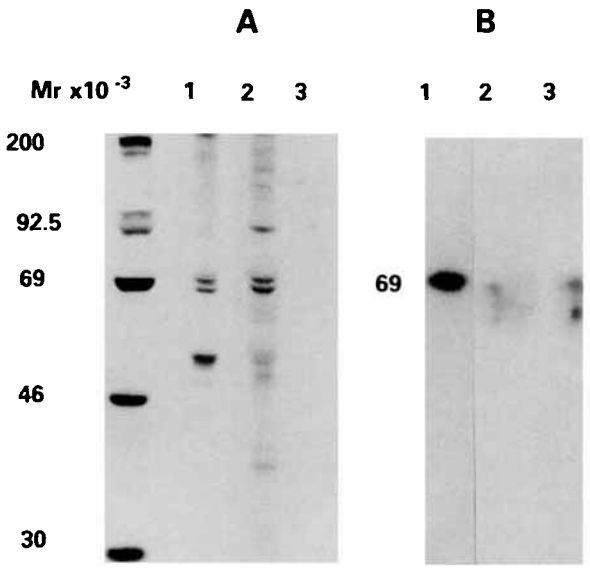

Fig. 2. Biochemical analysis of released 67LR. **A:** Immunoprecipitation of soluble cell extract (*lane 1,* 24-h exposure), conditioned medium (*lane 2,* 48-h exposure), from metabolically labeled A431 cells with mAb MLuC5, and negative control without the first antibody (*lane 3,* 48-h exposure). **B:** Western blot of 67LR molecules present in the conditioned medium of A431 cells (lane 1) and CaLu3 cells (lane 2) as detected with MLuC5 mAb. Lane 3, A431 cell conditioned medium without mAb.

arguing against a mechanism of 67LR release involving active secretion via the Golgi apparatus.

Membrane Binding of the Soluble 67LR

The 67LR-negative CaLu3 cells, incubated for 1 h with conditioned medium from A431 cells, became positive for MLuC5 binding (Fig. 3A). Flow cytometric analysis of CaLu3 cells cocultivated for 7 days with A431 cells also indicated an increase in 67LR membrane expression (Fig. 3B), whereas in both experiments, the expression of HLA class I antigens used as control was unchanged.

Treatment of A431 cells with laminin induced an increase in 67LR membrane expression (Fig. 4A left) with a concomitant decrease in the quantity of the free molecule in the conditioned medium (Fig. 4B). No changes in the expression of the EGF receptor were detected after laminin treatment (Fig. 4A right).

Lactose treatment, which has been proposed to dissociate the 67LR from laminin, reduced the amount of 67LR present on the A431 cell surface by about 30% (Fig. 5 left), with a corresponding increase in the shed 67LR (data not shown), whereas the treatment had no effect on

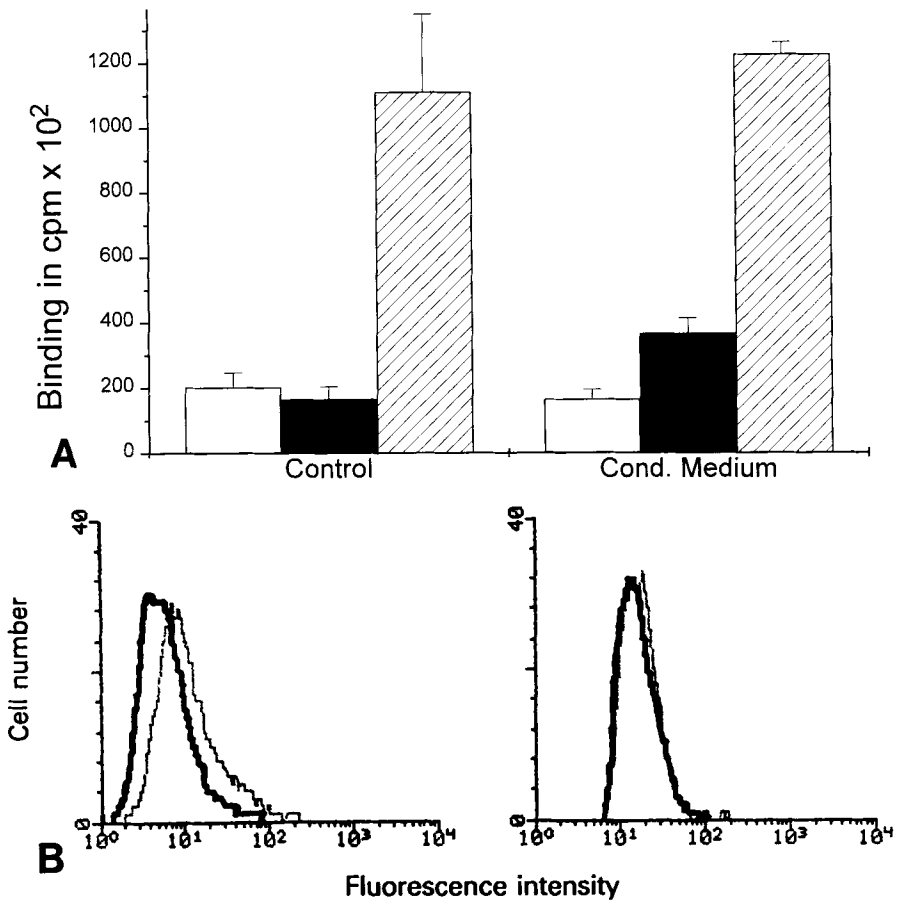

Fig. 3. Analysis of 67LR expression on CaLu3 cells. **A:** Binding of MLuC5 mAb (*filled bar*) and W6/32 mAb (*hatched bar*) on CaLu3 cells treated or not for 1 h with A431 conditioned medium. *Empty bar,* negative control without first mAb. **B:** Flow cytometry on CaLu3 cells after 7-day co-cultivation with A431 cells. **Left,** reaction with MLuC5 mAb; **right,** reaction with W6/32 mAb. *Black line:* CaLu3 cells, *gray line:* CaLu3 cells after co-cultivation with A431. Mean ±SD.

membrane expression of the EGF receptor (Fig. 5 right).

Complex of Soluble 67LR With Laminin

To examine the ability of soluble 67LR to bind laminin, the A431 cell conditioned medium was separated by high-performance liquid chromatography (HPLC) size exclusion chromatography and the fractions were tested by DDIRMA for the presence of the 67LR. Of the three different fractions corresponding to molecular mass higher than 1,000 kD, out of the range of separation of the column, around 1,000 kD and around 67 kD, the first and third fractions showed reactivity in DDIRMA (Fig. 6). To examine the possibility that the high-molecular-weight fraction contained insoluble molecules, the conditioned medium was ultracentrifuged for 1 h at 45,000 rpm and separated by HPLC. After centrifugation, the soluble 67LR was detected only in the low-molecular-weight fraction.

The addition of laminin to A431 cell conditioned medium resulted in decreased reactivity of soluble 67LR in DDIRMA, whereas addition of fibronectin did not affect soluble 67LR detection (Fig. 7). In parallel with DDIRMA, these samples were run in HPLC, and a 50% decrease of the 67-kD peak was found in the laminin-treated samples, whereas in the samples to which fibronectin was added, only a 18% reduction of the 67 kD peak was observed. On the other hand, when ^{125}I-labeled laminin was incubated for 2 h with conditioned medium from A431 cells and ultracentrifuged, 25% of the radioactivity was recovered in the insoluble fraction, whereas labeled laminin incubated with control medium showed only 3% insoluble molecules (data not shown).

DISCUSSION

Using a double determinant radioimmunoassay, we demonstrate that 67LR can be detected

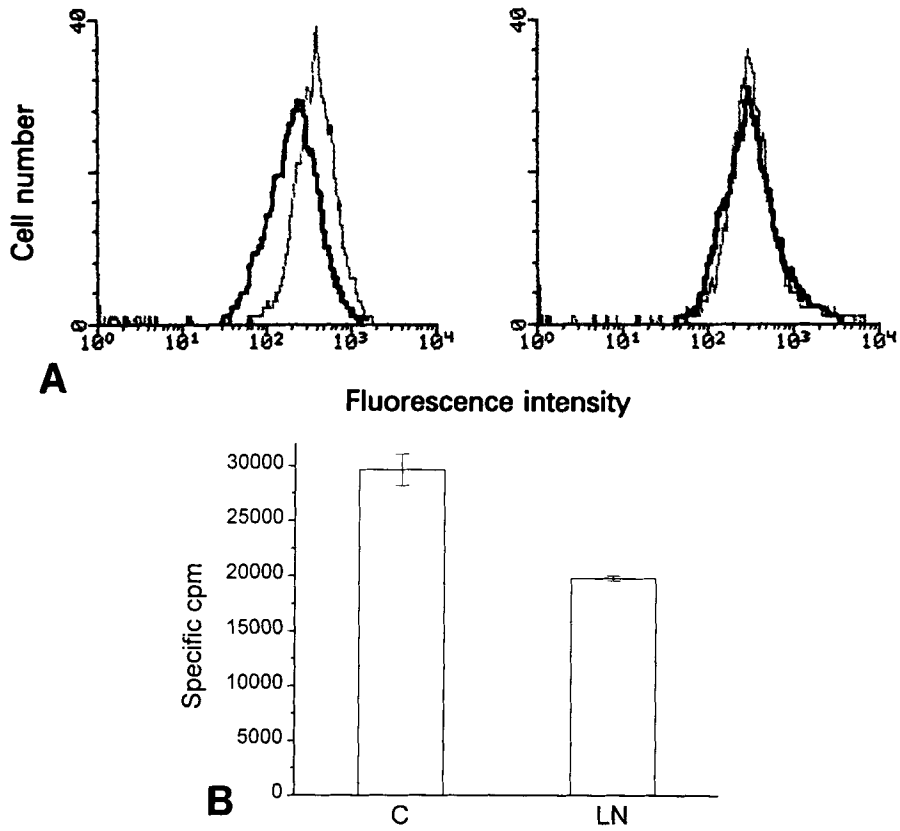

Fig. 4. **A:** Flow cytometric analysis of 67LR expression on laminin-treated A431 cells. **Left,** reaction with MLuC5 mAb; right, reaction with MGR1 mAb. *Black line:* control cells, *gray line:* laminin-treated cells. **B:** DDIRMA analysis of released 67LR from A431 cells treated (LN) or not (C) with laminin. Mean ±SD.

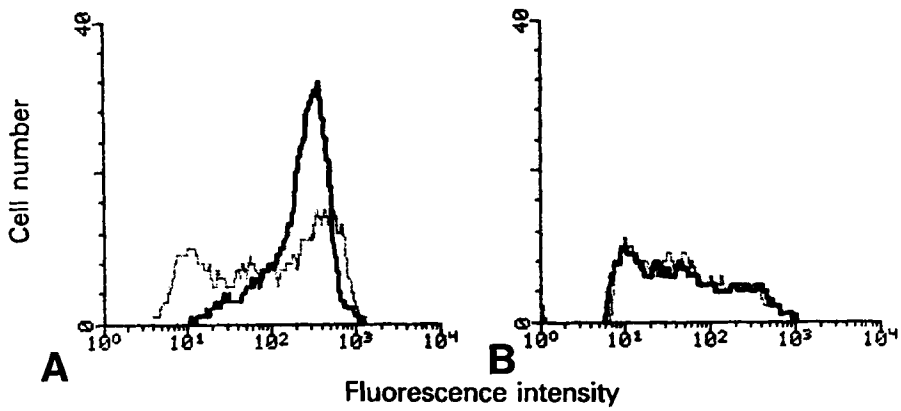

Fig. 5. Flow cytometric analysis of 67LR expression on A431 cells after incubation with 0.1 M lactose. **Left,** reaction of MLuC5 mAb; **right,** reaction of MGR1 mAb. *Black line:* control cells, *gray line:* lactose-treated cells.

in the culture medium of cancer cell lines expressing high levels of the receptor on their surface. The observation that the released 67LR exhibits the same molecular weight as the cell surface-associated 67LR and that the release is temperature-dependent indicates that the presence of 67LR in the culture medium does not result from a degradation of the cell surface receptor or release from dead cells. Since the complete structure of the 67LR is unknown, there are no indications of the mechanisms by which the 67LR is associated to the cell surface.

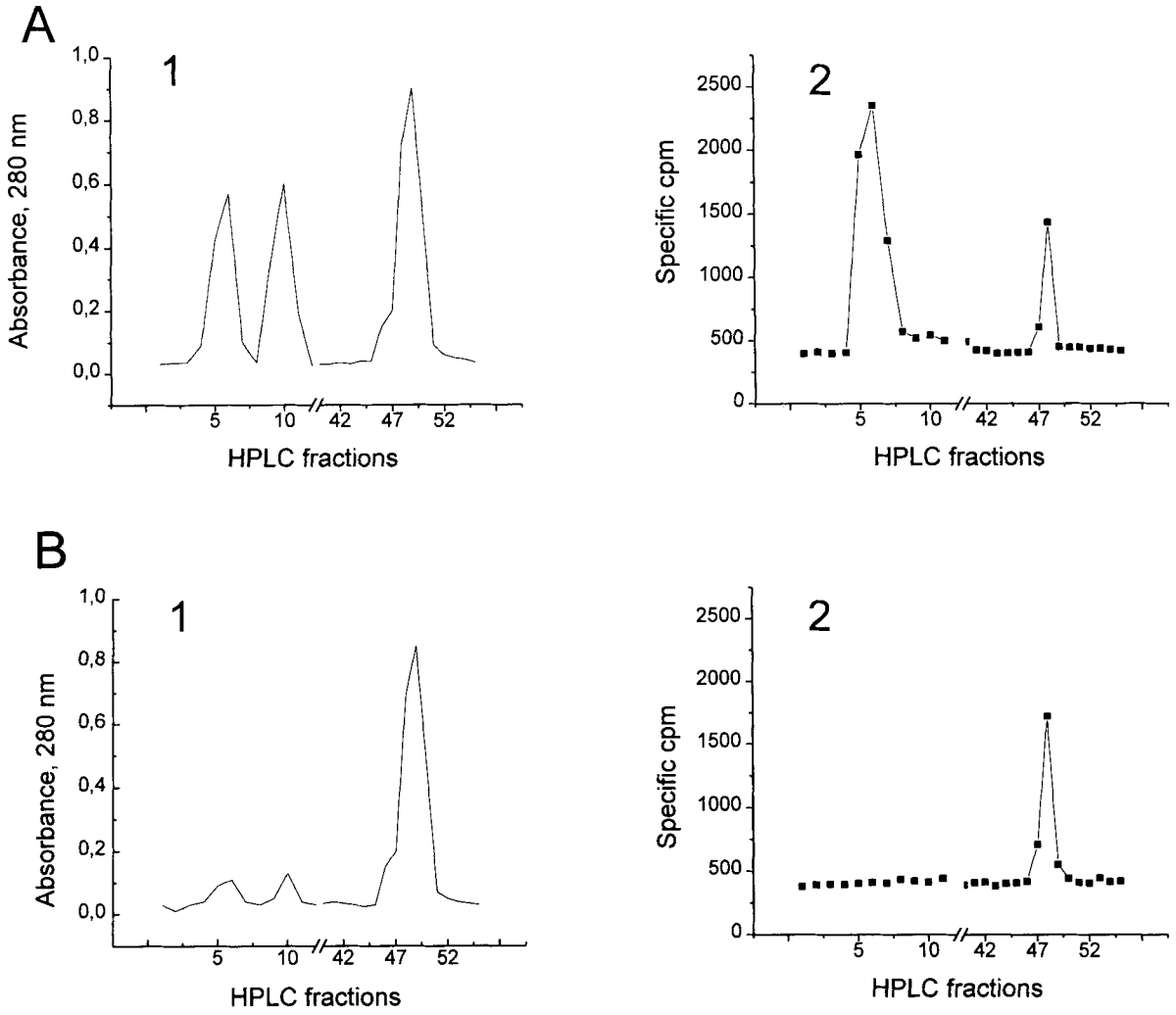

Fig. 6. HPLC separation of proteins in conditioned medium ultracentrifuged (B) or not (A) from A431 cells. 1, 2, absorbance and DDIRMA of HPLC fractions. Fractions 5, 10, and 50 correspond to molecular mass > 1,000 kD (void volume of the column), = 1,000 kD and 67 kD, respectively.

The 37 LRP contains a 16-hydrophobic amino acid stretch that could span the cell membrane [Rao et al., 1989]. Immunofluorescence experiments have demonstrated that epitopes that are N-terminal to this domains are inaccessible in nonpermeabilized cells, while epitopes carboxy-terminus to it bind their corresponding antibodies [Wewer et al., 1987]. This could be due to the association of those epitopes with the other putative polypeptide associated with the 37LRP rather than to the existence of a real transmembrane domain. Our analysis rules out the possibility that the 67LR is anchored to the cell membrane through a glycosyl–phosphatidylinositol bridge. Proteolytic cleavage of the cell surface 67LR can also be ruled out by the observation that the released 67LR does not exhibit a

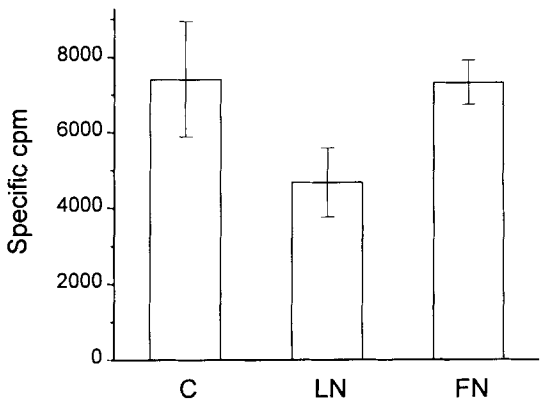

Fig. 7. DDIRMA analysis of unfractioned conditioned medium from A431 incubated for 30 min with 10 μg of laminin (LN) or 10 μg of fibronectin (FN) or neither (C).

reduced molecular weight. Some authors have suggested that the 67LR might be linked to the cell surface through a β-galactoside-binding lectin [Mecham et al., 1989; Castronovo et al., 1991a]. The increase in soluble 67LR in the culture medium after exposure of the cells to lactose favors such an anchoring mechanism. The 55-kD protein detected in association with the membrane might be the anchor protein for the 67LR [Mecham et al., 1989].

Neither brefeldin nor monensin, two drugs that block vesicular traffic at the endoplasmic reticulum and Golgi levels, inhibited the secretion or membrane expression of the 67LR. This suggests that the transport of the receptor to the cell surface occurs through an unusual secretory pathway, consistent with immunoelectron microscopy data indicating the cytoplasmic localization of the receptor [Romanov et al., 1994].

Many membrane receptors are shed by cells through mechanisms that depend on their membrane anchorage. For example, the high-affinity receptor for folate (FBP), which is bound to the membrane via glycosyl–phosphatidylinosytol, can be released by phospholipase C [Luhrs, 1989], whereas transmembrane receptors such as EGF-receptor or c-erbB-2 oncoprotein are shed through proteolytic cleavage [Petch et al., 1990; Pupa et al., 1993]. A soluble form of the membrane receptor implies physiological regulation of the ligand [Mosley et al., 1989; Colotta et al., 1993] or production of a constitutively activated truncated cell-associated receptor [Kris et al., 1985; Khazaie et al., 1988].

The role of the soluble 67LR in the metastatic process remains unclear. Although the prognostic significance of the 67LR membrane form, which is overexpressed in a variety of human carcinomas [Castronovo et al., 1990; Cioce et al., 1991; Martignone et al., 1992], has been suggested, and most of the hypotheses concerning the role of 67LR in tumor invasion are based on its function as a membrane receptor involved in cell adhesion to the extracellular matrix [Liotta, 1986], the molecule actually involved in tumor aggressiveness might be the soluble form. Indeed, peptide G, the laminin-binding domain of the 67LR enhances the metastatic potential of tumor cells [Taraboletti et al., 1993], which suggests that the free receptor might act in the same way by changing the conformation of laminin.

Whatever the mechanisms responsible for the 67LR shedding, the soluble 67LR that we have demonstrated retains its ability to bind laminin could play a role in the modulation of the interactions of cancer cells to laminin. Because the high aggressiveness of a cancer is consistently associated with high expression of the 67LR, it is possible that soluble 67LR can be detected in the serum of patients and thereby have diagnostic and/or prognostic value.

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