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Formation of the 67-kDa Laminin Receptor by Acylation of the Precursor

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Abstract Even though the involvement of the 67-kDa laminin receptor (67LR) in tumor invasiveness has been clearly demonstrated, its molecular structure remains an open problem, since only a full-length gene encoding a 37-kDa precursor protein (37LRP) has been isolated so far. A pool of recently obtained monoclonal antibodies directed against the recombinant 37LRP molecule was used to investigate the processing that leads to the formation of the 67-kDa molecule. In soluble extracts of A431 human carcinoma cells, these reagents recognize the precursor molecule as well as the mature 67LR and a 120-kDa molecule. The recovery of these proteins was found to be strikingly dependent upon the cell solubilization conditions: the 67LR is soluble in NP-40-lysis buffer whereas the 37LRP is NP-40-insoluble. Inhibition of 67LR formation by cerulenin indicates that acylation is involved in the processing of the receptor. It is likely a palmitoylation process, as indicated by sensitivity of NP-40-soluble extracts to hydroxylamine treatment. Immunoblotting assays performed with a polyclonal serum directed against galectin3 showed that both the 67- and the 120-kDa proteins carry galectin3 epitopes whereas the 37LRP does not. These data suggest that the 67LR is a heterodimer stabilized by strong intramolecular hydrophobic interactions, carried by fatty acids bound to the 37LRP and to a galectin3 cross-reacting molecule. J. Cell. Biochem. 69:244–251, 1998.

Key words: monomeric laminin receptor; receptor maturation; acylation

In 1983, three independent laboratories used affinity chromatography on laminin-Sepharose to isolate a protein with an apparent molecular mass of 67 kDa, designated as 67 laminin receptor (67LR) [Lesot et al., 1983; Malinoff and Wicha, 1983; Rao et al., 1983]. Using this partially purified molecule, polyclonal and monoclonal antibodies were produced to analyze the tissue distribution of the receptor [Liotta et al., 1985; Liotta, 1986]. By means of such reagents, 67LR expression was found to be increased in tumors [Cioce et al., 1991], and to be associated with tumor aggressiveness [Sanjuán et al., 1996; Martignone et al., 1993]. Nevertheless, the exact structure and role of this molecule has not yet been elucidated. One of the main obstacles is the amino-terminal block of the 67LR, which has impeded sequencing. An alternative strategy was then adopted: monoclonal antibodies previously raised against the 67LR were used to isolate the corresponding cDNA from a human expression library [Wewer et al., 1986]. Intriguingly, from these molecular studies, carried out in human as well as in other species [Grosso et al., 1991; Rao et al., 1989; Yow et al., 1988], a full-length cDNA encoding a 37-kDa polypeptide was isolated. This cDNA is homologous to the sequence of p40, a polypeptide associated with the 40S ribosomal subunit, suggesting multifunctions for the 37-kDa molecule [Auth and Brawerman, 1992]. Several polyclonal antibodies raised against synthetic peptides deduced from this cDNA sequence have been generated and pulse-chase experiments carried out on human melanoma cells demonstrated that the 37-kDa polypeptide is the precursor of 67LR [Rao et al., 1989]. The former was thus designated as 37 laminin receptor precursor (37LRP). Epitope-tagged transfection experiments have confirmed a precursor-product relationship between the 37LRP and

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the 67LR (Montuori and Sobel, unpublished data). Despite the use of these anti-peptide antibodies, the mechanism by which the 37-kDa polypeptide matures in the 67LR is still unknown.

To further investigate the 67LR maturation process, a new set of monoclonal antibodies directed against recombinant 37LRP were selected [Butò et al., 1997]. Using these reagents in combination, we investigated the mechanisms of 37LRP maturation to 67LR. The data reported here indicate that the 37-kDa precursor and the 67-kDa receptor have a different solubility, due to the presence of fatty acids, which are in turn responsible for the formation of a dimer that is the 67LR.

MATERIALS AND METHODS

Monoclonal Antibodies Directed Against the 37LRP

The 37LRP cDNA was cloned into prokaryotic expression vector pTrcHisB (Stratagene, San Diego, CA) so that it would be epitope-tagged at its amino-terminus (Montuori and Sobel, unpublished data). Purified His-tagged recombinant 37LRP was used as antigen to produce monoclonal antibodies against the precursor of laminin receptor (MPLR). The production and preliminary characterization of MPLRs have been previously reported [Butò et al., 1997].

Cell Culture and Differential Treatments

Human vulvar epidermoid carcinoma A431 cells (ATCC CRL 1555, ATCC, Rockville, MD) were cultured in RPMI 1640 (Sigma, St. Louis, MO) containing 10% fetal-calf serum (Sigma) and L-glutamine. Cells were grown at 37°C in air/CO_2 (19:1) and 95% humidity.

Cerulenin (Sigma) was first tested at different concentrations (10–200 µg/ml) and vitality of the cells controlled after a 16-h incubation. At 25 µg/ml concentration, the one reported to be active [Kuhajda et al.,1994], no toxicity was found. Cerulenin treatment was performed on subconfluent cells by addition of cerulenin, dissolved in DMSO, to the standard culture medium. After 16 h of treatment the cells were harvested and protein extraction was performed as described below.

Isolation of the 37LRP-Related Proteins

The 37LRP-related proteins were extracted by the method of Wewer et al. [Wewer et al., 1986], with some slight modifications. A431 cells were harvested by rinsing twice with calcium, magnesium-free PBS (CMF-PBS), 10 min each at 37°C. As already described, this harvesting technique was used to preserve membrane protein integrity. Cells were then centrifuged at 500*g* for 10 min. The pellet was resuspended in 10 volumes of CMF-PBS and centrifuged again under the same conditions. Cells were suspended in 2 volumes of ice-cold 25 mM Tris-HCl/300 mM sucrose/1 mM PMSF (Sigma), pH 7.4, and sonicated with 5 x 5 s bursts on ice using a Labsonic U sonicator (B. Braun, Burlingame, CA) at 50% power. Nuclei, cytoskeletal proteins, and unbroken cells were pelleted by centrifugation at 500*g* for 10 min at 4°C, and the supernatant was collected. The pellet was then resuspended in 5 volumes of the above Tris/sucrose buffer, sonication was repeated and this supernatant was added to the first one. These sequential steps were repeated three times. Centrifugation in a Ti65 rotor (Beckman Instruments, Palo Alto, CA) at 150,000*g* for 90 min at 4°C was then performed. The first solubilization step was performed in 25 mM Tris-HCl/150 mM NaCl/1 mM $CaCl_2$/3 mM $MgCl_2$/1% NP-40, pH 7.4 buffer: the solution was rotated end-over-end for 16 h at 4°C. After centrifugation in a Ti65 rotor at 150,000*g* for 60 min at 4°C, the supernatant was collected (NP-40 soluble extract). The resulting pellet was re-solubilized in 50 mM Tris pH 7.4 containing 1% β-octylglucoside, for 1 h at 4°C. The supernatant coming from a centrifugation at 4,000*g* was then collected (β-octylglucoside soluble extract).

Laminin Affinity Chromatography

Laminin affinity columns were prepared by coupling 500 µg laminin (Sigma) to 500 µl AffiGel 10 matrix (Bio-Rad, Richmond, CA), previously equilibrated in PBS. Incubation was performed for 4 h at 4°C under constant agitation. Remaining active esters were blocked by overnight incubation of AffiGel-laminin resin with 200 mM Tris-HCl, pH 7.4. Affinity chromatography was performed by loading the soluble extract, at a concentration of 1 mg/ml, onto the coupled matrix. Incubation was carried out in batch conditions for 3 h at 4°C. After this step, unbound material was removed and the matrix washed successively with: (1) 50 mM Tris-HCl, 0.1% NP-40, 100 mM NaCl, pH 7.4 (10 bed volumes); (2) 50 mM Tris, 0.1% NP-40, 400 mM NaCl, pH 7.4 (10 bed volumes); (3) 200 mM

glycine, 2.7 (5 bed volumes). In all the washing buffers the protease inhibitor PMSF was included, at 1 mM concentration. Eluates were precipitated by the addition of acetone to a final concentration of 50%, followed by centrifugation at 4,000*g* for 15 min at 4°C; the pelleted material was resuspended in 1 ml 70% acetone and centrifuged at 4,000*g* for 15 min at 4°C. Finally, the pellet was resuspended in 100 µl of 10 mM Tris-HCl, pH 7.4, and frozen.

SDS-Polyacrylamide Gel Electrophoresis

Samples were dissolved in reducing sample buffer (50 mM Tris-HCl/20% glycerol/2% 2-mercaptoethanol/5% SDS, pH 6.8) and boiled for 5 min at 95°C; proteins were separated on 8% or 10% SDS-PAGE gels. Prestained Rainbow [^{14}C]methylated (Amersham, Arlington Heights, IL) protein molecular mass markers (14.3–220 kDa range) were used.

Western Blotting Technique

The SDS-PAGE separated proteins were electrophoretically transferred to Hybond-C nitrocellulose membranes (Amersham). The membranes were then blocked with PBS containing 5% non-fat dry milk, and the ECL detection system (Amersham) was performed. The membranes were incubated with the pool of the eight MPLRs (as ascitic fluids, diluted 1:200 each), for 1 h at room temperature, washed and further incubated with biotinylated anti-mouse whole antibody (1:200 dilution) for 1 h. After washing, the membranes were incubated with streptavidin-biotinylated horseradish peroxidase (1:200 dilution) for 30 min and then autoradiographed. The anti-galectin3 serum used in some experiments is a rabbit polyclonal serum raised against the recombinant molecule (the kind gift of Dr. F.-T. Liu, Scripps Clinic, La Jolla, CA). In this kind of assay, the secondary antibody used in the detection system was a biotinylated anti-rabbit whole antibody diluted 1:200 (Amersham).

Hydroxylamine Treatment of A431 Cellular Extracts

One hundred micrograms of a A431 cytosolic extract were incubated for 4 h at room temperature in the presence of freshly prepared 1M hydroxylamine (Merck, Darmstadt, Germany), pH 10. As a control, the same amount of protein extract was incubated in 10 mM Tris, pH 10. Trichloroacetic acid (20% final concentration) was then added to the incubation mixture and the proteins were precipitated after a 30-min incubation at 0°C. Insoluble material was recovered by centrifugation at 4,000*g* for 15 min and the resulting pellet was resuspended in 100 µl of sample buffer and electrophoresis was performed as described above.

RESULTS

Solubility of the Different 37LRP-Related Molecules

Western blotting analysis was performed with MPLRs on two different and sequentially obtained A431 soluble extracts: a NP-40 soluble extract and the fraction solubilized by β-octylglucoside from the NP-40 insoluble fraction. As shown in Figure 1, MPLRs recognized both the precursor and the mature laminin receptor. As already observed (Montuori and Sobel, unpublished data), the precursor molecule shows an anomalous electrophoretic mobility, which accounts for an apparent molecular mass of 44 kDa. The two molecules demonstrated different solubility profiles. NP-40 extraction (Fig. 1, lane 1) solubilized mainly the 67-kDa protein whereas the 37LRP was not detected. The "doublet" band of the 67LR was reported previously [Castronovo, 1993] and was ascribed to differences in the phosphorylation status of the protein. The 37LRP was recovered together with the 67LR after a subsequent solubilization by β-octylglucoside (Fig. 1, lane 2). An additional 120-kDa protein was also detected after the

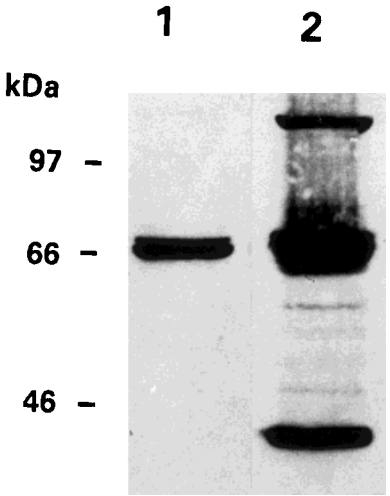

Fig. 1. Western blotting with MPLR pool on NP-40 (**lane 1**) and β-octylglucoside (**lane 2**) soluble extracts from A431 cells. The positions of the prestained molecular mass markers are shown on the left.

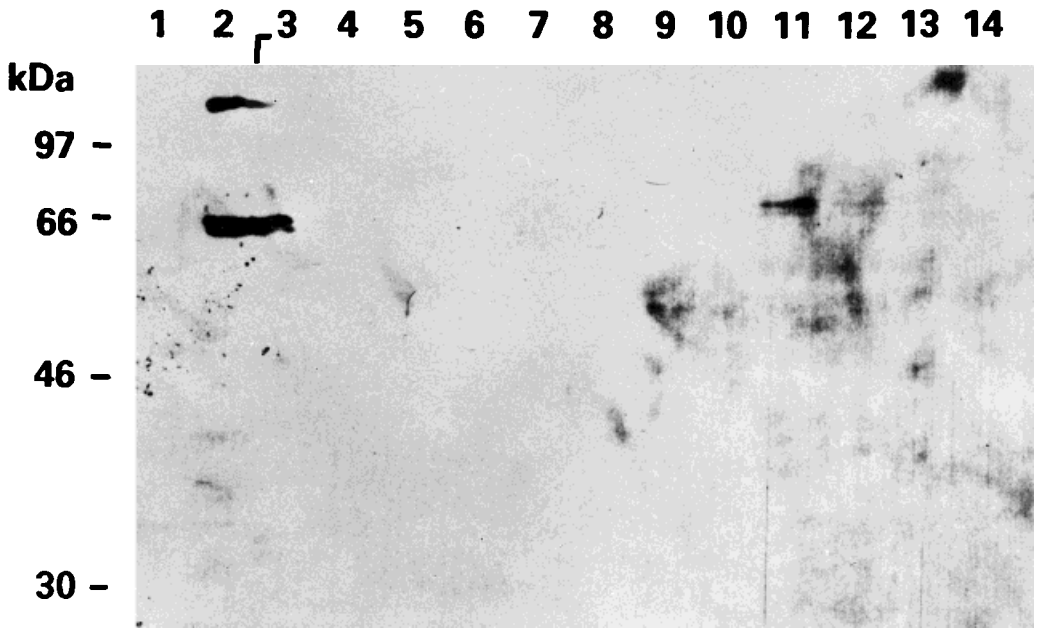

Fig. 2. Western blotting with MPLR pool on an A431 NP-40 soluble extract chromatographed on a laminin affinity column. **Lane 1**, 200 mM Tris-HCl pH 7.4 (active esters blocking buffer); **lane 2**, 100 µg of the material loaded on the matrix; **lane 3**, unbound material; **lanes 4–6**, fractions coming from the elution with 50 mM Tris, 100 mM NaCl, pH 7.4; **lanes 7–10**, fractions coming from the elution with 50 mM Tris, 400 mM NaCl, pH 7.4; **lanes 11–14**, fractions coming from the elution with 200 mM glycine, pH 2.7. The positions of the prestained molecular mass markers are shown on the left.

second solubilization. It should be noted that in some experiments, the 120-kDa protein was partially recovered after NP-40 extraction.

Laminin-Affinity Chromatography

To determine whether the 67-kDa molecule recognized by the anti-37LRP monoclonal antibodies was actually the reported 67-kDa protein able to bind laminin, laminin-affinity chromatography was performed on A431 cells. NP-40 soluble extract and the eluates were then immunoblotted with MPLRs (Fig. 2). Our findings indicate that of the two proteins of 67 and 120 kDa present in the protein extract (Fig. 2, lane 2), only the 67-kDa molecule was able to specifically bind to the laminin column and to be selectively eluted (Fig. 2, lane 11).

Inhibition of Cellular Fatty Acid Synthesis

To study the involvement of fatty acids on the processing of the 67LR we used cerulenin, a well-known inhibitor of fatty acid synthetase [Funabashi et al., 1989]. Figure 3 shows a Western blot analysis performed with MPLRs on cerulenin-treated and untreated A431 cells, solubilized with the two extraction methods. All the experiments were repeated thrice with good reproducibility. While both the 67-kDa and the 120-kDa molecules were present in the NP-40 soluble extract of untreated cells (Fig. 3, lane 1), only the precursor of 37 kDa could be detected in the corresponding soluble extract obtained after cerulenin-treatment (Fig. 3, lane 2). In the β-octylglucoside soluble extract of

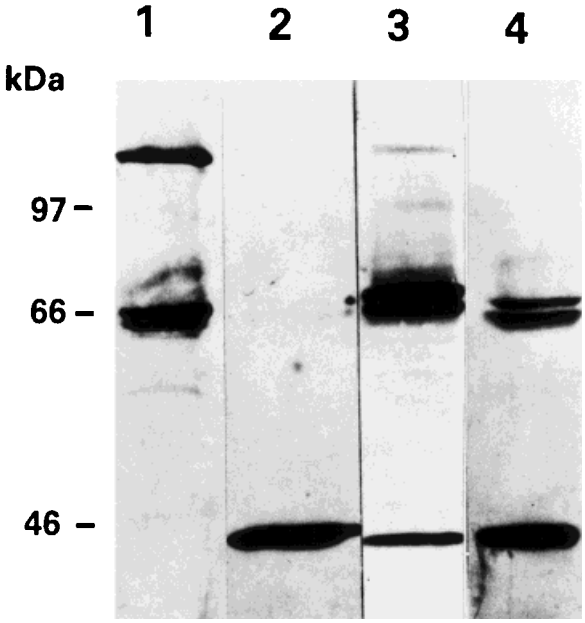

Fig. 3. Westen blotting with MPLR pool on NP-40 (**lanes 1** and **2**) and β-octylglucoside (**lanes 3** and **4**) protein extracts from A431 cells incubated in the absence (lanes 1 and 3) and presence (lanes 2 and 4) of cerulenin.

untreated A431 cells, the molecule migrating at 67 kDa was found in addition to the 37LRP band (Fig. 3, lane 3), whereas in cerulenin-treated cells the amount of 67LR decreased concomitantly with an increase in the 37LRP (Fig. 3, lane 4). Soluble extracts obtained from A431 cells incubated in the presence of DMSO alone gave the identical immunoblotting profile of the untreated cells (data not shown). The data reported suggest that inhibition of fatty acid addition to the 37LRP results in an inhibition of 67LR synthesis. The specificity of cerulenin treatment was confirmed by the finding that EGF receptor expression was unaffected in the treated cells (data not shown).

Hydroxylamine Treatment of Protein Extracts

To further investigate the involvement of acylation processes in the 67LR build up, an A431 NP-40 soluble extract was treated with hydroxylamine, which is reported to induce the release of ester-bound fatty acids from protein moieties by chemical cleavage of the corresponding bond [James and Olson, 1989]. In these conditions, the only fatty acids that can be removed are those linked to the protein via thioester or oxyester bonds (principally palmitate), whereas amide-linked fatty acids are not cleaved off. As shown in Figure 4, the untreated extract contains mainly the 67LR and a weak 120-kDa band (Fig. 4, lane 1). Hydroxylamine treatment induces the recovery of other proteins migrating at an apparent molecular mass ranging from 30 to 37 kDa (Fig. 4, lane 2). Even if hydroxylamine treatment led to a disturbed electrophoretic run, the electrophoretic mobility of the bands detected by MPLRs in this lane is consistent with the molecular weight reported for the 37LRP molecule. As for cerulenin treatment, EGF receptor migration pattern was used to assess the specificity of the hydroxylamine treatment (data not shown).

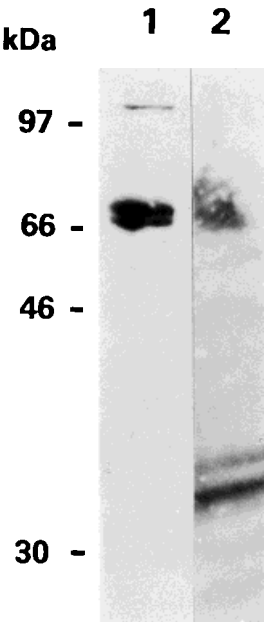

Fig. 4. Western blotting with MPLR pool on an A431 NP-40 soluble extract: untreated (**lane 1**) and treated (**lane 2**) with hydroxylamine. The positions of the prestained molecular mass markers are shown on the left.

Presence of a Lectinic Epitope in the 67LR

Galectin3 is an acylprotein involved in tumor invasion and metastasis whose lectin features confer upon it a laminin-binding activity [Van den Brûle et al., 1995]. Since it has been suggested [Castronovo, 1993] that the 67LR expresses epitopes cross-reacting with β-galactoside-binding lectins, immunoblotting of detergent extracts of A431 cells was performed using a polyclonal anti-galectin3 antiserum. The anti-galectin3 antibodies recognized the 30-kDa galectin3 in addition to a band of 67 kDa in both β-octylglucoside and NP-40 extracts and a 120-kDa molecule only in NP40-extracts (Fig. 5A and lane 1 of 5B). As expected, the 37LRP was not detected in any extract by the specific anti-galectin3 antiserum. On an NP-40 soluble extract obtained after treatment of A431 cells with cerulenin, there was a marked decrease in detectable 30-kDa (galectin3), 67-kDa, and 120-kDa molecules (Fig.5B).

DISCUSSION

The data presented herein indicate that acylation plays a key role in the processing of the laminin receptor from the 37-kDa precursor form to the 67-kDa mature form. Indeed, we have demonstrated that inhibition of acylation by cerulenin inhibits the formation of the mature form and leads to an accumulation of the precursor. The partitioning of the different 37LRP-related molecules between soluble and insoluble phases after different solubilization steps is likely dependent upon their acylation degree and the exposition of the acyl groups. The observation that the 37LRP is insoluble in NP-40 lysis buffer unless cells are treated with cerulenin demonstrates the involvement of acyl groups in the overall hydrophobicity of the protein. Consistent with this hypothesis is the observation that the large T antigen recovered

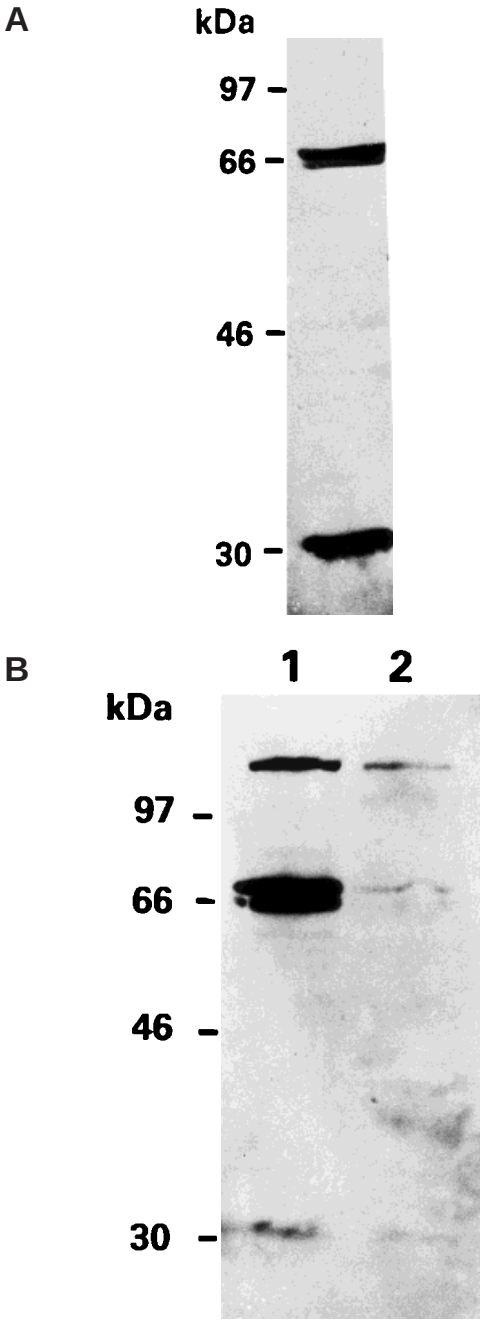

Fig. 5. Western blotting with anti-galectin3 serum on an A431 β-octylglucoside soluble extract (**A**) and NP-40 soluble extracts (**B**) from untreated (**lane 1**) and cerulenin-treated (**lane 2**) cells.

in the NP-40 soluble fraction is not acylated whereas the acylated form is associated with NP-40-resistant plasma membrane lamina from which it can only be released by rigorous treatment [Grand, 1989].

On the other hand, the fact that the 67LR is fairly soluble after NP-40 extraction suggests that the acyl groups in this molecule are in some way hidden. Actually, alkaline hydroxylamine treatment of an NP-40 soluble extract induces the formation of molecules migrating at the apparent mass of the precursor, with only a slight decrease in 67LR detection. This suggests that acyl groups present in the 67-kDa protein are not equally sensitive to hydroxylamine action. The cleavage by hydroxylamine indicates the presence of ester-bound fatty acids, namely palmitate molecules bound to cysteine, serine, or threonine residues [Towler and Gordon, 1988], in agreement with some data recently reported [Landowski et al., 1995]. Indeed, Landowski and coworkers for the first time reported data arguing a 67LR acylated status. In their model, laminin receptor is presented as a 37LRP homodimer. Instead, our data showing the presence of lectin epitopes present in the 67LR but not in the 37LRP suggest the existence of other processing pathways, such as a heterodimerization between the 37LRP and another acylated protein carrying galectin3 epitopes. Actually, if the 67-kDa protein is a heterodimer between the 37LRP and an unknown polypeptide, the latter should be a lectin since the 67LR can be eluted from laminin-affinity chromatography columns by lactose [Hinek et al., 1994].

In our experimental conditions, 67LR migrates at an apparent molecular mass that lies between 65 and 70 kDa. This value does not certainly match the value that could be expected based on the electrophoretic mobility of its precursor. However, acyl groups are frequently known to alter protein migration features. Indeed, Landowski and coworkers also [Landowski et al., 1995] report a 67LR that migrates nearby the 67-kDa electrophoretic position.

Even though the molecular weight of galectin3 corresponds to that expected for the unknown molecule participating in the 67LR formation, it is unlikely that galectin3 itself is involved in the maturation of the 67LR. Galectin3 mRNA and 37LRP mRNA are inversely expressed in most cancers; i.e., galectin3 mRNA is decreased routinely in tumors overexpressing the 67LR [Castronovo et al., 1992]. Since galectins belong to a wide family of cross-reacting molecules [Barondes et al., 1994], other members of this family might be involved in interacting with the 37LRP. Treatment with cerulenin induces the decrease of both the 67- and the 120-kDa molecules carrying the galectin3 epitope with no concomitant accumulation of galectin3 epitope-carrying molecules: the non-

acylated form could be unstable or the cross-reacting epitope recognized by the anti-galectin3 antibody could depend upon acylation. Indeed, we cannot exclude that the galectin3 epitope is given by the sole fatty acid moiety. The hypothesis of heterodimerization does not exclude the possibility of homodimer formation if an excess of acylated 37LRP is produced in an artificial system such as transfected cells, as described for CHO cells [Landowski et al., 1995].

The 120-kDa protein is likely a dimer of the 67LR since it follows the same behaviour as the 67LR. From the affinity chromatography experiments, this molecule does not appear to be able to bind laminin. However, since the molecule was present only in a small amount in the extract, its presence in the eluate might have been undetectable.

The two polypeptides that take part in dimer formation of the 67LR are linked by noncovalent interactions, e.g., a fatty acid-fatty acid interaction. It must be noted that SDS-PAGE conditions are not strong enough to denaturate this kind of bond. In fact, fatty acids could be regarded as sodium dodecyl sulphate-like molecules and, therefore, could help in maintaining intermolecular interactions even in a microenvironment rich in SDS.

The overexpression of the 67LR has been associated with an increase in tumor aggressiveness and, therefore, inhibition of its formation should decrease malignancy. Noteworthy, treatment of mice bearing xenotransplanted human 67LR-overexpressing ovarian carcinoma with cerulenin caused a reduction in ascites incidence, a delay in onset of ascites, and significant increased survival [Pizer et al., 1996].

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