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BIOSYNTHESIS OF THE 67 kDa HIGH AFFINITY LAMININ RECEPTOR

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High affinity interactions between cells and laminin are mediated, at least in part, by the 67 kDa laminin receptor (67 LR). A 37 kDa nascent polypeptide (37 LRP), predicted by a full length cDNA clone and obtained by in vitro translation of hybrid-selected laminin receptor mRNA, has been immunologically identified in cancer cell extracts as the putative precursor of the 67 LR. In this study, we used affinity purified antibodies developed against cDNA-deduced 37 LRP synthetic peptides in pulse chase experiments and demonstrated a precursor-product relationship between the 37 LRP and the 67 LR. Immunoblot, pulse chase and immunofluorescence experiments showed that transient transfection of the full length 37 LRP cDNA clone induced a dramatic increase in the synthesis of the 37 LRP but not of the mature 67 LR. We propose that the 67 LR results from the association of two gene products: the 37 LRP and a polypeptide yet to be identified.

Interactions between cells and laminin are mediated through specific membrane receptors, among which, a high affinity cell surface receptor with an apparent molecular weight of 67 kilodaltons (67 LR) is differentially expressed in neoplastic cells (1,2). The coding sequence predicted by full length cDNA clones of the laminin receptor is 295 amino acids (3,4). We have previously reported that the nascent translation product of selectively-hybridized human laminin receptor mRNA has an apparent molecular mass on SDS-polyacrylamide gels of 37 kDa (37 LRP)(4). Efforts to demonstrate a precursor-product relationship between the 37 LRP and the 67 LR have been hampered in the past by the inability of anti-laminin receptor antibodies to immunoprecipitate both the 37 LRP and the 67 LR. In the current study, we developed immunoprecipitating anti-37 LRP synthetic peptide antibodies and used them in pulse chase experiments to definitively demonstrate that the cDNA-predicted nascent 37 kDa polypeptide is the precursor of the 67 LR. Transient

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transfection of the full length 37 LRP cDNA into COS 7 cells, a simian renal cell line constitutively expressing 37 LRP and 67 LR, resulted in a dramatic increase in the expression of the 37 LRP, but not of the mature 67 LR. We therefore propose that the 67 LR results from the posttranslational association between the 37 LRP and another, yet to be identified, gene product.

MATERIALS AND METHODS

Preparation of antibodies to synthetic peptides. Peptides were synthesized according to the predicted amino acid sequence of the 37 LRP cDNA on a Biosearch 9600 peptide synthesizer. Antibodies 4099, 4056, 4162 and 4160 were each raised against a different synthetic peptide and were affinity purified and characterized as described (4). Antibody 4160 is known to recognize an extracellular domain of the receptor (2).

Pulse chase experiments. A2058 cells (4) or transfected COS 7 cells (5) were radiolabeled for 30 min with [^{35}S] methionine (1153 Ci/mmol, ICN) as described (4). For the pulse chase experiments, after radiolabeling, the cells were rinsed twice in Dulbecco's medium containing 10% fetal calf serum and then incubated in the same medium at 37°C for the designated periods of time. The radiolabeled cell layers were lysed and immunoprecipitated as described (4).

Construction of an expressing laminin receptor precursor cDNA clone. An expressing cDNA clone of the 37 LRP sequence, designated pPLR1.2, was constructed in two steps. First, prototype expression clone pPLR1.1 was constructed: EcoR1 and SalI recognition sequences were inserted at the 5' end of the murine 37 LRP coding sequence of clone pMLR11 (6), and the modified cDNA, including the 3' untranslated region and a short poly(A) tail with a BamHI recognition sequence at the 3' end, was inserted into the EcoRI/BamHI sites of vector pGEM4 (Promega, Madison, WI). Second, the SalI/BamHI restriction fragment of pPLR1.1 was inserted into the compatible XhoI/BamHI sites of vector pSVL (Pharmacia, Piscataway, NJ). The XhoI site in pSVL is downstream from the SV40 late promoter. Thus, laminin receptor mRNA can be transcribed from pPLR1.2 in T antigen-producing cells such as COS 7. The size of the recombinant transcript should be 350 bases longer than authentic laminin receptor mRNA.

Transient transfection. Increasing amounts of pPLR1.2 (or pSVL vector as a control) were transfected into COS-7 cells using the electroporation technique as described (5). The efficiency of the transfection was assessed by quantification of the level of 37 LRP mRNA in the transfected cells. Total cellular RNA was isolated from pPLR1.2- and pSVL-transfectants and hybridized to laminin receptor cDNA insert as described (1).

Immunoblots and immunofluorescence experiments. The effects of 37 LRP-cDNA transfection on the expression of 37 LRP and the mature 67 LR were evaluated by immunoblot, immunofluorescence, and pulse chase experiments performed on both pPLR1.2- and pSVL-transfected COS 7 cells. Total cell extracts of transfected and control COS 7 cells were prepared as described (4), and equal amounts of cell extract protein were electrophoresed on a 10% SDS-polyacrylamide gel and transferred onto PDVF membrane as described (4). The amount of 37 LRP and 67 LR were quantified by incubation of the blots with anti-37 LRP antibodies 4160 and 4099 as described (4). These antibodies recognize the 37 LRP or the 67 LR, respectively, on immunoblots. The sensitivity of the detection system was evaluated

for each antibody by testing reactivity with increasing amounts of cell extract protein. Immunofluorescence was performed as described (2) on permeabilized and nonpermeabilized transfected and control COS 7 cells using antibody 4160. The intensity of staining was evaluated according to a scale from + to +++++.

RESULTS AND DISCUSSION

Previous experiments have suggested that the 37 LRP cDNA clone may encode a precursor of the 67 LR that has a calculated molecular mass of 33 kDa and that has the mobility on SDS polyacrylamide gels of a polypeptide with an apparent mass of 37 kDa. The original 37 LRP cDNA clones were isolated from an expression library using a monoclonal antibody that recognized the 67 LR on immunoblots of tumor cell extracts and that inhibited laminin binding to cells and cell membranes (7). The anti-67 LR monoclonal antibody did not recognize any other cDNA clones out of a total of 1.5×10^6 plaques (7). There was complete sequence homology between the 37 LRP cDNA-predicted amino acid sequence and cyanogen bromide generated peptides of purified human 67 LR (7). When the 37 LRP cDNA probes were hybridized to laminin receptor mRNA on Northern blots, a single size class mRNA was detected even on overexposed radioautographs (4,7). Furthermore, primer extension experiments detected a single size mRNA transcript (7). It is thus likely that the 37 LRP and the 67 LR are encoded by the same mRNA. Indeed, the RNA transcript of the 37 LRP cDNA clone may encode a precursor of the 67 LR. If this were the case, it should be possible to detect a cellular protein with an apparent molecular mass of 37 kDa. To investigate this possibility, synthetic peptides deduced from the 37 LRP cDNA sequence were used to generate antibodies. Antibody 4056 as well as other anti-synthetic peptide antibodies recognized both the 37 LRP and the 67 LR on immunoblots of human fibrosarcoma cell extracts (4), providing evidence that there is an antigenic relationship between the 37 LRP and the 67 LR. Furthermore, antibody 4056 immunoprecipitated a protein from metabolically labeled A2058 cells with an apparent molecular mass on SDS polyacrylamide gels of 37 kDa. The mobility of the immunoprecipitated protein was identical to that of the in vitro translation product of mRNA that was hybrid-selected using the 37 LRP cDNA clone (4). The immunoprecipitation and immunoblot experiments confirm that the cDNA-predicted 37 LRP is indeed a cellular protein.

To test the hypothesis that the 37 LRP nascent protein is the precursor of the 67 LR, we performed pulse chase experiments using affinity purified anti-37 LRP synthetic peptide antibodies. Human melanoma A2058 cells were pulsed with [^{35}S] methionine for 30 min and then chased for various time intervals up to 5 hours, and total

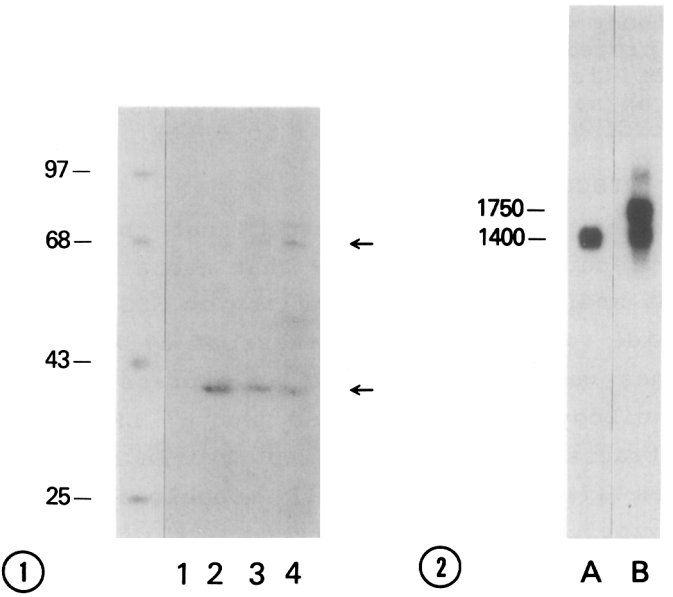

Figure 1. Precursor-product relationship between the 37 LRP and the 67 LR. A2058 cells were pulse labeled with [^{35}S] methionine and chased with methionine-containing media as described in Materials and Methods. At different time intervals, cell layers were lysed and immunoprecipitated with antibody 4160. Samples were run alongside [^{14}C] protein markers (Bethesda Research Laboratories) whose molecular masses in kDa are shown to the left. Lane 1: metabolically labeled cell extract incubated with protein A agarose only. Lanes 2-4: metabolically labeled cell extracts immunoprecipitated with antibody 4160 after 0 min (lane 2), 30 min (lane 3) and 90 min (lane 4) chase. The arrows designate the 37 LRP and the 67 LR.

Figure 2. Northern blot hybridization. Total cellular RNA was extracted from COS 7 cells transfected with pSVL (control, A) or with pPLR1.2 (B), electrophoresed on a 1.2% agarose gel containing formaldehyde, and hybridized with a 37 LRP cDNA probe as described in Materials and Methods. The length of the hybridized mRNAs (1400 bases for the physiological 37 LRP mRNA and 1750 bases for the mRNA transcribed from the pPLR1.2) was determined by comparison with the known size of rRNA and with the sizes of lambda DNA digested with *Hind* III.

cell extracts were immunoprecipitated with antibodies 4056, 4160, and 4162. A representative experiment is shown in Figure 1. Each of the antisera immunoprecipitated a 37 kDa pulse labeled product (lane 2) that was chased into a 67 kDa polypeptide within 30 minutes (lane 3). The intensity of the 37 kDa band decreased as the 67 kDa band increased (lanes 2, 3, and 4); the 37 kDa band completely disappeared after 3 hours (data not shown). Two minor bands, with apparent molecular masses of 70 kDa and 50 kDa, were also detected during the chase. The 70 kDa polypeptide was previously shown to be immunoreactive with polyclonal antibodies to the natural 67 kDa laminin receptor (8). It may be an intermediate form of the receptor that is synthesized from the 37 LRP and is later proteolytically cleaved to form the 67 LR. The 50 kDa band appears after a 90 min

chase (lane 4) and is most likely a degradation product of the 67 LR. Indeed, we have detected it in preparations of purified 67 LR that have been allowed to sit overnight (data not shown).

The pulse chase experiments demonstrate a precursor-product relationship between the 37 LRP and the 67 LR. It is not clear what biosynthetic mechanism(s) may be responsible for the molecular weight shift from 37 kDa to 67 kDa. Traditional mechanisms for posttranslational modifications such as glycosylation, acylation and phosphorylation are most likely not sufficient to account for this large molecular weight shift. No N-linked glycosylation sites are predicted by the cDNA clone (4), and appropriate N-linked endoglycosidases do not shift the mobility of purified 67 LR on SDS polyacrylamide gels (7). Although there are multiple serine and threonine residues predicted by the cDNA clone, O-linked glycosylation does not appear to play a role in the biosynthesis of the 67 LR. We have been unable to immunoprecipitate any proteins from A2058 cells that were metabolically labeled with [^{3}H] glucosamine or [^{3}H] galactosamine. Nor have we been able to stain purified 67 LR with Periodic Acid Shift reagent (data not shown).

One possible explanation for the rapid shift in molecular weight from 37 kDa to 67 kDa demonstrated in the pulse chase experiments might be the linkage of the 37 LRP to another polypeptide to form the 67 LR. Reduction and alkylation of the 67 LR does not convert it to a 37 kDa form. Therefore, linkage of the 37 LRP to another peptide is not disulfide bond mediated. One potential mechanism could be a transpeptidation reaction in which the 37 LRP is covalently linked to another gene product. A similar mechanism has been proposed for the synthesis of the human red cell glucose-6-phosphatase dehydrogenase (9). Similarly, the human Lyt-3 molecule must be incorporated into a heterodimer with CD8 for cell surface expression (10).

We attempted to study the biosynthesis of the 67 LR in experiments in which the 37 LRP could be overexpressed in COS 7 cells after transfection of the 37 LRP cDNA. COS 7 cells express the 37 LRP mRNA (Figure 2, lane A) and constitutively express both the 37 LRP and the 67 LR (Figure 3). Presumably, if traditional posttranslational mechanisms such as glycosylation or acylation are responsible for the 37 kDa to 67 kDa shift, then COS 7 cells should be able to overexpress the 67 LR when transfected with 37 LRP cDNA. On the other hand, if another gene product is required to form a 67 LR heterodimer, that gene product would be in limiting supply and the 67 LR would not be overexpressed. This strategy has been used to determine that three different gene products are required for expression of the high affinity IgE receptor in COS cells (11). We

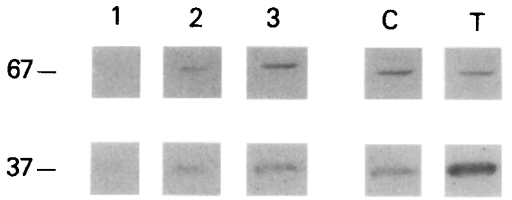

Figure 3. Transfection of COS 7 cells with pPLR1.2 increases the expression of 37 LRP but not of 67 LR. Increasing amounts of total COS 7 cell protein extracts (1, 2, and 3 correspond to 1 µg, 5 µg and 10 µg, respectively) were electrophoresed on 10% SDS-polyacrylamide gels, blotted onto PDVF membranes and incubated with antibodies 4099 to detect the 67 LR (67-) or 4160 to detect the 37 LRP (37-) as described in Materials and Methods. Equal amounts of protein extracts from COS 7 cells transfected with pSVL (C) or pPLR1.2 (T) were also electrophoresed as described above and immunoblotted using antibodies 4099 and 4160.

therefore subcloned the complete sequence of the murine laminin receptor in the pSVL vector, which contains the SV40 late promoter upstream of the insert. The resulting pPLR1.2 construct was transfected into COS 7 cells. Figure 2 shows that COS 7 cells transfected with pPLR1.2 transcribe both constitutive amounts of the 37 LRP mRNA as well as an RNA from the pPLR1.2 construct that is 350 bases longer.

Immunoblot experiments performed on increasing amounts of COS 7 cell extracts show that both the 37 LRP and the 67 LR can be detected immunologically (Figure 3). Compared to COS 7 cells transfected with the control pSVL vector, pPLR1.2-transfected COS cells contain significantly more 37 LRP (Figure 3). In contrast, there is no corresponding increase in expression of the 67 LR in the pPLR1.2 transfectants.

Similarly, pulse chase experiments showed that the pPLR1.2 transfectants synthesized more 37 LRP than did control cells, but there was no parallel increase in the 67 LR after chasing (data not shown). Immunofluorescence experiments performed using antibody 4160 showed a dramatic increase in the staining of permeabilized pPLR1.2-transfected COS 7 cells (+++++) compared to control cells (++), but no difference in the staining of nonpermeabilized cells (+). This suggests that the anti-37 LRP synthetic peptide can detect intracellular levels of 37 LRP (in permeabililzed cells) but can detect only cell surface-associated 67 LR when nonpermeabilized cells are used. Consistent with these data, pPLR1.2-transfected COS cells and control cells attached (1) to laminin-coated wells to the same extent (35% ± 6 versus 38% ± 4 respectively).

The transfection experiments presented here demonstrate that an increase in the synthesis of the 37 LRP does not result in increased expression of the 67 LR in either the cytosol or the cell membrane.

We therefore propose that the 67 LR is a chimeric protein composed of the 37 LRP and another gene product that is a rate-limiting factor for the synthesis of the mature 67 LR. The hypothetical second polypeptide may belong to the galactoside lectin family, since antibodies against other galactoside binding lectins can detect the 67 LR on immunoblots (manuscript submitted). We are currently purifying sufficient quantities of the 67 LR to permit extensive protein sequence determination that will confirm the existence of a second gene product in the 67 LR and determine its identity.

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