siRNA-MEDIATED SILENCING OF THE 37/67-kDa HIGH AFFINITY LAMININ RECEPTOR IN Hep3B CELLS INDUCES APOPTOSIS

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Abstract: The laminin-binding protein, variously called the 37/67-kDa high affinity laminin receptor or p40, mediates the attachment of normal cells to the laminin network, and also has a role as a ribosomal protein. Over-expression of this protein has been strongly correlated with the metastatic phenotype. However, few studies have investigated the cellular consequence of the ablation of this gene’s expression. To address this issue, the expression of the 37/67-kDa high affinity laminin receptor was knocked out with several siRNA constructs via RNA interference in transformed liver (Hep3B) cells. In each case where the message was specifically ablated, apoptosis was induced, as determined by annexin V/propidium iodide staining, and by double staining with annexin V and an antibody directed against the 37/67-kDa high affinity laminin receptor. These results suggest that this protein plays a critical role in maintaining cell viability.

Key words: siRNA, RNA interference, Laminin receptor, p40, Ribosomal, Liver, Silencing, LAMR1

INTRODUCTION

The multifunctional protein which we here designate the 37LBP/67LR protein has been variously called the 37/67-kDa high affinity laminin receptor protein, the 37-kDa laminin-binding protein (37LBP), the laminin receptor precursor...
(LRP), the 67-kDa laminin receptor (67LR), LAMR1, and the laminin-binding protein precursor p40 (LBP-p40). It was initially identified as a 67-kDa protein through its high affinity interaction with laminin [1-3], a predominant glycoprotein component of the extracellular matrix that mediates cell attachment, movement, growth and differentiation. The screening of human cDNA libraries using antibodies directed against the purified protein enabled the isolation of a full-length cDNA encoding a protein with a calculated molecular mass of 32 kDa and an apparent molecular weight of 37 kDa, after in vitro translation of hybrid-selected mRNA and SDS-PAGE analysis [4]. Highly homologous cDNAs were subsequently isolated from human colon cancer cell lines [5] and obtained in a study on the structure and sequence determination of the rat 40S ribosomal subunit [6]. LPB-p40 was localized on 40S ribosomes [7] and in the nucleus [8], while 67LR was located on the cell surface, where, in addition to its role as a high affinity laminin receptor, it was shown to function as the receptor for elastin [9] and as a positional marker for the differentiation of the fetal eye organ [10]. 37-kDa LRP was identified as a PrP^c accomplice [11], and 37LBP/67LR acts as a receptor for PrP^c [12] and infectious prions [13], as extensively reviewed elsewhere [14-16]. Additionally, 37LBP/67LR acts as a receptor for a number of viruses, including sindbis [17], dengue [18], and the adeno-associated virus serotypes 8, 2, 3, and 9 [19]. Although expression of the mature 67-kDa form of the protein was detected on many normal cells, the immature, 37-kDa form was identified as an oncofetal antigen [20, 21], and its over-expression directly correlated with the increased invasiveness and metastatic potential of a number of different tumours (reviewed in [22]). Although a clear precursor-product relationship between the 37-kDa (or 40-kDa) and 67-kDa forms was established [23], the exact mechanism by which the 37-kDa form gives rise to the 67-kDa form has yet to be established, although post-translational modification involving acylation [23], specifically via palmitoylation [24], has been implicated. Inhibiting 37LBP/67LR with a specific immunoreactive polyclonal antibody inhibited the attachment of a human fibrosarcoma cell line in a dose-dependent manner, and inhibited the formation of pulmonary metastases in a mouse model system [25]. Down-regulation of 37LBP using antisense cDNA constructs was shown to induce apoptosis in HeLa cells [26]. Down-regulation of 37LBP/67LR expression using an siRNA approach resulted in a reduction in PrP^c propagation in Scrapie-infected neuronal cells [27]. More recently, 37LBP/67LR expression was knocked down in the mouse brain using an antisense-LRP RNA approach [28]. One anecdotal report suggested that inhibiting 37LBP/67LR using small interfering RNAs (siRNAs) may induce apoptosis in several cell types [29]. Given the multifunctional nature of this protein, we sought to formally verify whether siRNA-mediated knock-down of expression resulted in the induction of apoptosis in transformed liver cells.
MATERIALS AND METHODS

Cell culture
The human hepatoma cell line Hep3B [30] was cultivated at 37°C under 5% CO2 in Dulbecco’s modified Eagle’s medium (DMEM; HyClone, Logan, Utah) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco BRL, Gaithersburg, MD) and 100 U of penicillin-streptomycin (HyClone) per ml.

siRNA design and generation
The target sites on the human 37LBP/67LR (GenBank accession number NM_002295) and the green fluorescent protein (GFP; GenBank accession number U50974) were determined using the online tool from Ambion, Austin, TX (http://www.ambion.com/techlib/misc/siRNA_finder.html). The selected sequences were subjected to siRNA template design to generate DNA oligonucleotide sequences for use with the Silencer™ siRNA Construction kit (Ambion). Six templates for siRNA generation were selected:

- siLRP1: 5’-AATTTCAGGGTGAATGGACTG-3’ (nt 762-782);
- siLRP2: 5’-AAATTTTCACAATGTCCGGAG-3’ (nt 75-95);
- siLRP3: 5’-AAATCTCAAGAGGACCTGGGA-3’ (nt 232-252);
- siLRP4: 5’-AACCTTCACTAACCAGATCCA-3’ (nt 403-423);
- siLRP5: 5’-AACAACAAGGGAGCTCACTCA-3’ (nt 575-595);
- siGFP: 5’-AAAGATGACGGGAACTACAAG-3’ (nt 295-315).

The numbering indicates the corresponding position of the selected 21-nucleotide sequence in the open-reading frame of NM_002295 (siLRP1 to siLRP5) or U50974 (siGFP). All the sequences were searched against the NCBI database to confirm specificity to human 37LBP/67LR or GFP. Sense and antisense DNA templates were chemically synthesized (BioBasic, Canada), and following the kit instructions based on \textit{in vitro} transcription, the siRNAs were produced and quantified by spectrophotometry. To confirm that the generated siRNAs were double-stranded, an aliquot of each siRNA was digested individually with RNaseIII or RNaseA. For RNaseIII treatment, 3 µg of siRNA, 1x MnCl2, 1x ShortCut reaction buffer (50 mM Tris-HCl, 1mM DTT, pH 7.5) and 3 µl of ShortCut® RNaseII (New England Biolabs, Inc. Ipswich, MA) were combined in a total volume of 20 µl, and incubated at 37°C for 20 min. The reaction was stopped with the addition of EDTA. For the RNaseA treatment, 3 µg of siRNA, 1x RNaseA buffer (300 mM NaOAC, 10 mM Tris-HCl, pH 7.5 and 5 mM EDTA) and 0.01 µg/µl of RNaseA were mixed in a total volume of 20 µl, and the reaction mixture was incubated at 37°C for 5 min before being terminated on ice. All the samples were analyzed by gel electrophoresis.

siRNA labeling
GAPDH-siRNA provided in the Silencer™ siRNA Labeling kit (Ambion) was end-labeled with the provided Cy3 by mixing the GAPDH-siRNA with the provided 10x labeling buffer and Cy3 labeling reagent. The reaction mixture was then incubated in the dark at 37°C for 1 h, and excess label was removed via
ethanol precipitation by the addition of 0.1 volumes of 5 M NaCl and 2.5 volumes of 100% ethanol, with incubation at -20°C for 1 h. The Cy3-labeled GAPDH-siRNA was pelleted by centrifugation at 10,000 g for 20 min, and the pellet was washed with 70% ethanol. Finally, the pellet was air-dried and dissolved in nuclease-free water. The concentration and base:dye ratio of the labeled siRNA was measured by spectrophotometry.

**siRNA transfection**

Hep3B cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum without antibiotics. Reverse transfections were performed with Lipofectamine™RNAiMAX (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocols, by mixing the respective siRNA and 1.2 µl of Lipofectamine™RNAiMAX, and adding to a single well of a 24-well plate. After 20 min of incubation at room temperature, a suspension of 5x10^4 Hep3B cells was added, and the cell:complex mixtures were incubated under standard conditions. Mock transfections (lipofectamine only) were performed in parallel. To assess transfection efficiency using Cy3-labeled GAPDH-siRNA, a glass cover slip was placed in the well prior to the transfection mix. All the transfections were undertaken in a final volume of 600 µl with siRNA at a final concentration of 50 nM. The cells transfected with Cy3-labeled GAPDH-siRNA were analyzed via fluorescent microscopy at 24 h post-transfection, while other transfections were harvested 1 to 4 days post-transfection. For the analysis of apoptosis, a total of 1x10^5 Hep3B cells were reverse-transfected in a 6-well plate using the same final siRNA concentration and 2.4 µl of the transfection agent. All the transfections were undertaken independently in triplicate.

**Fluorescent microscopy**

After 24 h, the cells transfected with Cy3-labeled GAPDH-siRNA were fixed with 4% paraformaldehyde in PBS at room temperature for 30 min, and then permeabilized by incubation with 0.3% TritonX-100 in PBS for 5 min at room temperature. The coverslips were incubated with 1:500 DAPI in 0.3% TritonX-100/PBS for 10 min at room temperature, followed by two washes with PBS. They were then mounted on glass slides with Vectashield (Vector Laboratories, Inc.) mounting medium. The fluorescent signal was visualized under an Olympus BX61 fluorescent microscope.

**RNA extraction and RT-PCR analysis**

Transfected cells from a single well of a 24-well plate were homogenized in 0.5 ml Trizol reagent (Molecular Research Center, Cincinnati, OH) and allowed to stand at room temperature for 5 min. The cell lysate was vigorously shaken for 15 s in the presence of 0.1 ml chloroform, and allowed to stand at room temperature for 3 min, followed by centrifugation at 12,000 g and 4°C for 15 min. The aqueous phase solution was transferred to a new tube and precipitated with 0.25 ml isopropanol at room temperature for 10 min, followed
by centrifugation at 12,000 g and 4°C for 10 min. The RNA pellets were washed with 75% ethanol with subsequent centrifugation at 7,500 g and 4°C for 5 min. They were air-dried. Finally, the RNA was dissolved in DEPC-treated water. For the RT-PCR analysis, an oligo(dT)$_{17}$ primer was used to synthesize the first strand cDNA using ImpromII™ reverse transcriptase (Promega, Madison, WI). The cDNA was then amplified in a multiplex reaction with 2 specific primer pairs for 37LBP/67LR (LRPf: 5’-TCACTCAGTGGTTTGATGTG-3’; LRPr: 5’-TTTACAGACCAGTCTGCAACCTC-3’), with GAPDH (GAPDHf: 5’-TTG GTATCGTGGAGGACTCA-3’; GAPDHR: 5’-ACCACCTGGGTGCTCAG TGTA-3’) as an internal control. The expected products were 343 bp (GAPDH) and 247 bp (37LBP/67LR). The cycle conditions were 94°C for 3 min, followed by 20 cycles of 94°C for 30 s, 58°C for 45 s and 72°C for 45 s, followed by a final extension of 72°C for 7 min. The PCR products were analyzed on 1.8% agarose gels containing ethidium bromide.

**Protein extraction and Western blot analysis**

A total of 2.5x10$^5$ Hep3B cells were reverse transfected in 6-well plates using a final siRNA concentration of 50 nM and 6 µl of transfection agent. On days 3 and 4 post-transfection, the transfected cells were harvested by scraping from the tissue culture plates, and transferred into 1.5-ml tubes followed by centrifugation at 1,500 rpm for 5 min to pellet the cells. The culture medium was then discarded, and the cell pellets were resuspended in ice-cold 1x PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na$_2$HPO$_4$ and 1.4 mM KH$_2$PO$_4$). After centrifugation at 1,500 rpm for 5 min, 1x PBS was removed, and the cells were lysed by vigorous vortexing in RIPA lysis buffer (1x PBS with 1% Nonidet P-40, 0.5% Sodium Deoxycholate and 0.1% SDS), followed by sonication for 15 min on ice. Finally, the supernatant was collected after centrifugation at 12,000 rpm for 15 min at 4°C, and the protein concentration was determined using the Bradford assay (Bio-Rad). A total of 60 µg of total proteins was subjected to electrophoresis through 12% sodium dodecyl sulfate-polyacrylamide gels in Tris-glycine buffer (25 mM Tris-Cl, pH 8.3, 192 mM glycine, 0.1% SDS) at a constant voltage of 100 volts at room temperature. The separated samples were then transferred to nitrocellulose membranes in a transfer buffer (15.6 mM Tris Base, 120 mM glycine) at a constant voltage of 30 volts at 4°C for 16 h. The membrane containing the transferred proteins was blocked with 5% skimmed milk in TBS (20 mM Tris-Cl, pH 7.5, 140 mM NaCl) at room temperature for 1 h, and incubated with a mixture of a goat polyclonal antibody against the 37/67-kDa high-affinity laminin receptor (SC-21534, Santa Cruz Biotechnology, Inc., Santa Cruz CA) at a dilution of 1:500, and with a 1:500 dilution of goat polyclonal antibody against actin (SC-1616, Santa Cruz Biotechnology Inc.) in 5% skimmed milk in TBS at room temperature for 2 h. After three washes with TBS-T, the membrane was further incubated with a 1:3000 dilution of HRP-conjugated rabbit anti-goat IgG (31402, Pierce,
Rockford Il.) in 5% skimmed milk in TBS for 1 h at room temperature. The signal was developed using an ECL Plus™ Western blotting detection kit (Amersham Biosciences), followed by exposure to autoradiography film.

**Flow cytometry analysis**

Transfected cells in a single well of a 12-well plate were collected by treatment with trypsin (0.25% trypsin/1 mM EDTA in Hank's balance salt solutions) for 3 min at 37°C, and transferred to a new tube. The collected cells were pelleted by centrifugation at 2,000 rpm for 3 min, and the supernatant was discarded. An ApoAlert® AnnexinV Apoptosis kit (Clontech, PaloAlto, CA) was used to assess the level of apoptosis in the LRP-silenced Hep3B cells. Following the manufacturer’s protocols, the cell pellets were resuspended in 400 µl 1x Binding buffer and incubated for 15 min with 5 µl FITC-conjugated annexinV and 10 µl propidium iodide at room temperature in the dark. Finally, the cells were analyzed by flow cytometry using a FacsScan equipped with Cell Quest software (Becton-Dickinson). For double staining with an antibody against LRP and annexinV, the cell pellets were washed with 200 µl ice-cooled FACS buffer (1x PBS with 2% FBS, freshly prepared) followed by centrifugation at 2,000 rpm for 3 min. Then they were incubated with a 1:50 dilution of rabbit polyclonal antibody against the 37/67-kDa high-affinity laminin receptor (SC-20979, Santa Cruz Biotechnology Inc.) for 1 h on ice. The cells were then washed twice in FACS buffer and labeled with PE-conjugated donkey anti-rabbit secondary antibody (711-116-152; Jackson ImmunoResearch Laboratories, Inc. West Grove, PA) diluted 1:100 in FACS buffer for 45 min on ice in the dark. After two washes, the cells were resuspended in 400 µl FACS buffer, incubated with 5 µl FITC-conjugated annexinV for 15 min in the dark, and subjected to flow cytometry analysis.

**RESULTS AND DISCUSSION**

Small interfering RNAs (siRNAs) are double-stranded RNAs (dsRNAs) of approximately 19 to 21 bp in length that specifically induce the degradation of cellular mRNAs containing complementary nucleotide sequences [31] by activating the cellular RNA interference (RNAi) pathway [32]. As such, they can be used to specifically ablate the expression of single target genes. To down-regulate the expression of 37LBP/67LR, 5 different siRNAs against the human 37LBP/67LR gene (GenBank accession number NM_002295) were generated using in vitro transcription, together with 1 siRNA targeted to the green fluorescent protein gene (GFP; GenBank accession number U50974) for use as a control. To confirm that all the siRNAs were double-stranded, an aliquot of each siRNA was treated with RNaseII, which digests double-stranded RNA, or RNaseA, which digests single-stranded RNA. All the siRNA constructs were confirmed to be of the appropriate size and to consist of dsRNA. Examples (siLRP2 and siLRP4) of the RNase treatment are shown in Fig. 1A. The optimal
siRNA transfection conditions using lipofectamine were established using commercially available siRNAs directed against GAPDH and subsequently labeled with Cy3. The optimal transfection conditions routinely resulted in transfection efficiencies of 80 to 90% (Fig. 1B).

Fig. 1. siRNA analysis and transfection. A – Examples (siLRP2 and siLRP4) of RNAaseA- and RNaseIII-treated siRNAs. In both, lane M: 100-bp ladder, lane 1: dsDNA 21 bp, lane 2: siRNA, lane 3: RNaseIII-treated siRNA, lane 4: RNaseA-treated siRNA. B – Merged images of Hep3B cells transfected with Cy3-labeled siGAPDH (red signal). The nuclei are stained with DAPI (blue). Four representative individual fields are shown.

**Silencing human 37LBP/67LR in cultured Hep3B cells**

To silence the expression of 37LBP/67LR, 5 different siLRPs were transfected into Hep3B cells in parallel with transfections of siGFP and lipofectamine alone (mock control). On days 1 to 4 post-transfection, the cells were harvested and the RNA extracted. Multiplex RT-PCR was done to detect messages from GAPDH and 37LBP/67LR simultaneously, and the results were analyzed by agarose gel electrophoresis. The experiments were done independently in triplicate. The results (Fig. 2) showed a constant signal for GAPDH and 37LBP/67LR for the mock, siGFP and siLRP1 transfections. By contrast, a significant reduction in the level of expression for both genes was observed in the siLRP2 to siLRP5 transfections by day 3 to 4 post-transfection, with transfections for siLRP2 and siLRP4 showing a complete silencing of both genes, suggesting that cell death was occurring in response to the silencing of 37LBP/67LR.
Fig. 2. siLRP-silencing profiles. Multiplex PCR products of GAPDH (upper band) and LRP (lower band), respectively (top to bottom) from mock, siGFP-, siLRP1-, siLRP2-, siLRP3-, siLRP4- and siLRP5-transfected Hep3B cells, from days 1 to 4 post-transfection. M: 100-bp ladder. The transfections were done independently in triplicate.

Fig. 3. Western blot analysis of the expression of 37LBP/67LR and actin. The expression levels of 37LBP/67LR and actin were simultaneously assessed by Western blot analysis for mock, siGFP-, siLRP2- and siLRP4-transfected Hep3B cells on days 3 and 4 post-transfection. The molecular weight in kDa is indicated. A representative gel of a duplicate experiment is shown.
Down-regulation of the 37LBP/67LR protein on days 3 and 4 post-transfection for mock, siGFP, siLRP2 and siLRP4 was investigated by Western blot analysis using a mixture of antibodies directed against actin and 37LBP/67LR. The experiment was done independently in duplicate. The results (Fig. 3) show a very significant reduction in the level of 37LBP/67LR protein in the cells transfected with siLRP2 and siLRP4 compared to the level seen in mock and siGFP-transfected cells. Notably, the level of actin was also reduced in siLRP2- and siLRP4-transfected cells as compared to mock and siGFP-transfected cells, again consistent with a loss of cells as a result of transfection with siLRP2 and siLRP4.

**Observation of apoptosis in 37LBP/67LR knock-down Hep3B cells**

To investigate whether silencing 37LBP/67LR was triggering apoptosis, both siLRP2 and siLRP4 were again transfected into Hep3B cells in parallel with a mock transfection as a control. On days 1 to 4 post-transfection, the samples were again harvested, and this time double-labeled with FITC-labeled annexinV and propidium iodide followed by analysis by flow cytometry (Fig. 4). The experiment was done independently in triplicate. The results showed a significant increase in Annexin V/propidium iodide-positive cells in cultures transfected with either siLRP2 or siLRP4 as compared to the mock transfected cultures, confirming that silencing 37LBP/67LR induces apoptosis in Hep3B cells. To confirm that the increase in the amount of apoptotic cells was associated with a loss in 37LBP/67LR protein, mock, siGFP-, siLRP2- and siLRP4-transfections were again undertaken, and this time, the cells were analyzed by flow cytometry on days 1 to 4 post-transfection by double staining with annexinV and an antibody directed against the 37LBP/67LR protein. This experiment was done independently in triplicate. The results (Fig. 5) show a significant increase in the percentage of AnnexinV+/37LBP/67LR- cells in the siLRP2 and siLRP4 transfections as compared to the mock and siGFP transfections, confirming that the cells undergoing apoptosis are those that have had the 37LBP/67LR protein down-regulated.

Besides its normal roles in mediating cellular adhesion and functioning as a member of the ribosomal translational machinery, the 37LBP/67LR protein has been implicated in a number of pathological processes including metastasis (reviewed in [22]). It also functions as a receptor protein for a number of pathogenic agents including the prion protein [12] and several viruses [17-19]. As such, modulating the expression of 37LBP/67LR is an attractive prophylactic or therapeutic target. However, the induction of apoptosis in response to complete silencing of 37LBP/67LR, as shown here and previously for HeLa cells [26], suggests that this may not be a viable approach without specific targeting to avoid inducing apoptosis in non-tumorigenic cells. However, it should be noted that the induction of apoptosis in response to the down-regulation of 37LBP/67LR is limited to a small number of cell lines investigated, and this may not be the case for all cell types. In particular, the down-regulation...
Fig. 4. Apoptosis detection by flow cytometry. A – Scattergrams of Hep3B cells 4 days after mock, siLRP2- or siLRP4-transfection, with double staining with annexinV and propidium iodide and analysis by flow cytometry. B – The results over days 1 through 4 of the flow cytometry analysis of Hep3B cells transfected with siLRP2 (dotted line) or siLRP4 (dashed line with one dot), or mock transfected (solid line), and stained with annexinV and propidium iodide. The graphs show the percentage of positive cells against the time point. The error bars represent the SEM of three experiments.

The over-expression of 37LBP/67LR in metastatic tumours [29] is involved in maintaining a non-apoptotic state. As such, therapies that specifically target the cell surface-expressed protein [25], or that use viruses such as Sindbis, with 37LBP/67LR as their specific receptor protein [17], as the therapeutic agent [33] may well provide novel strategies for suppressing the growth of metastatic tumors.
Fig. 5. Flow cytometric analysis of AnnexinV+/37LBP/67LR- cells. Flow cytometry was used to assess the percentage on days 1 through 4 of AnnexinV+/37LBP/67LR- cells of experimental populations that had been mock transfected, or transfected with siGFP, siLRP2 or siLRP4, and doubly stained with Annexin V and an antibody directed against the human 37LBP/67LR protein.

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