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Reelin is overexpressed in the liver and plasma of bile duct ligated rats and its levels and glycosylation are altered in plasma of humans with cirrhosis

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Abstract

Reelin is an extracellular matrix protein secreted by a variety of cell types in both embryonic and adult tissues, including the liver. However, the physiological significance of Reelin in normal and cirrhotic liver has thus far not been elucidated. We have investigated Reelin levels in the liver and plasma of bile duct ligated (BDL) rats. We observe a 115% increase in full-length Reelin and its 310- and 180-kDa fragments in liver extracts from BDL rats, compared to sham-operated controls ($p = 0.005$). The overall increase in protein levels was associated with a 30% increase of Reelin transcripts ($p = 0.03$). Immunohistochemical analysis demonstrated that hepatic stellate cells are the major source of Reelin in the injured liver. Increased liver Reelin in BDL rats leads to a pronounced 165% increase in the plasma levels ($p < 0.001$), particularly in the less abundant 180-kDa fragment (300% increase; $p < 0.001$). The data provides evidence that a fraction of plasma Reelin is synthesized in the liver. In human subjects suffering liver cirrhosis the level of the 180-kDa fragment was also increased by 140% in the plasma ($p < 0.001$). Analysis of Reelin glycosylation by lectin binding demonstrated that the 180- and predominant 310-kDa Reelin fragments in the plasma of cirrhotic patients are differentially glycosylated compared to non-diseased control subjects. The data show that Reelin is up-regulated in experimental liver cirrhosis and that its levels and glycosylation are altered in plasma from patients with cirrhosis, thereby supporting that Reelin is involved in the pathogenesis of liver disease.

Keywords: Liver fibrosis; Plasma; Hepatic stellate cells; Glycosylation; Proteoglycan

Abbreviations: BDL, bile duct ligation; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; LC, liver cirrhosis; LCA, Lens culinaris agglutinin; NL, non-ligated; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; RCA_{120}, Ricinus comunis agglutinin; RT-PCR, reverse transcripts polymerase chain reaction.

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1. Introduction

Reelin is a secretory glycoprotein structurally resembling extracellular matrix proteins. It is generally accepted that Reelin and its signaling pathway play a central role during brain development and maturation (Rice & Curran, 2001; Tissir & Goffinet, 2003). The Reelin pathway involves a cascade of intracytoplasmic events initiated by its binding to the transmembrane apolipoprotein E receptor 2 (ApoER2) and very-low-density lipoprotein receptor (VLDLR) (D'Arcangelo et al., 1999; Hiesberger et al., 1999), which transduce the Reelin signal though the intracellular protein adapter disabled-1 (Dab1) (Bar & Goffinet, 1999; Cooper & Howell, 1999; Trommsdorff et al., 1999). While great progress has been made in identifying the role of Reelin and its receptors in neuronal embryogenesis, little is known about the role of these proteins in the adult brain and in peripheral tissues.

Several studies have described the distribution of Reelin in the liver of humans and rodents (Kobold et al., 2002; Samama & Boehm, 2005; Smalheiser et al., 2000), and Reelin mRNA transcripts have been observed in both fetal and adult liver (DeSilva et al., 1997; Ikeda & Terashima, 1997; Smalheiser et al., 2000). However, the Dab1 protein is not detected within the liver suggesting that liver-derived Reelin is not retained but is secreted into the blood for action on distal targets (Smalheiser et al., 2000). Reelin is also expressed in other peripheral tissues including kidney, testis, ovary, adrenal and pituitary glands, and blood cells (Ikeda & Terashima, 1997; Smalheiser et al., 2000; Underhill, 2003). Thus, the potential physiological role of Reelin in the liver and whether liver is an important source for serum Reelin are still unclear.

Primary biliary cirrhosis is a chronic cholestatic liver disease, characterized by the destruction of interlobular bile ducts. Persistent hepatic injury results in the differentiation of nonparenchymal cells into myofibroblasts, which then produce and accumulate large amounts of extracellular matrix proteins leading to liver fibrosis (Friedman, 2003). Reelin has been described as one of the extracellular matrix proteins upregulated in the fibrotic liver (Kobold et al., 2002; Magness, Bataller, Yang, & Brenner, 2004), although it is not currently clear whether cell types other than hepatic stellate cells (HSC) contribute to this increase in the injured liver.

In the present study, Reelin levels were investigated in rat liver and plasma after chronic bile duct ligation (BDL) and levels were compared to sham-operated controls. We also investigated the cellular origin of Reelin in the BDL liver to determine whether the liver is an important source for plasma Reelin. We further investigated changes in the levels and glycosylation of the Reelin protein in the plasma of patients suffering liver cirrhosis.

2. Materials and methods

2.1. Bile duct ligation and tissue preparation

Liver injury was induced in male Sprague-Dawley rats (~250 g) by common bile duct ligation as previously described (García-Ayllón et al., 2006; Jover et al., 2006). In sham non-ligated (NL) controls, the bile duct was identified, manipulated, and left *in situ*. Rats were sacrificed 21 days after surgery. Animals were halothane-anaesthetized and blood was drawn by cardiac puncture. Plasma was separated from blood cells by centrifugation. Cirrhosis was routinely confirmed by blinded examination of hematoxylin-eosin stained sections. Liver damage was also confirmed by analysis of conventional serum markers (see García-Ayllón et al., 2006; Jover et al., 2006). For molecular analysis the liver was rapidly removed, washed exhaustively in saline and stored at −80 °C for later analysis. All animal procedures were approved by the Animal Care and Use Committee of the Miguel Hernández University.

2.2. Human plasma

Plasma samples from patients with liver cirrhosis (LC; 3 female/5 male; 57 ± 3 years) and age-matched controls (6 female/6 male; 60 ± 3 years) were provided by the Hospital General Universitario de Alicante (Spain). The diagnostic evaluation of all patients included clinical examination, liver biopsy and observation by physical examination, results of laboratory tests, and imaging studies. Causes of cirrhosis were alcoholism ($n = 5$), Hepatitis C virus (HCV) infection ($n = 1$) and both alcoholism and HCV ($n = 3$). Plasma was separated from whole blood by centrifugation, aliquoted and frozen at −80 °C until use. The study was approved by the local ethic committee and was carried out in accordance with the Declaration of Helsinki.

2.3. Protein extraction from liver tissue

Tissues stored at −80 °C were thawed gradually at 4 °C and small pieces of liver were homogenized (10%, w/v) in ice-cold 50 mM Tris–HCl, pH 7.4/150 mM NaCl/0.5% Triton X-100/0.5% Nonidet P-40 supplemented with a cocktail of proteinase inhibitors (Botella-López et al., 2006). The homogenates were sonicated and centrifuged at 20,000 × *g* at 4 °C for 20 min;

the supernatant was collected, aliquoted and frozen at −80 °C until use.

2.4. Detection of Reelin by Western Blotting

Reelin expression was determined as previously described (Botella-López et al., 2006). Liver extracts (20 μg) or plasma samples (0.7 μL) were boiled for 3 min, then resolved on 6% polyacrylamide slab gels. Electrophoresis was allowed to proceed at low voltage to minimize excessive heat (Lugli et al., 2003). Proteins were blotted onto nitrocellulose membranes, blocked with 5% non-fat milk and incubated in monoclonal mouse anti-Reelin antibodies G10 [1:500 dilution; Chemicon International, Temecula, CA] for rat samples, and 142 [1:200 dilution; Chemicon International] for human samples. The antibodies recognize epitopes located in the region of amino acids 164–189 (clone 142) and 164–496 (clone G10) (de Bergeyck, Naerhuyzen, Goffinet, & Lambert de Rouvroit, 1998) (see also manufacturer's information). Immunoblots were then developed with enhanced chemiluminescence (ECL) using the ECL-Plus kit (GE Healthcare Limited, Buckinghamshire, UK) in a Luminescent Image Analyzer LAS-1000 Plus (FUJIFILM, Stamford, CT). The intensity of Reelin bands was measured by densitometry using Science Lab Image Gauge v 4.0 software provided by FUJIFILM. Total protein concentrations were determined using the bicinchoninic assay (Pierce, Rockford, IL).

2.5. Lectin binding analysis

Aliquots (100 μL) of plasma (diluted 1:20 in Tris-buffered saline) were mixed with 40 μL of immobilized lectins [Lens culinaris agglutinin (LCA) or Ricinus comunis agglutinin (RCA_{120}), Sigma–Aldrich Co]. After overnight incubation at 4 °C, Reelin–lectin complexes were separated from free (unbound) Reelin by centrifugation and examined by Western blotting.

2.6. RNA isolation and analysis by semi-quantitative PCR

Liver RNA was extracted using TRIzol® Reagent (Invitrogen™ Life Technologies, Grand Island, NY) according to the manufacturer's instructions. Liver Reelin mRNAs were identified by reverse transcriptase polymerase chain reaction (RT-PCR) with selected primers. First-strand cDNAs were obtained by reverse transcription using SuperScriptTM III Reverse Transcriptase (Invitrogen™ Life Technologies) according to the manufacturer's instructions using 1 μg of total RNA and oligo (dT)12-18. Oligonucleotide primers used for PCR analysis were Reel+: 5′ ATACGTGGATCCCTGTATCTACTTGCTGTGTTGC 3′; Reel−: 5′ ATACGTCTAGACAAGTCACTTTGTTACCACAG 3′. For semiquantitative RT-PCR, aliquots of the cDNA samples were incubated with Taq DNA Polymerase (1 U) in the presence of primers (0.2 μM each) and a dNTP Mix (0.2 mM). The number of cycles (25–35) was chosen to assure that quantification was performed in the linear amplification phase. All cDNAs were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

2.7. Reelin immunocytochemistry and laser confocal microscopy

Sections of paraffin-embedded tissues were stained with hemotoxylin-eosin and examined by light microscopy for necrosis and other structural changes. For rat Reelin immunohistochemistry 20 μm-thick frozen sections were cut on a sliding microtome. Frozen sections were rinsed and double immunostaining was performed at room temperature overnight with antibody G10 (1:1000 dilution) and monoclonal anti-glial fibrillary acidic protein (GFAP) antibody (1:500 dilution, Calbiochem, San Diego, CA, USA). Sections were then incubated with either biotinylated anti-mouse IgG (for G10) or anti-rat IgG (for anti-GFAP) from Vector (Burlingame, CA, USA). This was followed by rat or mouse fluorescein or Texas Red conjugated to streptavidin (Vector). Dual immunofluorescence images were captured simultaneously using a Leica laser-scanning confocal microscope (TCS SL, Leica Microsystems, Ernst-Leitz-Strasse, Germany) with a 40× oil immersion objective.

2.8. Isolation of hepatocytes

Liver cells were isolated by sequential digestion with collagenase, as described previously (García-Ayllón et al., 2006). Briefly, the liver suspension was centrifuged for 5 min at 50 × *g* and the pellet, containing the hepatocytes, was resuspended in Hanks's balanced salt solution and centrifuged at 400 × *g* (10 min, 4 °C) over a 60% Percoll solution (GE Healthcare Limited) to obtain purified hepatocytes.

2.9. Statistical analysis

All data were analyzed in SigmaStat (Version 2.0; SPSS Inc.) by a Student's *t*-test (two-tailed) or

Mann–Whitney *U* test (when variances were significantly different between groups) for single pair-wise comparisons and determination of exact *p* values, as stated. Results are presented as means ± S.E.M. *p* values < 0.05 were considered significant.

3. Results

3.1. Reelin is up-regulated in experimental liver fibrosis in rats

Rat liver extracts were analyzed by SDS-PAGE under fully reducing conditions, followed by Western blotting using the anti-Reelin antibody G10. Interestingly, Reelin is cleaved in vivo at two sites resulting in the production of several fragments whose relative abundance differs in distinct tissues (Ikeda & Terashima, 1997; Smalheiser et al., 2000). Fig. 1A shows the three typical Reelin-immunoreactive bands in liver homogenates, as observed in previous studies (Smalheiser et al., 2000). The predicted structure of Reelin and its fragments, recognized by the antibody, are shown in Fig. 1B. In all cases, the 420-kDa band corresponding to full-length Reelin displayed higher immunoreactivity than the 310-kDa fragment. The smaller 180-kDa band appeared faintly stained only in some samples from NL-control rats and only after long exposure times (not shown). Western blot semi-quantitative analyses showed an increase in full-length Reelin and its fragments in the BDL liver compared to NL liver (Fig. 1C). The differential detection of different Reelin fragments by the antibody cannot be excluded; nonetheless we estimated total Reelin content as the sum of all three bands. The total amount of Reelin was increased in the BDL liver compared to controls ($p = 0.005$, 115% increase; Fig. 1C).

A semi-quantitative PCR assay was further designed to determine whether changes in Reelin protein corresponded to alterations in mRNA expression (Fig. 1D). Reelin mRNA levels shows a 31% increase in the BDL liver compared to controls ($p = 0.03$; Fig. 1E).

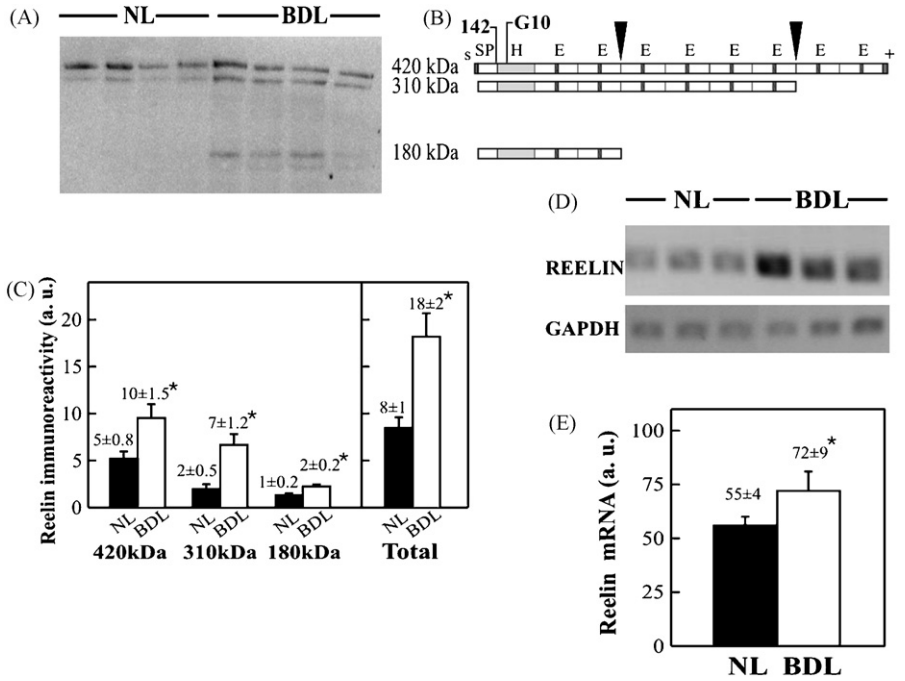

Fig. 1. Bile duct ligation (BDL) induces an increase of liver Reelin. (A) Representative immunoblot of Reelin in liver of BDL and non-ligated (NL) rats. (B) Schematic representation of the Reelin protein and its fragments generated by the two main processing sites (arrow-heads), and recognized by the 142 and G10 antibodies (the epitopes are approximately located as indicated). s, signal peptide; SP, spondin similarity region; H, unique region; E, EGF-like motifs which separate two related sub-domains in the eight internal repeats; "+" terminal basic region. (C) Densitometric quantification of the Reelin immunopositive bands from eight BDL and eight control rats. Accumulative total immunoreactivities of the three Reelin bands are also shown. (D) Reelin transcripts were detected by semiquantitative RT-PCR and the products identified according to their sizes (~342 bp in both, NL and BDL liver extracts). (E) Graphic representation of the Reelin mRNA levels normalized to GAPDH. The data represent the means ± S.E. (duplicate determinations). (*) Significantly different ($p < 0.05$) from the NL group, as assessed by the Student's *t* test ($n = 8$ for each group).

3.2. Cellular source of Reelin in fibrotic rat livers

The distribution of Reelin-positive cells in the rat liver was assessed by immunocytochemistry using the G10 antibody. Similar to a previous report (Samama & Boehm, 2005), Reelin immunostaining was found in hepatic stellate cells (HSC) (Fig. 2A and D) with no significant staining seen in hepatocytes. Stellate cells in the liver appear as regularly distributed polygonal cells expressing GFAP (Fig. 2B), as previously shown (Buniatian, 1997; Neubauer, Knittel, Aurisch, Fellmer, & Ramadori, 1996). Very few HSC (∼15–20%) co-express Reelin protein, as demonstrated by double immunofluorescence labelling (Fig. 2C). In BDL livers the hepatic parenchyma was severely disrupted. Although the morphology of GFAP-expressing HSC was normal, they were more abundant and distributed in clusters in the cirrhotic tissue (Fig. 2E). Hepatic zones abundant with HSC showed that a majority of these cells (∼85–90%) co-express Reelin (Fig. 2D and F).

In situ hybridization has previously shown positive staining for mRNA Reelin in rat liver hepatocytes (Kobold et al., 2002). We therefore isolated hepatocytes and determined if these cells were also a cellular source of increased Reelin in rat BDL liver. Hepatocytes were isolated, proteins extracted and analyzed by SDS-PAGE followed by Western blotting using the G10 antibody and an antibody specific for the α-smooth muscle isoform of actin (clone 1A4 from DakoCytomation, Denmark). Immunoblot analysis of hepatic α-smooth muscle actin-expression confirmed that isolated hepatocyte samples were free of detectable amount for this activation marker for HSC (not shown). Trace amounts of the three major Reelin bands were detected in hepatocyte homogenates (Fig. 2G). However, the levels of Reelin were not significantly different in hepatocytes from NL and BDL rats,

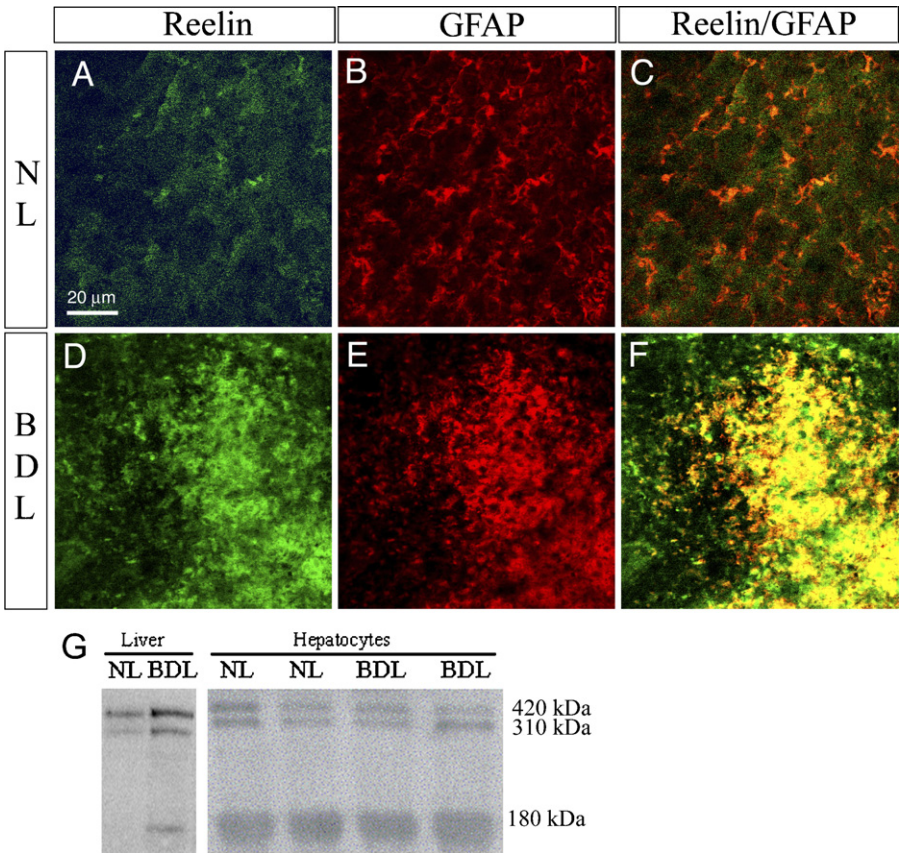

Fig. 2. Expression of Reelin and GFAP in liver stellate cells. (A–C) Control (non-ligated, NL) rat liver sections showing few HSC expressing GFAP (B) co-express Reelin (A, C). In BDL rats the number of HSC (red) significantly increases (E) with a concomitant increase in Reelin expression (green; D). (F) Co-localization of Reelin and GFAP in the experimental liver. (G) Comparison of the immunoreactive Reelin-banding pattern in hepatocytes isolated from NL and BDL liver examined by Western Blotting (equivalent amounts of protein were loaded in each lane). Total liver extracts were used to indicate the migration of the major Reelin fragments.

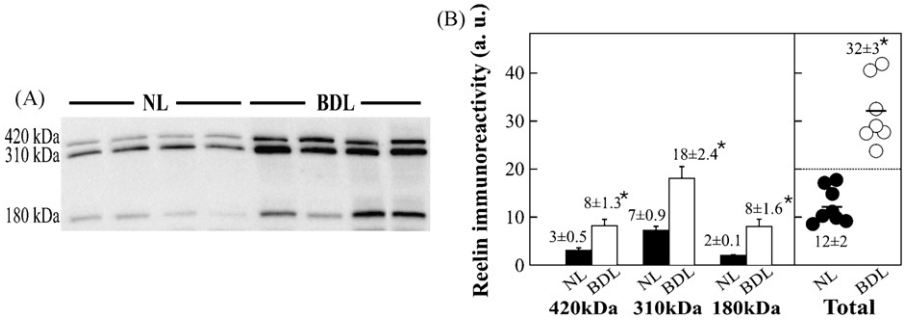

Fig. 3. Bile duct ligation (BDL) induces an increase in plasma Reelin. (A) Reelin immunoreactivity in plasma samples of BDL compared to non-ligated (NL) rats. (B) Densitometric quantification of the Reelin-immunoreactive bands from 8 BDL and 8 NL rats. Accumulative immunoreactivity of the three Reelin bands was calculated and corresponding scatterplots is also shown. Dashed line represents an arbitrary cutoff. *$p < 0.005$.

indicating that the change in BDL liver Reelin is not due to this cell type.

3.3. Changes in Reelin plasma levels in BDL rats

In order to determine whether changes in plasma Reelin levels correlate with hepatic expression in livers with advanced fibrosis, we examined the levels of this protein in the plasma of control and BDL rats. The relative abundance of Reelin bands differed between plasma and liver as determined by immunoblotting (Fig. 3A). The 310-kDa band was the most abundant, followed by the full-length 420-kDa band and the 180-kDa fragment. The immunoreactivity of the major Reelin bands was increased in the plasma from BDL compared to NL animals (Fig. 3B), indicating that circulating Reelin levels are affected during cirrhosis. Interestingly, the smaller 180-kDa Reelin fragment, which represents the least intense band, was the Reelin species which shows the major increase in BDL plasma (∼305% increase; $p < 0.001$). Analysis of the total amounts of Reelin thus enables us to fully discriminate between BDL and NL animals (165% increase in Reelin in the sum of the three bands; $p < 0.001$) (Fig. 3B).

3.4. Changes in Reelin plasma levels in liver cirrhosis

Analysis of Reelin levels in humans (Fig. 4A and B) indicated that plasma from cirrhotic patients had higher 180-kDa Reelin than controls (∼140% increase; $p < 0.001$), while no difference was observed in the content of either the 420-kDa ($p = 0.20$) or predominant 310-kDa ($p = 0.29$) species, nor in total Reelin content (the sum of the three bands; $p = 0.75$). We defined, for each sample, a quotient (420 kDa + 310 kDa)/180 kDa Reelin, as the sum of the immunoreactivity of the full-length and larger 310-kDa Reelin fragments divided by the immunoreactivity of the proteolytically generated 180-kDa fragment (Fig. 4C). This quotient

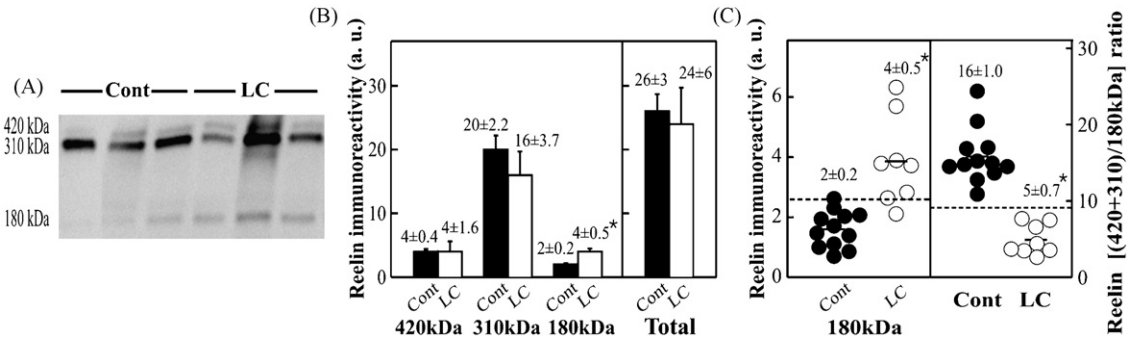

Fig. 4. Concentration and gel mobility of Reelin fragments in plasma from patients with liver cirrhosis. (A) Representative blot of Reelin in plasma samples from cirrhotic liver (LC) and control individuals. (B) Densitometric quantification of the Reelin-immunoreactive bands from 8 LC and 12 controls. Accumulative immunoreactivity of the 3 Reelin bands is shown. (C) Scatterplots for 180-kDa Reelin and for the (420 kDa + 310 kDa)/180 kDa Reelin ratio. Dashed lines show values that discriminate between groups. *$p < 0.001$.

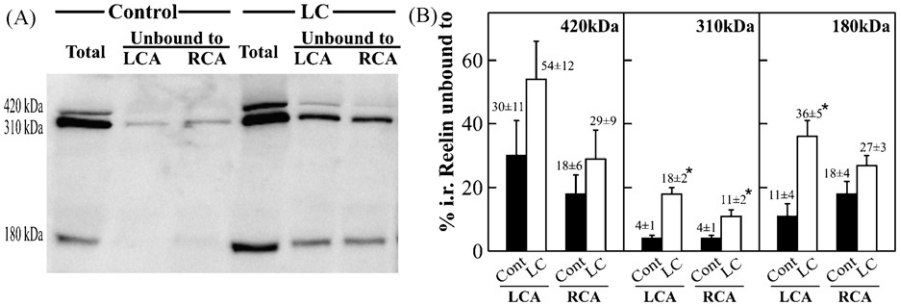

Fig. 5. Glycosylation of plasma Reelin. Plasma samples from six cirrhotic liver (LC) and 6 control individuals were incubated with immobilized lectins LCA and RCA. Attempts to determine the total amount of Reelin bound to each lectin by resuspension and boiling of the resin resulted in only 60–80% of recovery of the glycoprotein. (A) Unbound Reelin, assayed by Western blotting, in the supernatant fraction was therefore used to compare differences in lectin binding between LC and control groups. (B) The graph represents the percentage (%) of unbound Reelin immunoreactivity (i.r.) for each band. Determinations in duplicate. *$p \leq 0.01$.

provided greater discrimination between the two groups ($p < 0.001$).

3.5. Changes in plasma Reelin lectin binding in liver cirrhosis

To study the alterations in the glycosylation of Reelin during liver cirrhosis, plasma samples from six controls and six cirrhosis cases were incubated with immobilized lectins (Fig. 5A). The percentage of unbound Reelin was calculated for each fragment by Western blotting (Fig. 5B). A significant difference between the LC cases and controls was observed in the binding of the 180-kDa fragment to LCA ($p = 0.005$). We also observed a non-statistically significant increase in the proportion of the 180-kDa fragment that does not bind to RCA ($p = 0.09$) in LC compared to controls. In addition, there was also a change in the lectin-binding ability of the 310-kDa fragment assayed in LC compared with controls, for both LCA ($p < 0.001$) and RCA ($p = 0.01$). We were not able to detect any lectin-binding differences in full-length glycosylated Reelin.

4. Discussion

Our results demonstrated that Reelin levels are significantly increased in the cirrhotic liver. We observe a marked increase in the Reelin protein in cirrhotic livers of BDL rats accompanied by an increase in mRNA transcripts. We also confirm that Reelin is present in HSC and demonstrate that changes in liver Reelin are a consequence of altered Reelin expression in this cell type.

Several laboratories have reported the expression of Reelin mRNA in the adult rodent liver and that isolated liver cells secrete full-length Reelin *in vitro* (DeSilva et al., 1997; Ikeda & Terashima, 1997; Smalheiser et al., 2000). To date, there is little agreement as to which cell type contributes to liver Reelin content in normal and pathological conditions. In contrast with early reports on the localization of liver Reelin mRNA to sinusoid endothelial cells (Ikeda & Terashima, 1997), studies have shown Reelin expression in both HSC and hepatocytes (Kobold et al., 2002; Magness et al., 2004; Samama & Boehm, 2005). The increase in Reelin transcripts has been attributed to hepatocytes during acute injury using CCl_4 (Kobold et al., 2002). In agreement with a previous immunohistochemical study (Samama & Boehm, 2005), the Reelin protein was not detected in hepatocytes. A marked increase in Reelin staining was also in BDL-HSC cells. In addition, we cannot completely discard that isolated liver hepatocytes are contaminated by trace amount of other cell types, nonetheless the detection of Reelin from isolated liver hepatocytes by western blotting analyses of protein extracts shows that Reelin levels remain unchanged in hepatocyte extracts from BDL rats compared to NL rats. Our data thus indicate that HSC, rather than hepatocytes, are the major source of the Reelin in the injured liver. In normal liver, HSC are mainly involved in the storage of vitamin A in lipid droplets and in the synthesis of extracellular matrix proteins, matrix degrading metalloproteinases, cytokines and growth factors. Following liver injury activated HSC proliferate and migrate to sites of injury and play a role in tissue repair, a process where Reelin may be involved. If injury persists, enhanced extracellular matrix protein deposition can lead to fibrosis and cirrhosis.

This is the first study to reliably demonstrate that liver disease has an effect on the levels of Reelin in plasma. To date, it has been suggested that the liver synthesizes and secretes Reelin in amounts that may be important in maintaining blood Reelin levels, although it cannot be

excluded that other peripheral tissues may also secrete the glycoprotein (Smalheiser et al., 2000). We demonstrate a correlation between increased Reelin protein and mRNA expression (as a consequence of the experimental BDL in rats) with elevated blood Reelin levels. However, only the levels of the shorter 180-kDa Reelin fragment are increased in the plasma of patients suffering cirrhosis.

In contrast with brain and cerebrospinal fluid, where the 180-kDa Reelin fragment was predominant fragment, in both liver and blood, this shorter N-terminal fragment was less abundant than the 310-kDa and the unprocessed form of Reelin (Botella-López et al., 2006; Smalheiser et al., 2000). Interestingly, in several psychiatric disorders such as schizophrenia and autism (Fatemi, Kroll, & Stary, 2001; Fatemi, Stary, & Egan, 2002), the detection of the 180-kDa band in blood band did not parallel the largest 410 and 320-kDa Reelin bands, suggesting the possibility of specific biological activities for the different Reelin fragments. As the 180-kDa Reelin fragment is the least abundant fragment, it is also possible that differential proteolytic processing of Reelin in cirrhosis may contribute to the increase of this band observed in our western blotting studies. In patients with severe liver disease, blood levels of many coagulation and fibrinolytic factors are lower due to a decrease in the synthetic capability of the liver. Recent evidence indicates that plasminogen activators and plasmin can convert full-length 420-kDa Reelin into smaller fragments (Lugli et al., 2003). While proteolytic processing may play a role in the generation of the smaller fragment, the net increase in the smaller Reelin fragment does not appear to be derived from an increased degradation of the 420 and 310-kDa Reelin forms.

Is Reelin expression altered in humans undergoing liver cirrhosis? Reelin is a highly glycosylated protein, however, little is known about the number and localization of its glycosylation sites. After signal peptide cleavage, full-length Reelin is predicted to be ~385 kDa in size. The higher molecular weight protein detected by electrophoretical analysis (~420 kDa) is thought to be a result of glycosylation (D'Arcangelo et al., 1997; Tissir & Goffinet, 2003). By exploiting the ability of lectins to bind different carbohydrate moieties, we demonstrate that most Reelin from blood reacts with RCA_{120}, which has an affinity for terminal β-D-galactosyl residues, and LCA, which binds to terminal α-D-mannosyl and α-D-glucosyl residues. The three Reelin fragments exhibit similar patterns of binding, in agreement with a previous report (Botella-López et al., 2006). Many pathological states, including liver cirrhosis (García-Ayllón et al., 2006; Gravel et al., 1996), cause characteristic changes in the glycosylation pattern of specific glycoproteins. Here, we demonstrate an altered pattern of Reelin-lectin binding of both the 180-kDa and 320-kDa fragments from plasma, although net protein levels remain unaltered. These results show that in plasma of patients suffering cirrhosis the 180-kDa fragment is not the only fragment affected. Changes in the glycosylation pattern of proteins may be due to an altered glycosylation mechanism, as well as differences in protein glycoforms from different cell types. Recently, we demonstrated that human Reelin from different sources (plasma and cerebrospinal fluid) is differentially glycosylated, and that the pattern of Reelin-lectin binding differed between the cerebrospinal fluid of control subjects and in patients with Alzheimer's disease (Botella-López et al., 2006). Thus, the cellular origin and whether altered glycosylation patterns in cirrhosis is a direct consequence of altered metabolism or reflects changes in the expression of different Reelin glycoforms to the circulating pool warrants further study. Moreover, we cannot completely discount that changes in the proportion of Reelin fragments reflect these changes in glycosylation, since abnormal glycosylation can increase protein degradation, affecting Reelin stability and secretion.

The physiological significance of Reelin in liver and plasma and how this protein acts locally and peripherally (and where it acts) have thus far not been elucidated. It is widely accepted that Reelin functions in developmental processes such as cellular differentiation and migration, and activates signaling cascades, similar to those in neural development, in the adult (Chen et al., 2005). Nonetheless, essential components of the Reelin-mediated signaling are absent in the liver. The two Reelin receptors, ApoER2 and VLDLR, bind Reelin with similar affinities and share an internalization motif that is also required for Dab1 binding (Beffert et al., 2004). However, only one of these receptors, the VLDLR, is expressed at low levels in adult rodent liver (Takahashi, Kawarabayasi, Nakai, Sakai, & Yamamoto, 1992; Tiebel et al., 1999). Mouse liver does not express Dab1 protein (Smalheiser et al., 2000), implying that the classical Reelin-Dab1 pathway may not be activated in the adult liver. The lack of Dab1 expression in liver suggests that Reelin is not stored within the liver and is rapidly secreted into the blood for action on distal targets (Smalheiser et al., 2000). Thus, the role of Reelin in liver and blood and its potential mechanisms of action are still unclear. Recently, reports describe that Reelin is upregulated following injury to the retina and cornea, suggesting that Reelin may play an important role in regulating stem cell trafficking in neuronal and nonneuronal tissues following injury, similar to its role in normal organogenesis (Pulido, Sugaya, Comstock, & Sugaya, 2007). Simi-

larly, upregulation of Reelin following nerve crush injury suggests that it may play a role in peripheral nervous system repair by regulating Schwann cell-axon interactions (Pantera et al., 2006). Reelin was also found in glial somata and in small astrocytic processes in post-mortem human brain suggesting that in the adult human Reelin has a role in synaptic remodelling (Roberts, Xu, Roche, & Kirkpatrick, 2005). In this context and as mentioned earlier, it is also unclear if altered Reelin expression during liver injury results in changes in the expression pattern of matrix proteins associated with extensive fibrosis, which may in turn influence plasticity and surrounding connective tissue re-organization. The *in vitro* interaction of Reelin with lipoprotein receptors is inhibited in the presence of ApoE (D'Arcangelo et al., 1999), and polymorphisms in this gene may confer susceptibility to liver disease (Corpechot et al., 2001; Wozniak et al., 2002). Moreover, an expression shift from ApoE to Reelin has been described upon plasma cell differentiation and is proposed to regulate cell migration and cell interactions (Underhill, George, Bremer, & Kansas, 2003). Thus, impairment of the Reelin/ApoE balance may contribute to a progression in liver pathology.

Our studies show that liver Reelin expression is affected during severe liver injury and that a fraction of plasma Reelin is synthesized in the liver. The potential use of increased levels of the plasma 180-kDa Reelin fragment and altered glycosylation of the protein as markers of liver cirrhosis warrant further study. Further work will help elucidate how Reelin synthesis and release are regulated physiologically and pathologically in the human liver.

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