IGF2BP1 enhances HCV IRES-mediated translation initiation via the 3'UTR

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ABSTRACT

The positive-strand RNA genome of the Hepatitis C virus (HCV) contains an internal ribosome entry site (IRES) in the 5'untranslated region (5'UTR) and structured sequence elements within the 3'UTR, but no poly(A) tail. Employing a limited set of initiation factors, the HCV IRES coordinates the 5'cap-independent assembly of the 43S pre-initiation complex at an internal initiation codon located in the IRES sequence. We have established a Huh7 cell-derived in vitro translation system that shows a 3'UTR-dependent enhancement of 43S pre-initiation complex formation at the HCV IRES. Through the use of tobramycin (Tob)-aptamer affinity chromatography, we identified the Insulin-like growth factor-II mRNA-binding protein 1 (IGF2BP1) as a factor that interacts with both, the HCV 5'UTR and 3'UTR. We report that IGF2BP1 specifically enhances translation at the HCV IRES, but it does not affect 5'cap-dependent translation. RNA interference against IGF2BP1 in HCV replicon RNA-containing Huh7 cells reduces HCV IRES-mediated translation, whereas replication remains unaffected. Interestingly, we found that endogenous IGF2BP1 specifically co-immunoprecipitates with HCV replicon RNA, the ribosomal 40S subunit, and eIF3. Furthermore eIF3 comigrates with IGF2BP1 in 80S ribosomal complexes when a reporter mRNA bearing both the HCV 5'UTR and HCV 3'UTR is translated. Our data suggest that IGF2BP1, by binding to the HCV 5'UTR and/or HCV 3'UTR, recruits eIF3 and enhances HCV IRES-mediated translation.

Keywords: HCV-IRES; IGF2BP1; eIF3; translation initiation; HCV-3'UTR; RNA-protein interaction

INTRODUCTION

HCV, the main causative agent of non-A and non-B hepatitis, belongs to the *Flaviviridae* family. The plus-strand RNA genome of HCV contains one open-reading frame (ORF) encoding a polyprotein, which is flanked by a 5'UTR and a 3'UTR. These UTRs are conserved between HCV genotypes and required for viral replication and translation (Binder et al. 2007). Four domains are located in the 5'UTR, of which domains II to IV form a highly structured IRES that bears an initiator AUG in domain IV

(Brown et al. 1992; Reynolds et al. 1996; Kieft et al. 1999). Translation of the polyprotein encoding ORF is initiated by a 5'cap-independent mechanism at the HCV IRES (Tsukiyama-Kohara et al. 1992; Wang et al. 1993; Reynolds et al. 1996; Pestova et al. 1998). Cell-free translation experiments and reconstitution of HCV IRES-mediated initiation revealed that first a binary complex consisting of the IRES and the ribosomal 40S subunit is constituted at domain III near to the initiator AUG. Secondly, eIF3 and the ternary complex (eIF2/GTP/Met-tRNA^{Met}) are recruited to form the 43S pre-initiation complex, again aided by domain III. Finally, after GTP hydrolysis and eIF2 release, the ribosomal 60S subunit joins, and formation of the 80S ribosome mediated by domain II and portions of domain III takes place (Pestova et al. 1998; Hellen and Pestova 1999; Ji et al. 2004; Otto and Puglisi 2004). Mutations in the basal part of domain III abolish binding of the ribosomal 40S subunit (Kieft et al. 2001), whereas mutations in the apical loops of domain III affect eIF3 recruitment and 43S pre-initiation complex assembly (Ji

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et al. 2004; Otto and Puglisi 2004). Domains II and IV also contact the ribosomal 40S subunit. Domain II interacts with ribosomal protein S5 (Fukushi et al. 2001) and protrudes into the ribosomal start site, causing a conformational change in the ribosomal 40S subunit (Spahn et al. 2001). Proper positioning of the initiation codon is controlled by the sequence (Fletcher et al. 2002) and stability (Honda et al. 1996) of domain IV. Deletion of domain II does not alter the recruitment of eIF3 and the ternary complex, but reduces translational activity fivefold by blocking 80S ribosome formation (Pestova et al. 1998; Kolupaeva et al. 2000; Ji et al. 2004; Otto and Puglisi 2004).

The HCV 3'UTR, which lacks a poly(A) tail, is composed of a variable region, a poly(U-C) motif, and a conserved sequence, the 3'X region, which forms a structure consisting of three stem-loops (Kolykhalov et al. 1996, 2000; Tanaka et al. 1996; Yamada et al. 1996; Yanagi et al. 1999; Blight et al. 2000; Friebe and Bartenschlager 2002; Yi and Lemon 2003a,b). By transfection of monocistronic reporter RNAs into the human liver-derived cell line Huh7, it has recently been shown that the HCV 3'UTR stimulates translation at the HCV IRES (Song et al. 2006).

Here we address the molecular mechanism for this enhancement. We established an *in vitro* translation system derived from Huh7 cells and show that the 3'UTR directly stimulates 43S pre-initiation complex formation at the HCV 5'UTR. Through the use of the Tob-aptamer technique, we identified IGF2BP1 as a protein, which specifically interacts with both the HCV 5'UTR and HCV 3'UTR. Employing *in vitro* RNA binding assays, *in vitro* translation experiments, as well as RNA interference (RNAi) studies, we further characterized the function of IGF2BP1 and show that this protein specifically enhances HCV IRES-mediated translation, but does not affect replication. Interestingly, endogenous IGF2BP1 could be co-immunoprecipitated with the HCV replicon RNA, the ribosomal 40S subunit, and eIF3. Furthermore, eIF3 comigrates with IGF2BP1 in 80S ribosomal complexes when a reporter mRNA bearing both the HCV 5'UTR and 3'UTR is translated. Our data implicate that IGF2BP1, by binding to the HCV 5'UTR and/or HCV 3'UTR, recruits eIF3 and enhances HCV IRES-mediated translation. Furthermore, binding of IGF2BP1 to the variable region and the poly(U-C) motif of the HCV 3'UTR might facilitate the 5'-to-3' interaction of HCV RNA. Both processes can enhance translation at the HCV IRES.

RESULTS

The HCV RNA 3'UTR stimulates 43S pre-initiation complex formation at the HCV IRES

To gain more insight in the mechanism of HCV 3'UTR-dependent enhancement of HCV IRES-mediated translation initiation, we established an *in vitro* translation system based on cytoplasmic extracts of Huh7 cells. This

system resembles the 3'UTR stimulatory effect, which was shown earlier by transfection of monocistronic reporter RNAs into Huh7 cells (Song et al. 2006). The [³²P] trace-labeled luciferase (LUC) reporter mRNAs bearing the HCV 5'UTR and HCV 3'UTR (HCV Luc) or no HCV 3'UTR (HCVΔ3'UTR Luc) were translated in Huh7 cell extract. Translation rate (Fig. 1A) and mRNA stability (Fig. 1B) were analyzed at 0, 15, 30, and 45 min. HCV Luc mRNA translation was stimulated eightfold compared to HCVΔ3'UTR Luc mRNA after 45 min (Fig. 1A). Reduced Luc expression from the HCVΔ3'UTR Luc mRNA was not a result of enhanced mRNA degradation as demonstrated by mRNA isolation from the translation reaction (Fig. 1B). These data indicate that the HCV 3'UTR stimulates HCV IRES-mediated translation *in vitro*.

Next, we aimed to identify the mechanism underlying the enhancing effect of the HCV 3'UTR. To investigate whether or not the initiation step of HCV IRES-mediated translation is augmented by the 3'UTR, we performed translation initiation reactions, which were analyzed by sucrose gradient centrifugation. To optimize the resolution of translation initiation complexes, we generated shortened reporter mRNAs by replacing the Luc ORF with a synthetic short ORF (sORF) (Fig. 1C). The [³²P]-labeled mRNAs (HCV sORF or HCVΔ3'UTR sORF) were incubated in translation competent Huh7 cell extract in the presence of cycloheximide, which inhibits the peptidyltransferase reaction and thereby stalls the 80S ribosome at the AUG (Obrig et al. 1971). Time course experiments from 0.5 to 15 min revealed that the HCV 3'UTR kinetically enhances 80S complex formation (Supplemental Fig. 1A). We further examined these results by a direct comparison of translation initiation reactions performed for 1 and 15 min. After 15 min, 80S complexes did accumulate at the HCV sORF mRNA, but 80S complex formation was reduced at the HCVΔ3'UTR sORF mRNA (Fig. 1C, cf. left and right panels).

To analyze whether the 3'UTR directly enhances 43S pre-initiation complex formation, in addition to cycloheximide, the non-hydrolyzable GTP analog GMP-PNP, which stalls 43S ribosomal pre-initiation complexes at the initiator AUG, was added (Hershey and Monro 1966; Anthony and Merrick 1992).

After 15 min, 43S pre-initiation complexes accumulated at the HCV sORF mRNA. In contrast, at the HCVΔ3'UTR sORF mRNA 43S pre-initiation complex formation was significantly inhibited, demonstrating directly that the 3'UTR stimulates 43S pre-initiation complex assembly (Fig. 1D, cf. left and right panels; Supplemental Fig. 1B).

The position of ribosomal complexes and RNPs in the gradient fractions was analyzed by the distribution of 18S and 28S rRNA (Fig. 1C,D). 80S ribosomes accumulated in fractions 5–8, while fractions 11–14 contained mainly 18S rRNA of the ribosomal 40S subunit (Fig. 1C,D, left and right panels). The ribosomal protein rpS3 that is associated

FIGURE 1. (Legend on next page)

with the ribosomal 40S subunit accumulates in 43S and 80S ribosomal complexes containing fractions (Fig. 1C,D, bottom panel). To localize the position at which the ribosomal 60S subunits migrate in the gradient, we analyzed the distribution of eIF6 that is bound to the ribosomal 60S subunit prior to 80S ribosome formation (Ceci et al. 2003). EIF6 could mainly be detected in fractions 9 and 10 (Fig. 1C,D, bottom panel). These data indicate that the 3'UTR stimulates translation initiation by promoting an early step of 80S ribosome formation.

Tob-aptamer affinity purification of HCV 5'UTR and 3'UTR complexes from Huh7 cell extract

Since we observed that the 3'UTR enhances HCV IRES-mediated translation initiation, we wanted to analyze the composition of the HCV IRES-associated translation initiation complex assembled on an mRNA bearing both the HCV 5'UTR and HCV 3'UTR. For this purpose, we made use of the Tob-aptamer affinity chromatography. Under physiological conditions, the Tob-aptamer binds with high affinity ($K_D = 5$ nM) to the amino glycoside antibiotic tobramycin (Hamasaki et al. 1998). This technique has proved to be highly efficient and specific in the analysis of the pre-spliceosomal protein complex composition (Hartmuth et al. 2002), the HCV RNA-replication complex from Huh7 cell extract (Isken et al. 2007), and HCV IRES-associated 80S ribosome complexes from HeLa cell extract (Boehringer et al. 2005).

To purify proteins under translation initiation conditions, we applied the HCV sORF mRNA, which contained the Tob-aptamer upstream of the 5'UTR (Fig. 2A, Tob-HCV sORF). We first pre-incubated the Tob-HCV sORF mRNA with the tobramycin-affinity matrix to allow efficient mRNA binding. Next the pre-coupled mRNA was incubated with cytoplasmic Huh7 cell extract under translation initiation condition in the presence of cycloheximide. 80S ribosomal complex formation was not disturbed by the Tob-aptamer (data not shown). As a specificity control, the RNA bearing no Tob-aptamer (HCV sORF)

(Fig. 2A) or beads alone (data not shown) was used. Proteins specifically eluted from the affinity matrix by tobramycin were analyzed by SDS-PAGE and silver staining (Fig. 2B shows a representative gel). Following extraction and tryptic digestion, liquid chromatography–tandem mass spectrometry was performed to identify the proteins. Only those parts of the Tob-HCV sORF lane in which distinct differences to the control lanes could be detected (Fig. 2B, slices 1–17) were analyzed. Mass spectrometry identified 39 proteins bound to Tob-HCV sORF mRNA. Among others, we found hnRNP C, polypyrimidine tract binding protein 1, and nucleolin, previously described to interact with the HCV 5'UTR (Gontarek et al. 1999; Ito and Lai 1999; Lu et al. 2004). We also identified NF90/NFAR-1, NF45, and RNA helicase A, which interact with both the HCV 5'UTR and HCV 3'UTR, and function in HCV replication (Isken et al. 2007).

Interestingly, we also isolated IGF2BP1 that was identified before as an HCV IRES-interacting protein (CRDBP) (Lu et al. 2004), but not characterized regarding its function in HCV IRES-mediated translation. A role of IGF2BP1 in the regulation of IGF-II mRNA and β -actin mRNA translation was described before (Nielsen et al. 1999; Hüttelmaier et al. 2005).

We performed the affinity purification three times and included control purification experiments with an mRNA bearing the HCV 3'UTR, but no HCV 5'UTR (Tob-sORF-HCV-3'UTR). When that mRNA was used, besides IGF2BP1, four proteins (NF90/NFAR-1, hnRNP Q, polypyrimidine tract binding protein 1, and RNA helicase A) were enriched in comparison to the Tob control RNA or beads alone (data not shown).

IGF2BP1 binds to both the HCV 5'UTR and HCV 3'UTR in vitro

To analyze the function of IGF2BP1 for HCV IRES-mediated translation, we first investigated whether IGF2BP1 directly interacts with the HCV 5'UTR and/or HCV 3'UTR. For this purpose, we performed UV-cross-linking with

FIGURE 1. HCV IRES-mediated translation initiation is enhanced by the HCV 3'UTR in vitro. (A,B, upper panel) Schematic representation of HCV 5'UTR and HCV 3'UTR bearing Luc reporter mRNAs. (Lower panel) Cytoplasmic Huh7 cell extract was used in cell-free translation reactions programmed with the [32 P] trace-labeled mRNAs HCV Luc or HCV Δ 3'UTR Luc for the times indicated. Luc activity is expressed as Luc units. Error bars denote the standard deviation from the mean of three different experiments. [32 P] trace-labeled mRNAs were extracted from the translation reaction at the time points indicated. [32 P] trace-labeled 5'cap sORF mRNA was used as an extraction control. Extracted mRNAs were analyzed by agarose gel electrophoresis. The incorporation of [32 P] was determined by phosphorimaging (Storm 860 Phosphor Imager; Molecular Dynamics, Image Quant software) and expressed as a percentage of signal intensity. Error bars denote the standard deviation from the mean of three different experiments. (C) [32 P]-labeled HCV sORF mRNA (left panel) or HCV Δ 3'UTR sORF mRNA (right panel) was incubated in Huh7 cell extract in the presence of 2 mM cycloheximide for 1 and 15 min, as indicated. (D) [32 P]-labeled HCV sORF mRNA (left panel) or HCV Δ 3'UTR sORF mRNA (right panel) was incubated in Huh 7 cell extract in the presence of 2 mM cycloheximide and 5 mM GMP-PNP for 1 and 15 min, as indicated. (C,D) Translation initiation complexes were allowed to assemble for the time indicated and subsequently resolved by centrifugation on 5%–25% linear sucrose gradients. After fractionation from the bottom to the top of the gradient, the radioactivity was monitored, expressed as a percentage of incorporation, and plotted against the fraction number. RNA extracted from 22 fractions was analyzed on agarose gels and visualized by ethidium bromide staining. The positions of 18S and 28S rRNA are indicated. The distribution of rpS3 and eIF6 in the sucrose gradient fractions, determined by Western blotting, indicates the position of 80S ribosomes and ribosomal 60S and 40S subunits, respectively.

FIGURE 2. Tob-aptamer affinity purification of HCV 5'UTR and HCV 3'UTR interacting proteins from cytoplasmic Huh7 cell extract. (A) Schematic representation of the transcripts applied in the RNA affinity purification. (B, left panel) Silver stain of affinity-purified proteins (SDS-PAGE): (lane 1) purification with Tob-HCV sORF; (lane 2) control purification with HCV sORF lacking the Tob-aptamer; (lane 3) protein standard. Numbers at the left indicate the position of gel slices used for the identification of proteins shown at the right.

recombinant IGF2BP1 and the [32 P]-labeled 5'UTR (nucleotides 1–375) or 3'UTR (nucleotides 1–234) transcripts, respectively (Fig. 3A,B). IGF2BP1 was bound directly to both the 5'UTR (Fig. 3A, lane 1) and 3'UTR (Fig. 3B, lane 1). The nonlabeled 5'UTR did compete for the interaction of IGF2BP1 with the 5'UTR (Fig. 3A, lane 3) and the 3'UTR (Fig. 3B, lane 3). The nonlabeled 3'UTR competed with IGF2BP1 binding as well (Fig. 3A,B, lane 5), but the 5' leader of the β -globin mRNA, which served as a negative control, did not affect the interaction (Fig. 3A,B, lanes 6,7). We performed filter binding assays to measure the affinity of IGF2BP1 to the different RNAs (Fig. 3C). IGF2BP1 was bound with nearly the same affinity to the HCV 5'UTR (20.5 nM) and HCV 3'UTR (18 nM). Consistent with our results in the UV-cross-linking competition assays, the 5' leader of the β -globin mRNA showed a significantly weaker affinity for IGF2BP1 (Fig. 3C, 146.70 nM).

To narrow down the interacting sequence elements, we subdivided the 5'UTR (375 nt) into three fragments: (1) nucleotides 1–128, consisting of domain I, which contains a short stem-loop and is otherwise mostly single stranded, and domain II, which forms a stem with several internal

loops; (2) nucleotides 128–315, representing domain III, a pseudoknot connected to a four-helix junction; and (3) nucleotides 315–375, containing domain IV, in which the HCV IRES AUG-start codon is located. The 3'UTR (234 nucleotides [nt]) was divided into two fragments: (1) nucleotides 1–151, consisting of the variable region and the poly(U-C) motif; and (2) nucleotides 151–234, which

FIGURE 3. IGF2BP1 binds to the HCV 5'UTR and 3'UTR in vitro. (A) UV-cross-linking of [32 P]-labeled 5'UTR and recombinant IGF2BP1 (lane 1) in the presence of 10- or 100-fold molar excess of unlabeled competitor RNAs: (lanes 2,3) 5'UTR; (lanes 4,5) 3'UTR; and (lanes 6,7) β -globin 5'-leader. (B) UV-cross-linking of [32 P]-labeled 3'UTR and recombinant IGF2BP1 (lane 1) in the presence of 10- or 100-fold molar excess of unlabeled competitor RNAs: (lanes 2,3) 5'UTR; (lanes 4,5) 3'UTR; and (lanes 6,7) β -globin 5'-leader. (C) Filter binding assay performed with [32 P]-labeled (black) HCV 5'UTR; (red) HCV 3'UTR; or (yellow) β -globin 5'-leader and increasing amounts of purified recombinant IGF2BP1. (Inset) K_D values. (D, left panel) UV-cross-linking of [32 P]-labeled 5'UTR and recombinant IGF2BP1 (lane 1) in the presence of 100-fold molar excess of unlabeled competitor RNAs: (lane 2) complete 5'UTR (nucleotides 1–375); (lane 3) fragment (1) (nucleotides 1–128); (lane 4) fragment (2) (nucleotides 128–315); (lane 5) fragment (3) (nucleotides 315–375); or (lane 6) β -globin 5'-leader. (Right panel) UV-cross-linking of [32 P]-labeled 3'UTR and recombinant IGF2BP1 (lane 1) in the presence of 100-fold molar excess of unlabeled competitor RNAs: (lane 2) complete 3'UTR (nucleotides 1–234); (lane 3) fragment (1) (nucleotides 1–151); (lane 4) fragment (2) (nucleotides 151–234); or (lane 5) β -globin 5'-leader.

contains the X region. To determine the binding site in the 5'UTR in detail, we again performed UV-cross-linking with the [32 P]-labeled 5'UTR and added the 5'UTR fragments as non-labeled competitor RNAs (Fig. 3D, left panel). The 5' leader of β -globin mRNA served as a negative control. Only the complete 5'UTR and the 5'UTR fragment (3) (nucleotides 315–375) competed with the IGF2BP1-5'UTR interaction (Fig. 3D, left panel, lanes 2,5), while the 5'UTR fragments (1) and (2) and the 5' leader of β -globin mRNA had no effect (Fig. 3D, left panel, lanes 3,4,6).

The formation of the IGF2BP1-3'UTR complex was reduced as well, when the complete 3'UTR or the 3'UTR fragment (1) (nucleotides 1–151) was added (Fig. 3D, right panel, lanes 2,3), while the 3'UTR fragment (2) (nucleotides 151–234) and the 5' leader of β -globin mRNA had no effect (Fig. 3D, right panel, lanes 4,5).

From these results, we conclude that IGF2BP1 preferentially binds to stem-loop IV of the 5'UTR and to the variable region and the poly(U-C) motif of the 3'UTR.

IGF2BP1 is required for HCV IRES-mediated translation, but not for 5'cap-dependent translation in vivo

To investigate the specificity of IGF2BP1 for HCV IRES-mediated translation, we performed RNAi experiments in Huh7 cells in combination with translation assays (Fig. 4). At the 72-h time point at which the expression of IGF2BP1 was reduced most efficiently employing two independent siRNAs (Fig. 4A, lanes 3,4) in comparison to no siRNA or control siRNA (Fig. 4A, lanes 1,2), we transfected Huh7 cells with in vitro synthesized HCV Luc mRNA, HCV Δ 3'UTR Luc mRNA or a 5'cap and 3'poly(A) tail bearing Luc mRNA (5'cap Luc) (Fig. 4B). The contribution of the 3'UTR was 10-fold (data not shown), comparable to the eightfold contribution in the in vitro translation system based on Huh7 cells (Fig. 1A). To investigate the effect of the reduced IGF2BP1 level on the 5'cap-dependent translation and on the contribution of the HCV 3'UTR simultaneously, the translation of the three mRNAs was set to 100% in mock transfected cells. In sharp contrast to the 5'cap-dependent translation that remained unaffected by all transfected siRNAs, the HCV IRES-mediated translation was significantly reduced to 33% or 26% by the two independent IGF2BP1 siRNAs (Fig. 4B), which decreased the IGF2BP1 level to 49% or 40%, respectively (Fig. 4A). The HCV IRES-mediated translation was further reduced to 21% or 17%, respectively, when the 3'UTR was lacking (Fig. 4B). Levels of all reporter mRNAs remained unaffected by IGF2BP1 knockdown, as determined by quantitative RT-PCR (Fig. 4C). Hence, changes in Luc reporter mRNA stability were not responsible for the observed specific inhibition of HCV IRES-mediated translation (Fig. 4B,C). These results indicate that IGF2BP1 functions specifically in HCV IRES-mediated translation in vivo.

FIGURE 4. IGF2BP1 is required for HCV IRES-mediated translation, but not for 5'cap-dependent translation in vivo. (A) Huh7 cells were transfected with (lane 1) no siRNA; (lane 2) control siRNA; or (lanes 3,4) two independent siRNAs against IGF2BP1. The RNAi effect on protein expression was analyzed using antibodies against IGF2BP1 and vinculin to monitor RNAi specificity. (B, upper panel) Schematic representation of HCV Luc, HCV Δ 3'UTR Luc, and 5'cap Luc mRNAs. (Lower panel) To quantify the influence of reduced IGF2BP1 expression on HCV IRES-mediated or 5'cap-dependent translation, the cells were transfected with the reporter mRNAs indicated. Luc activity was measured and expressed as percentage (average of three experiments with standard deviations). (C) Total RNA was isolated, and the integrity of Luc RNA levels was analyzed by qRT-PCR. Relative RNA amounts were determined by the $\Delta\Delta$ Ct method using endogenous cyclophilin A mRNA for normalization.

IGF2BP1 enhances HCV IRES-mediated translation initiation in extracts of rat primary hepatocytes

Employing RNAi, we did not observe a complete reduction of IGF2BP1 with two independent siRNAs (49% or 40% reduction, respectively) (Fig. 4A); therefore, we used a cell extract that does not contain IGF2BP1 to further study the 3'UTR-dependent mechanism of IGF2BP1 function in HCV IRES-mediated translation. Whereas IGF2BP1 is present in cytoplasmic extract of Huh7 cells, it is not detectable in total or cytoplasmic extracts of rat primary hepatocytes (Fig. 5A, lanes 2–4). We prepared translation

FIGURE 5. IGF2BP1 enhances HCV IRES-mediated translation via the 3'UTR in rat primary hepatocyte extract. (A) Detection of IGF2BP1 and vinculin (control) in Western blot assays: (lane 1) recombinant IGF2BP1; (lane 2) cytoplasmic Huh 7 cell extract; (lane 3) cytoplasmic rat primary hepatocyte extract; (lane 4) total rat primary hepatocyte extract; and (lane 5) total extract of rat testis (antibody control). (B) Cytoplasmic extract of rat primary hepatocytes was used in cell-free translation reactions, programmed with [³²P] trace-labeled reporter mRNAs HCV Luc or HCVΔ3'UTR Luc for the times indicated. Luciferase activity is expressed as relative Luc units. Error bars denote the standard deviation from the mean of three different experiments. (C) [³²P] trace-labeled mRNAs were extracted from the translation reaction at the time points indicated. [³²P] trace-labeled 5'cap sORF mRNA was used as an extraction control. Extracted mRNAs were analyzed by agarose gel electrophoresis. The incorporation of [³²P] was determined by phosphorimaging (Storm 860 PhosphorImager; Molecular Dynamics Image Quant software) and expressed as a percentage of signal intensity. Error bars denote the standard deviation from the mean of three different experiments. (D) Reporter mRNAs HCV Luc or HCVΔ3'UTR Luc were incubated in cytoplasmic extracts of rat primary hepatocytes in the presence of increasing amounts of recombinant IGF2BP1. Luciferase activity is expressed as relative Luc units. Error bars denote the standard deviation from the mean of three different experiments. Translation was set to 100% in the absence of recombinant IGF2BP1. In the presence of recombinant protein, data were analyzed with the *t*-test (two-tailed), (***) *p* < 0.0005.

competent extracts from these primary hepatocytes and analyzed the in vitro translation of [³²P] trace-labeled reporter mRNAs HCV Luc and HCVΔ3'UTR Luc for 0, 15, 30, and 45 min (Fig. 5B). Expression from the HCVΔ3'UTR Luc mRNA was reduced to 0.8×10^6 Luc units after 45 min compared to 2.1×10^6 Luc units from HCV Luc mRNA (Fig. 5B). Notably, both mRNAs were equally stable during translation (Fig. 5C). To investigate the effect of exogenously added IGF2BP1, the translation of both mRNAs was set to 100% (Fig. 5D). Following addition of increasing amounts of IGF2BP1 to primary hepatocyte extract, only the translation of HCV Luc mRNA was significantly increased, but not translation of HCVΔ3'UTR Luc (Fig. 5D). These results indicate that the interaction of IGF2BP1 with both the HCV 5'UTR and HCV 3'UTR is required to enhance translation in vitro.

Reduced expression of IGF2BP1 in HCV RNA replicon containing Huh7 cells does not affect HCV RNA replication, but HCV IRES-mediated translation

Next, we addressed the question whether IGF2BP1, besides affecting HCV IRES-mediated translation, also participates in the viral RNA replication process. For this purpose, we applied Huh7 cells that were stably transfected with an HCV RNA replicon (Lohmann et al. 1999; Grassmann et al. 2005). The HCV RNA replicon is a bi-cistronic, subgenomic viral RNA construct. It encodes a neomycin resistance gene as a selection marker to generate the stable cell line, and the HCV nonstructural proteins NS3, NS4A, NS4B, NS5A, and NS5B that form the viral replicase complex (Fig. 6A). That complex, together with host factors, is capable of replicating the HCV RNA (Lohmann et al. 1999; Grassmann et al. 2005). Translation of the neomycin-resistance gene is under the control of the HCV IRES, while the expression of the viral proteins NS3 to NS5B is initiated at the EMCV IRES (Fig. 6A).

First, we investigated the expression level of IGF2BP1 in these cells (Fig. 6B). Interestingly, the IGF2BP1 level was increased more than twofold in the HCV replicon containing Huh7 cells in comparison to naive Huh7 cells as determined by Western blot analysis (Fig. 6B).

Thus, we performed RNAi against IGF2BP1 in Huh7 replicon cells and measured the level of HCV RNA by quantitative RT-PCR. Interestingly, we found that when IGF2BP1 expression was suppressed by RNAi (Fig. 6C, upper panel), no reduction of HCV replication could be observed (Fig. 6C, middle panel). To prove the function of IGF2BP1 for HCV IRES-mediated translation in these cells, we investigated the HCV IRES-mediated expression of neomycin using Western blot assays (Fig. 6C, bottom panel). As a control, we measured the expression of NS5A that is translated via the EMCV IRES. When the expression of IGF2BP1 was reduced by RNAi only neomycin expression was significantly inhibited, but not NS5A

FIGURE 6. Reduced expression of IGF2BP1 in HCV RNA-replicon containing Huh 7 cells does affect HCV IRES-mediated translation, but not HCV RNA replication. (A) Schematic representation of the bi-cistronic replicon RNA. (B) Detection of IGF2BP1 and vinculin (control) in (lane 1) cytoplasmic extracts of Huh7 and (lane 2) Huh7 replicon cells in Western blot assays. (C) Huh7 replicon cells were transfected with (ctrl.) control or (IGF2BP1/1) IGF2BP1 siRNAs. IGF2BP1, vinculin, neomycin phosphotransferase II (NPT2), and NS5A were detected by specific antibodies. HCV RNA replication was measured by qRT-PCR. (D, lane 3) IGF2BP1 was immunoprecipitated with a specific IGF2BP1 antibody from cytoplasmic extracts of Huh7 replicon cells. Control immunoprecipitations were performed with (lane 5) a control antibody (Fyn) or (lane 7) beads alone. Coimmunoprecipitated HCV RNA was detected by RT-PCR. Cyclophilin A mRNA served as specificity control. (E) IGF2BP1 was immunoprecipitated with a specific IGF2BP1 antibody from cytoplasmic extracts of Huh7 replicon cells in the (lane 4) absence and (lane 5) presence of RNase A. Control immunoprecipitations were performed with (lanes 2,3) beads alone or (lanes 6,7) a control antibody (Fyn). Immunoprecipitated proteins were resolved on SDS-PAGE and analyzed with antibodies against IGF2BP1, eIF3c, rpS3, or eIF6.

expression (Fig. 6C, bottom panel). From these results we conclude that IGF2BP1 does affect HCV IRES-mediated translation, but not HCV RNA replication.

Since we could detect the inhibition of HCV IRES-mediated translation, we next investigated whether endogenous IGF2BP1 interacts with the HCV replicon RNA. Hence, IGF2BP1 was immunoprecipitated from cytoplasmic extract of replicon-transfected cells and, consisting with the purification data (Fig. 2), HCV RNA coprecipitated specifically with the IGF2BP1 antibody, but not with a control antibody or beads alone (Fig. 6D, lanes 3,5,7).

Because IGF2BP1 preferentially interacts with stem-loop IV of the IRES, where also the ribosomal 40S subunit binds (Kieft et al. 1999) and eIF3 enhances the recruitment of the 43S preinitiation complex (Pestova et al. 1998), we asked whether IGF2BP1 co-immunoprecipitated with the ribosomal 40S subunit and eIF3, and whether this interaction is RNA-dependent (Fig. 6E). Only in the absence of RNase A we specifically detected rpS3 and eIF3, but not eIF6 (Fig. 6E, lanes 4,5), suggesting that IGF2BP1, by binding to the HCV RNA, facilitates the recruitment of the ribosomal 40S subunit and initiation factor eIF3.

eIF3 and IGF2BP1 comigrate with 80S ribosomes assembled on HCV 5'UTR and 3'UTR bearing mRNA in Huh7 extract

To further analyze the specific effect of IGF2BP1 on HCV IRES-mediated, but not 5'cap-dependent translation (Fig. 4), we performed translation initiation reactions in micrococcal nuclease treated Huh7 cell extracts in the presence of cycloheximide comparing [³²P]-labeled HCV sORF and 5'cap sORF-poly(A) (5'cap sORF) mRNAs (Fig. 7, left and right panels, respectively). The distribution of rpS3 indicates the position of 80S ribosomes and 43S pre-initiation complexes, whereas eIF6 comigrates with ribosomal 60S subunits. The distribution of IGF2BP1 between 80S ribosome and 43S pre-initiation complex containing fractions did not differ significantly between HCV sORF and 5'cap sORF mRNA besides an additional sedimentation into heavier complexes with 5'cap sORF mRNA. In contrast, eIF3 comigrated with IGF2BP1 to 80S ribosome containing fractions only when HCV sORF mRNA was translated. These results suggest that IGF2BP1, by binding to the HCV 5'UTR and/or HCV 3'UTR, enhances HCV IRES-mediated translation by recruiting eIF3.

DISCUSSION

In this study, we demonstrate that the HCV 3'UTR has a strong stimulatory effect on HCV IRES-mediated translation in vitro. A stimulatory effect of the HCV 3'UTR has been described for transfected monocistronic mRNAs in Huh7 cells (Song et al. 2006). The question of how the HCV RNA 3'UTR enhances translation via the HCV IRES has not been addressed. Employing a newly established in vitro translation system based on Huh7 cell extract that shows a contribution of the HCV 3'UTR to HCV IRES-mediated translation

FIGURE 7. eIF3 and IGF2BP1 comigrate with 80S ribosomes assembled on HCV 5'UTR and 3'UTR bearing mRNA in Huh7 extract. [^{32}P]-labeled (*left* panel) HCV sORF mRNA or (*right* panel) 5'cap sORF mRNA was incubated in micrococcal nuclease treated Huh7 cell extract in the presence of 2 mM cycloheximide for 15 min. (A,B) Translation initiation complexes were allowed to assemble and subsequently resolved by centrifugation on 5%–25% linear sucrose gradients. After fractionation from the *bottom* to the *top* of the gradient, the radioactivity was monitored, expressed as the percentage of incorporation, and plotted against the fraction number. The distribution of rpS3, eIF6, IGF2BP1, or eIF3c in the sucrose gradient fractions was determined using specific antibodies.

(Fig. 1A,B), we investigated the underlying mechanism. We show that 80S complex assembly is stimulated by the HCV 3'UTR on an HCV IRES-bearing mRNA; hence the 3'UTR enhances translation initiation. Our data reveal that the 3'UTR promotes translation initiation at an early step by increasing the recruitment of 43S pre-initiation complexes (Fig. 1C,D). To shed light on the molecular requirements for the enhancing function of the 3'UTR, we adapted the Tob-aptamer RNA chromatography combined with liquid chromatography–tandem mass spectrometry to identify factors that interact with the HCV 5'UTR and/or HCV 3'UTR and contribute to the increased translation initiation at the HCV IRES in translational active Huh7 cell extract (Fig. 2). By using an mRNA that bears both the HCV 5'UTR and HCV 3'UTR as an affinity matrix, we specifically purified proteins that have been shown to interact with either the HCV 5'UTR or HCV 3'UTR, or both. The proteins IGF2BP1, hnRNP C, hnRNP D, hnRNP Q, polypyrimidine tract binding protein 1, PAI-1 mRNA binding protein 1, nucleolin, NF90/NFAR, RNA helicase A, and five subunits of eIF 3 (out of nine identified) match with proteins isolated from cytoplasmic Huh7 cell extract employing an HCV 5'UTR that lacks domain I and represents the HCV IRES consisting of domains II to IV (Kim et al. 2004; Lu et al. 2004). HnRNP

C, YB-1, polypyrimidine tract binding protein 1, DDX3, NF90/NFAR, and RNA helicase A have been identified as HCV 3'UTR binding proteins in Huh7 extract (Gontarek et al. 1999; Harris et al. 2006; Isken et al. 2007). Only for some of these interactions has a functional relevance been shown. The impact of NF90/NFAR, NF45, and RNA helicase A on HCV RNA replication has been characterized by Isken et al. (2007). An enhancement of HCV IRES-mediated initiation by hnRNP Q has been described by Kim et al. (2004). The function of polypyrimidine tract binding protein 1 in HCV IRES-mediated translation is controversially discussed (Ali and Siddiqui 1995; Ito and Lai 1999; Ostareck-Lederer et al. 2005; Brocard et al. 2007).

In our study, we evaluated the function of IGF2BP1 for HCV IRES-mediated translation. IGF2BP1 belongs to a family of RNA binding proteins, also termed IGF-II mRNA binding proteins (IMPs), zipcode mRNA binding proteins (ZBPs), or coding region instability determinant binding proteins (CRDBPs), which are involved in the regulation of RNA localization, translation, and turnover (Yisraeli 2005). Members of that protein family are expressed early in development, strongly decline in the postnatal phase, but show de novo synthesis in neoplasias (Nielsen et al. 1999; Yaniv and Yisraeli 2002; Kobel et al. 2007).

It has been shown that IGF2BP1 controls the translation of β -actin mRNA and IFG-II mRNA. In the case of IGF-II mRNA association of IGF2BP1 with the 5'UTR is suggested to be a prerequisite of translation inhibition (Nielsen et al. 1999). In contrast β -actin mRNA translation is blocked by IGF2BP1 binding to the 3'UTR. That interaction mediates translation inhibition at a late step of initiation at the 5'UTR (Hüttelmaier et al. 2005).

Our purified complex also shares proteins, which are common to IGF2BP1 granules, purified from human embryonic kidney 293 cells (HEK293) and human bone osteosarcoma epithelial cells (U2OS), like RNA helicase A, nucleolin, hnRNP Q, hnRNP D, and YB-1 (Jønson et al. 2007; Weidensdorfer et al. 2009).

Employing RNA-protein interaction techniques, we show that recombinant IGF2BP1 specifically interacts with both, the HCV 5'UTR and the 3'UTR (Fig. 3). This interaction is in the lower nanomolar range as shown for the binding of IGF2BP1 to the zipcode motif that has been identified as the binding region in the β -actin mRNA 3'UTR (Farina et al. 2003). We could not identify a distinct sequence element, like the zipcode motif, in either UTR. For the tau mRNA 3'UTR, it was shown that IGF2BP1 does not recognize a specific mRNA sequence, but a three-dimensional structure (Atlas et al. 2007). Furthermore, IGF2BP1 (CRDBP) (Doyle et al. 1998) binds to the CRD region located in the last 249 nt of the c-myc coding sequence, which has closely been defined to nucleotides 1763–1777 (Coulis et al. 2000). The CRD has some similarity with the zipcode (ACACCC) (Ross et al. 1997) and the IGF2BP1-binding consensus sequence (UUCACGUU CACA) (Nielsen et al. 1999), but no common motif required for RNA binding is defined.

The competition studies suggest that the HCV 5'UTR fragment that represents domain IV of the HCV IRES, but not domains I–III, is the major site of interaction. In the 3'UTR, the variable region and poly(U-C) motif contribute to IGF2BP1 binding (Fig. 3).

To investigate the specificity of the IGF2BP1 function in HCV IRES-mediated translation in vivo RNAi against IGF2BP1 was performed in Huh7 cells. While control siRNA has no effect on HCV IRES-mediated and 5'cap-dependent Luc expression, a reduction of IGF2BP1 does result in a specific decrease of HCV IRES-mediated Luc expression, which was further reduced in the absence of the 3'UTR and had no impact on 5'cap-dependent translation (Fig. 4). This suggests that IGF2BP1 has a specific function on HCV IRES-mediated translation and is not a general translational regulator. The incomplete reduction of HCV IRES-mediated Luc translation might not only be due to the limited IGF2BP1 knockdown, but could also be a result of compensatory effects of other IGF2BP isoforms. Although we have only identified IGF2BP1 in the affinity purification assay, two other members of the IGF2BP family (IGF2BP2 and IGF2BP3) are present in Huh7 cells.

Furthermore, we have purified several HCV 5'UTR and HCV 3'UTR interacting proteins (YB-1, nucleolin, hnRNP C, hnRNP D). Besides the significant impact of IGF2BP1 on HCV IRES-mediated translation, these factors might also play a role. This was shown for the stability of the c-myc mRNA that is regulated by an IGF2BP1-associated cytoplasmic RNP consisting of hnRNP U, syncrin (hnRNP Q), YB-1, and DHX9 (Weidensdorfer et al. 2009). A focus of further studies will be the function of other identified factors in the context of IGF2BP1. Interestingly, compared to an individual IGF2BP1 knockdown, a double knockdown with one of the additional factors shows no additive effect on the stability of c-myc mRNA. This suggests that stabilization is likely to occur via one regulatory complex (Weidensdorfer et al. 2009).

By employing an in vitro translation system generated from primary hepatocytes, which do not express IGF2BP1, the requirement of both the HCV 5'UTR and 3'UTR for efficient HCV IRES-mediated translation can be demonstrated. When the extract is supplemented with recombinant IGF2BP1, the translation of an mRNA that bears the HCV 5'UTR and HCV 3'UTR is enhanced in contrast to an mRNA, which lacks the HCV 3'UTR (Fig. 5). The observed intensity of this effect might be due to the fact that we added recombinant IGF2BP1, which showed a high affinity binding to the HCV 5'UTR and HCV 3'UTR (Fig. 3C), but possibly IGF2BP1 has to be modified post-translationally in order to enhance HCV IRES-mediated translation. Furthermore, IGF2BP1 has several mRNA targets (Yisraeli 2005) and might act in different cellular complexes concurrently. Therefore, the protein that is added can be incorporated in other functional complexes, which limits the impact on HCV Luc mRNA translation.

Because in HCV infected cells translation of the plus-strand RNA HCV genome precedes replication, we investigated whether IGF2BP1 supports the replication of a bicistronic reporter RNA stably expressed in Huh7 cells (Fig. 6). We first noticed a twofold increased IGF2BP1 level in the Huh7 replicon cells. This might be due to an activation of phosphoinositide 3-kinase mediated by the nonstructural HCV protein NS5A, which leads to the stabilization of β -catenin, resulting in an increased β -catenin dependent transcription of the IGF2BP1 gene (Street et al. 2005; Gu et al. 2008). Interestingly, the knockdown of IGF2BP1 resulted in an inhibition of the HCV IRES-mediated translation, but did not influence replication. In contrast, knockdown of RNA helicase A, DDX5, or HuR, which interact with the HCV 5'UTR and/or HCV 3'UTR, resulted in a reduced replication (Harris et al. 2006; Isken et al. 2007). For hnRNP Q, an influence on HCV IRES-mediated translation and HCV replication has been shown (Kim et al. 2004; Liu et al. 2009).

To get an initial insight into how IGF2BP1 specifically functions on HCV IRES-mediated translation, we performed immunoprecipitation experiments and found that

IGF2BP1 specifically coprecipitates with eIF3 and rpS3, a component of the ribosomal 40S subunit, in an RNA dependent manner (Fig. 6E). Furthermore, it colocalizes specifically with eIF3 in 80S ribosome containing fractions when an mRNA bearing both the HCV 5'UTR and HCV 3'UTR is translated (Fig. 7, cf. left and right bottom panels).

This could explain why only HCV IRES-mediated translation, but not 5'cap-dependent translation, is inhibited when the expression of IGF2BP1 is reduced (Fig. 4). EIF3 increases 43S pre-initiation complex formation and is required for 80S ribosome formation on the HCV IRES (Pestova et al. 1998; Sizova et al. 1998). IGF2BP1 bound to the HCV 5'UTR and/or HCV 3'UTR might enhance HCV IRES-mediated translation by recruiting eIF3. Furthermore, the interaction of IGF2BP1 with both UTRs can support mRNA circularization because the protein has been shown to dimerize upon RNA binding (Nielsen et al. 2004).

It will be interesting to study in the future whether, together with the recruitment of eIF3, the dimerization facilitates an increase in translation as it was shown for the formation of a closed loop structure and a synergistic interplay between the 5'cap and the 3'poly(A) tail (Gallie 1991; Jacobson 1996; Wells et al. 1998; Amrani et al. 2008).

MATERIALS AND METHODS

Plasmids

All plasmid constructs used for in vitro transcription of HCV RNAs derive from the plasmid HCVwt-fLuc-3'UTR (HCV Luc) or the plasmid HCVwt-fLuc (HCVΔ3'UTR Luc), which contain genomic sequences of Hepatitis C genotype 1b encoding the UTRs and the firefly luciferase ORF (Ostareck-Lederer et al. 2005). The plasmids HCV sORF and HCVΔ3'UTR sORF were generated from HCV Luc and HCVΔ3'UTR Luc by replacing the luciferase-coding sequence by a Flag-tag sequence between Hind III and BamHI (Supplemental Table). Tob-HCV sORF was generated by cloning the J6f1 aptamer (Tob) (Hartmuth et al. 2002; Jiang and Patel 2004) into KpnI upstream of the HCV 5'UTR in the plasmid HCV sORF (Isken et al. 2007). The sequences from the HCV 5'UTR and HCV 3'UTR used in the UV-cross-linking assay were generated by subcloning them into pBSII SK (Stratagene). The HCV 5'UTR fragment (1) was generated using KpnI and SmaI, fragment (2) using SmaI, and fragment (3) using SmaI and Hind III. The HCV 3'UTR was separated into fragments (1) and (2) using DdeI. The β-globin 5'leader (β-globin) (Hellen et al. 1993) was subcloned into pBSII SK using XhoI. CAT control RNA (pGEM-CAT) and 5'cap sORF poly(A) have been described by Naarmann et al. (2008); the latter was linearized with XhoI to generate a poly(A) tail. The 5'cap Luc-poly(A) have been described by Iizuka et al. (1994). GST-ZBP1 in pGEX6P1 (Amersham) was used for expression of recombinant IGF2BP1 (Hüttelmaier et al. 2005).

Cell culture and cytoplasmic extract preparation

Huh7 cells and Huh7 replicon cells were grown in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum (FBS; Biochrom), 1% non-essential amino acids, and 1%

penicillin streptomycin (Invitrogen). Rat primary hepatocytes prepared according to Seglen (1976) were a kind gift of M. Sgodda and H. Aurich (Martin-Luther-University Halle-Wittenberg). Huh7 S10 extract was prepared according to Barton and Flanagan (1993). Briefly, 1×10^8 of subconfluent Huh7 cells were harvested using Trypsin-EDTA (Invitrogen), washed three times with 10 volumes of isotonic buffer (35 mM HEPES/KOH at pH 7.6, 146 mM NaCl, 11 mM glucose) and collected by centrifugation at 220g. The pellet was resuspended in 1.5 volumes of hypotonic buffer [20 mM HEPES/KOH at pH 7.6, 10 mM KCl, 1.5 mM Mg(CH₃CO₂)₂, 1 mM DTT, protease inhibitors] and incubated for 20 min on ice. The cells were broken with 25 strokes in a dounce homogenizer, and 0.2 volumes of 5× S10 buffer [100 mM HEPES/KOH at pH 7.6, 600 mM K(CH₃CO₂)₂, 20 mM Mg(CH₃CO₂)₂, 25 mM DTT, protease inhibitors] were added. Nuclei were removed by centrifugation at 10,000g for 10 min at 4°C. Aliquots of the extract were stored at −80°C.

Protein expression and purification

Expression of recombinant IGF2BP1 was performed according to Farina et al. (2003) with the following modifications: BL21 (DE3) cells were induced for 4 h at 23°C with 1 mM IPTG in minimal medium (72 mM Na₂HPO₄ × 7 H₂O, 22 mM KH₂PO₄, 8.5 mM NaCl, 18.7 mM NH₄Cl, 0.4% casamino acids, 11 mM Glucose, 1 mM MgSO₄, 0.3 mM CaCl₂). Bacteria were lysed in lysis buffer (PBS, 1% Triton X-100, 850 mM NaCl, 10 mM EDTA, 10 mM EGTA, 1:1000 β-mercaptoethanol, protease inhibitors). After sonication the lysate was cleared for 30 min at 8000g at 4°C. Glutathione Sepharose beads 4B (GE Healthcare) were incubated with the cleared lysate for 1.5 h at 4°C. After washing, the GST tag was cleaved with precision protease (GE Healthcare) in 50 mM Tris (pH 7.6), 300 mM NaCl, 1 mM EDTA, and 3 mM β-mercaptoethanol, protease inhibitors overnight at 4°C. Next the protein was loaded onto a HiTrap Heparin HP column (GE Healthcare) in binding buffer (50 mM Na₂HPO₄, 25 mM NaCl at pH 7.2) and eluted in 50 mM Na₂HPO₄, 1 M NaCl (pH 7.2). Purified fractions were pooled and desalted into storage buffer (20 mM HEPES at pH 7.6, 150 mM NaCl, 10% glycerol) via PD-10 columns (Amersham) and stored at −80°C. Protein concentrations were determined by the BCA assay (Thermo Scientific), SDS-PAGE, and Coomassie staining using bovine serum albumin as a standard.

In vitro transcription

RNAs were transcribed with a T7 MEGAscript Kit (Applied Biosystems/Ambion) according to the manufacturer's instructions using 7 mM ApppG-cap (KEDAR). RNAs were purified by phenol and phenol/chloroform extraction, filtration (Chromaspin-100 column, Takara), and ethanol precipitation and quantified spectrometrically. For tobramycin-affinity coupling reactions, in vitro translation assays, UV-cross-linking assays, filter binding assays, and sucrose density gradient analysis, the RNAs were labeled with 10–30 μCi α-[³²P] UTP (3000 Ci/mmol, 10 mCi/mL; Hartmann Analytic). RNA yield was determined by Čerenkov counting.

Tobramycin affinity purification

The tobramycin affinity purification was carried out as described by Isken et al. (2007) with the following modifications: 28 pmol of

Tob-HCV sORF or HCV sORF were precoupled to the tobramycin-matrix for 1.5 h by rotation at 4°C in 400 µL of binding buffer (BB) (20 mM Tris-HCl at pH 8.1, 1 mM CaCl₂, 1 mM MgCl₂, 0.2 mM DTT), and the matrix was washed three times in BB. Translation initiation reactions (400 µL) containing 2 mM cycloheximide were loaded onto the matrix and incubated for 30 min at 30°C. Subsequently, the matrix was washed three times in BB, supplemented with 75 mM KCl and 5 mM MgCl₂. Bound complexes were eluted with 250 µL of BB, supplemented with 5 mM tobramycin, 145 mM KCl, and 2 mM MgCl₂ for 30 min by rotation at room temperature (RT). Proteins were recovered by methanol-chloroform precipitation (Jiang et al. 2004) and analyzed by 5%–15% SDS-PAGE and silver staining (Shevchenko et al. 1996).

Mass spectrometry

Protein bands were cut out, washed, and digested with trypsin (Promega) in 30 µL of 10 mM ammonium bicarbonate (pH 8.0) overnight at 37°C (Shevchenko et al. 1996). Tryptic peptides were extracted, and liquid chromatography–tandem mass spectrometry (LC/MS/MS) analyses were performed using a Micromass CapLC column 75 µm × 15 cm, 3 µm dC18 Atlantis NanoEase (Waters) coupled to a quadrupole time-of-flight mass spectrometer (Q-TOF2; Micromass). Peptide fragment ion spectra were searched against the non-redundant protein database for exact matches using MASCOT (<http://www.matrixscience.com>).

UV cross-linking

For UV cross-linking, 0.5 or 5 pmol of competitor RNA were pre-incubated for 15 min at 30°C with 1.6 µM recombinant IGF2BP1, 1 µg of rRNA (*Escherichia coli*) in 10 mM HEPES (pH 7.6), 100 mM KCl, 2 mM MgCl₂, 5% glycerol, 1 mM DTT, and 40 U RNasin (Promega). Fifty fmoles of [³²P]-labeled RNA (specific activity: 10,000 cpm/pmol) were added and incubated for 15 min. After addition of 5.5 µg of heparin, the samples were exposed to ultraviolet light (254 nm) for 10 min at 4°C followed by treatment with 7 µg of RNase A and 20 U of RNase T1 for 60 min at 37°C. Proteins were analyzed by 12% SDS-PAGE and autoradiography.

Filter binding

A 40 µL binding reaction contained 50 mM Tris (pH 8.0), 100 mM KCl, 1 mM EDTA 0.5 mM DTT, 10% glycerol, 0.2 mg/mL BSA, 0.01% Ipegal, 5 fmol of [³²P]-labeled RNA (specific activity: 500,000 cpm/pmol), and increasing amounts (0.7 pM to 1.6 µM) of recombinant IGF2BP1. After 10 min of incubation at RT, the reactions were applied to cellulose nitrate membrane filters (NC 20, 0.2 µm; Whatman) and washed in 50 mM Tris (pH 8.0), 100 mM KCl, and 1 mM EDTA. The filter-bound radioactivity was measured by scintillation counting. Apparent *K_D* values were calculated using nonlinear regression applied by SigmaPlot.

In vitro translation

Translation reactions contained 40% Huh7 S10 or rat primary hepatocyte extract, 100 µM amino acids, 16 mM HEPES (pH 7.6), 2–3 mM Mg(CH₃CO₂)₂, 100–160 mM KCH₃CO₂, 80 µg/mL tRNA, 0.8 mM ATP, 0.1 mM GTP, 40 µg/mL creatine kinase, 20 mM creatine phosphate, and 70–350 fmol of [³²P] trace-labeled

HCV reporter mRNAs. Translation reactions were incubated up to 45 min at 37°C and stopped on ice. Luciferase activity was measured using the luciferase assay system (Promega) in a tube Luminometer (Berthold Technologies).

Sucrose gradient analysis

Huh7 cell extract was incubated with 560 fmol of [³²P]-labeled HCV RNA in the presence of 2 mM cycloheximide (Sigma-Aldrich) and 5 mM GMP-PNP (Jena Biosciences) for the indicated time points at 30°C. Translation initiation complexes were resolved on linear 5%–25% sucrose density gradients (prepared in 20 mM HEPES at pH 7.6, 150 mM KCH₃CO₂, 5 mM MgCl₂, 1 mM DTT) by centrifugation at 100,000g for 225 min. Fractions (500 µL each) were collected from the bottom to the top of the gradient and analyzed by Čerenkov counting. For RNA analysis, 400 µL of each fraction were extracted with Trizol (Invitrogen) and separated on a 1% agarose gel. For protein analysis, 400 µL of each fraction were methanol-chloroform precipitated (Jiang et al. 2004), separated on a 5%–18% SDS gradient PAGE, and analyzed by Western blot assay.

Micrococcal nuclease treatment

To remove endogenous RNAs, Huh7 extract was micrococcal nuclease-treated prior to the translation reaction. Four microliters of Huh7 extract were treated with 0.08 U of micrococcal nuclease (Roche) and 0.8 mM CaCl₂ for 15 min at 27°C. The reaction was stopped with 1.6 mM EGTA and used for in vitro translation.

Antibodies and Western blotting

The monoclonal antibody against IGF2BP1/ZBP1 has been described by Hüttelmaier et al. (2005). All other antibodies were purchased from Santa Cruz Biotechnologies (eIF3c, sc-28858, Fyn, sc-434), Abnova (eIF2S1, H00001965-M01), BD Transduction Laboratories (eIF6, 611120), NEB (rpS3, 2579S), Sigma-Aldrich (vinculin, V9131), and Abcam (Neomycin phosphotransferase II, ab 60018). NS5A antiserum has been described by Isken et al. (2007). HRP conjugated secondary antibodies were purchased from GE Healthcare (NA931; NA934), infrared fluorescence labeled antibodies (926-32212; 926-32223) from LI-COR Biosciences.

PVDF membrane (Fisher Scientific) was blocked in 5% milk, 2% BSA, TBST (5 mM Tris-HCl at pH 7.4, 10 mM Glycin, 300 mM NaCl, 0.1% Tween 20) for 1 h at RT. Antibodies were incubated according to the manufacturer's instructions. HRP-conjugated antibodies (1:3000) were incubated for 1 h at RT, and membranes were developed with ECL Western blotting substrate (Thermo Scientific) or infrared antibodies (1:10,000) for 30 min at RT following visualization with an LI-COR infrared scanner (Odyssey software).

RNAi

Huh7 or Huh7 replicon cells (0.6 × 10⁶ cells in Dulbecco's Modified Eagle's Medium without FBS and antibiotics) were transfected by electroporation (270 V, 950 µF, GenePulser II; Bio-Rad) with 500 pmol of IGF2BP1 siRNA or 500 pmol of nonspecific control siRNA (Supplemental Table 1) (MWG-Biotechnology). Cells were harvested 24–72 h post-transfection for Western blot

assays. Cell pellets were lysed in RIPA buffer (50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 1% Igepal, 0.5% sodiumdesoxycholate, protease inhibitors) for 15 min on ice following 10 min of centrifugation at 10,000g at 4°C. Protein concentration was determined by BCA assay (Thermo Scientific).

RNA transfection

Seventy-two hours after siRNA transfection, 1×10^6 Huh-7 cells were transfected in cytomix containing 1.25% DMSO (van den Hoff et al. 1992) by electroporation with 17.5 pmol of reporter mRNAs, and harvested after 3 h. Cells were counted and washed twice in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 2 mM KH₂PO₄ at pH 7.4). To measure luciferase activity, cells were resuspended in luciferase lysis reagent (Promega). RNA stability was analyzed by quantitative RT-PCR.

Quantitative RT-PCR

Total RNA was extracted using Trizol (Invitrogen). For reverse transcription, random primers and 2 µg of RNA (Fig. 4C) or oligo(dT) primers and 0.5 µg RNA (Fig. 6C), or specific primers (Luciferase, HCV replicon, cyclophilin A) (see Supplemental Table 1), M-MLV RT (Fermentas) were used. QRT-PCR was performed using SYBRgreen (Taq-Master mix; Promega) in an MX3000p cycler (Stratagene). RNA amounts were determined by the $\Delta\Delta C_t$ method using cyclophilin A mRNA for internal normalization (Livak and Schmittgen 2001).

Immunoprecipitation

Twenty microliters of protein A-sepharose coupled with 1.5 µg of IGF2BP1 or Fyn antibody were incubated with 50 µg of Huh7 replicon S10 extract for 4 h at 4°C in BB (20 mM HEPES at pH 7.6, 100 mM KCl, 5 mM MgCl₂, 0.5% Igepal, 1 mM DTT, RNasin, protease inhibitors). Beads were washed twice with BB and boiled in SDS sample buffer. The supernatant was analyzed in Western blot assays. RNase A treatment (20 µg/mL) was performed during the last washing step in BB for 15 min at RT. For RT-PCR, co-immunoprecipitated RNA in the pellet was isolated with Trizol (Invitrogen). For RT, equal volumes of RNA, random primers (Promega), a HCV replicon specific primer, and 150 U of M-MLV-RT (Promega) were used. Aliquots (2 µL) of the reverse transcription were used for PCR with GoTaq-FlexiDNA polymerase (Promega) and the respective primer pairs (Supplemental Table 1).

SUPPLEMENTAL MATERIAL

Supplemental material can be found at <http://www.rnajournal.org>.

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