The Journal of Microbiology, December 2005, p.529-536 Vol. 43, No. 6

Hepatitis C Virus Non-structural Protein NS4B Can Modulate an Unfolded Protein Response

Yi Zheng, Bo Gao, Li Ye, Lingbao Kong, Wei Jing, Xiaojun Yang, Zhenghui Wu and Linbai Ye*

State Key laboratory of virology, College of Life Sciences, Wuhan University, Wuhan, Hubei 430072, P.R.China

(Received August 28, 2005 / Accepted September 27, 2005)

Viral infection causes stress to the endoplasmic reticulum (ER). The response to endoplasmic reticulum stress, known as the unfolded protein response (UPR), is designed to eliminate misfolded proteins and allow the cell to recover. The role of hepatitis C virus (HCV) non-structural protein NS4B, a component of the HCV replicons that induce UPR, is incompletely understood. We demonstrate that HCV NS4B could induce activating transcription factor (ATF6) and inositol-requiring enzyme 1 (IRE1), to favor the HCV subreplicon and HCV viral replication. HCV NS4B activated the IRE1 pathway, as indicated by splicing of X box-binding protein (Xbp-1) mRNA. However, transcriptional activation of the XBP-1 target gene, EDEM (ER degradation-enhancing α-mannosidase-like protein, a protein degradation factor), was inhibited. These results imply that NS4B might induce UPR through ATF6 and IRE1-XBP1 pathways, but might also modify the outcome to benefit HCV or HCV subreplicon replication.

Keywords: hepatitis C virus (HCV), non-structural protein NS4B, unfolded protein response (UPR)

The hepatitis C virus (HCV) is a major health problem worldwide. HCV causes acute and chronic hepatitis, which can lead to chronic active hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) (Farci *et al*., 2000). The HCV viral genome consists of positive-strand RNA, 9.6 kb in length, encoding a 3,000 amino acid polyprotein which is cleaved into at least three structural proteins (core, E1, and E2) and six non-structural proteins (NS2 to NS5A/B). Many of the nonstructural protein products are required for productive viral replication (Bartenschlager and Lohmann, 2000; Rosenberg, 2001). The function of NS4B, one of the nonstructural proteins of HCV, is incompletely understood. NS4B localizes in membrane-associated manner (Hugle *et al*., 2001; Lundin *et al*., 2003). The precise topology of NS4B with respect to the membrane in which it resides is not clear. However, it is predicted to harbor at least four transmembrane domains (Hugle *et al*., 2001; Lundin *et al*., 2003), which are believed to be responsible for conferring the membrane association of the protein. Apparent activities in translation inhibition (Florese *et al*., 2002; Kato *et al*., 2002), modulation of NS5B enzymatic function (Piccininni *et al*., 2002), transformation (Park *et al*., 2000), and interaction with CREB-RP/ATF6 (Tong *et al*., 2002) have been reported for NS4B. NS4B has also recently been implicated in the perturbation of intracellular membranes and in the formation of the membranous web structures (Egger *et al*., 2002) postulated to harbor the HCV replication complex (Gosert *et al*., 2003). Recently, Elazer *et al*. reported that NS4B mediates membrane association, correct localization of replication complex proteins, and HCV RNA replication (Elazar *et al*., 2004).

The endoplasmic reticulum (ER) is an extensive membranous network that provides a unique environment for the synthesis, folding, and modification of secretory and cell proteins. To ensure the completion of these processes in the ER, a quality control mechanism exists to select proteins that have been improperly folded or modified. Accumulation of unfolded or misfolded proteins in the ER (i.e. ER stress) triggers the UPR, which acts to release cells from stress conditions. ER stress and the UPR are induced by physiological conditions known to cause protein misfolding, expression of mutant proteins, and viral infection. The UPR can also be induced using drugs such as tunicamycin (Tu), which inhibits N-linked glycosylation, and dithiothreitol (DTT), a strong reducing agent which prevents the formation of disulfide bonds. Three transmembrane proteins located in the ER have been identified as sensors of ER stress: activating transcription factor-6 (ATF6), inositol requiring enzyme-1 (IRE-1), and PER-like ER kinase (PERK). Under normal conditions, the ER chaperone immunoglobulin heavy-chain-binding protein (Bip), also referred to as glucose-regulated protein-78 (GRP78), is bound to the ER luminal domain of

* To whom correspondence should be addressed.
(Tel) 86-27-68752372; (Fax) 86-27-68752372
(E-mail) linbaiye@whu.edu.cn

each sensor. However, when misfolded proteins accumulate in the ER, Bip is pulled away from these sensors to bind to misfolded proteins. Upon sensing ER stress, the transcription of genes downstream of ATF6 is upregulated by the translocation of the cytoplasmic domain of ATF6 to the nucleus. ATF6 has also been shown to increase the amount of Xbp-1 mRNA, providing a link between the ATF6 and IRE-1 pathways. IRE-1-dependent transcription is upregulated when the endoribonuclease domain of the activated IRE-1 molecules catalyzes the removal of a small (26-nucleotide) intron from the mRNA of the gene X-box-binding protein (XBP1). The splicing of the mRNA produces an active transcription factor which induces the expression of a subset of genes which encode chaperones, as well as the expression of genes which encode protein degradation enzymes (e.g., EDEM). PERK activation leads to the phosphorylation of the α subunit of the eIF2 translation initiation factor, which inhibits the assembly of the 80S ribosome and results in general inhibition of protein synthesis. Insufficient adaptive responses to UPR result in the initiation of apoptosis, leading to the activation of JNK, caspase-7, and caspase-12 (Rutkowski and Kaufman, 2004). HCV replicons induce ER stress, which results from a decline in protein glycosylation and prevents the assembly of MHC class I molecules (Tardif *et al.*, 2002); HCV subreplicons could also induce IRE1-XBP1 expression, but represss XBP1 transactivation activity (Tardif *et al.*, 2004). Hence, it may be advantageous for HCV to modulate the UPR, inhibiting the effects that would be detrimental to the infection, while maintaining those that may be beneficial. It remains unknown which protein is associated with IRE1-XBP1 signal pathway in the context of the subreplicon. In the current report, we focus on the relationship between NS4B and the UPR.

Materials and Methods

Plasmid construction

The expression plasmid, pcDNA3.1 (-) NS4B, was constructed by inserting the PCR products of the HCV-NS4B region of HCV-k isolate (genotype1b) into the mammalian expression vector, pcDNA3.1 (-) (Invitrogen), which contains human cytomegalovirus (CMV) immediate-early promoter. The following primers were synthesized to amplify the HCV-NS4B gene: sense primer, 5'-GGATCCTCTAGAACCATGGCTCAGCACTTACCGTAC-3' (*Xba*I digestion site underlined); antisense primer, 5'-GAATTCAAGCTTTTAGCATGGAGTGGTACAC-3' (*Hind*III digestion site underlined). The PCR products of HCV-NS4B were digested with *Xba*I and *Hind*III and inserted into pcDNA3.1 (-) vector by T4 DNA ligase; the recombinant was confirmed by sequencing. The pEGFP-NS4B plasmid was constructed using a method similar to that described above, except that the primers were changed.

Cell culture and transfection

HeLa and Huh-7 (human hepatoma cell line) cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM), pH 7.4, supplemented to contain 25 mM $NaHCO_3$, 10 μg/ml streptomycin sulfate, 100 ug/ml penicillin G, and 10%(V/V) fetal bovine serum. Cells were maintained at 37°C in a 5% CO_2, 95% air incubator. HeLa and Huh-7 cells were seeded in DMEM containing 10% fetal calf serum at a density of 3×10^5 cells per 35-mm plate. After incubation for 12 h, the cells were transfected with 0.5 μg of pcDNA3.1 (-), which served as the control, or with pcDNA3.1 (-) NS4B using the Lipofectamine™ 2000 (Life Technologies, USA). For the HeLa cell line that stably expressed NS4B, the cells were cultured at 24 h post-transfection in 1mg/ml G418 until drug-resistant colonies appeared.

Western blot analysis

Cell lysates were prepared as previously reported. In brief, cells were lysed with cell lysis buffer [0.3% NP40, 1 mM EDTA, 50 mM Tris-CL (pH 7.4), 2 mM EGTA, 1% Triton X-100, 150 mM NaCl, 25 mM NaF, 1 mM Na_3VO_3, 10 μg/ml PMSF] for 30 min on ice, and the lysates were classified after centrifugation at 12,000×g for 15 min at 4°C. Protein samples were separated by SDS-PAGE and transferred onto Immobilon membranes (Millipore, USA). HCV NS4B and XBP1 proteins were identified using anti-NS4B monoclonal antibody (kindly provided by Prof. Luo, Kentucky University, USA) and anti-XBP1 primary antibodies (Santa Cruz Biotechnology, USA), respectively.

Indirect immunofluorescence assay

Cells were grown on coverslips and transfected according to the method described above. For detection of NS4B, cells were fixed and permeabilized with methanol-acetone (V/V, 1:1) for 3 min. Immunostaining with the primary antibody from the mixed sera of several patients was done at room temperature for 1 h, after which the cells were washed three times with PBS, each wash lasted for 5 min. Secondary antibody incubation was performed at room temperature for 30 min to 45 min, after which the cells were washed as described above. All antibodies were diluted in 3% bovine serum albumin (BSA), and cells were subsequently mounted in Mowiol (Calbiochem, USA). Immunostained samples were analyzed by fluorescence microscopy (Nikon Eclipse E800, Japan).

RT-PCR for XBP1

Total RNA was extracted from HeLa cells using an RNA extraction kit. Aliquots of 20 μg of total RNA were treated with M-MLV reverse transcriptase (Invitrogen, USA), and were then amplified with Ex-Taq polymerase (TaKaRa, Japan) using a pair of primers which corresponded to nucleotides: sense primer, 5'-CCTTGTAGT-

TAGAACC-AGG-3'; anti-sense primer, 5'-GGGGCTT-GGTATATATGTGG-3'.

Real-time Quantitative RT-PCR for ATF6, Grp78, caspase-12, and EDEM

Total RNA was extracted using Trizol reagent (Invitrogen, USA) from HeLa cells stably transfected and Huh-7 cells transiently transfected by pcDNA3.1(-) NS4B and pcDNA3.1(-). We carried out real-time quantitative PCR to quantify the mRNA levels and the copy numbers of ATF6 and Grp78. The primers were: for ATF6, ATF6-F, 5'-TCCGTGACTAAACCTGTCCT-3'; ATF6-R, 5'-AC-CATCTGATGTGTCCTG-3'; for Grp78, Grp78-F, 5'-TGGATGCACCATGAAGCTC-3'; Grp78-R, 5'-ACTC-GAGCTACAACTCATCTTT-3'; for caspase-12, caspase-12-F, 5'-GTCATTCTGTGTGTGTATTAATTGC-3'; caspase-12-R, 5'-CCTATAATATCATACATCTTGCTC-3'. For EDEM, EDEM-F, 5'-TACATGGCTCACGCCTTCCC-3'; EDEM-R, 5'-GAGGCTTCCCAGGACCCTT-3'. GADPH was used as an internal control for an equal amount of RNA used. GADPH primers were: Sense, 5'-ATCACTGCCAC-CCAGAAGAC-3'; Anti-sense, 5'-ATGAGGTCCACCAC-CCTGTT-3'. To quantify the mRNA levels, we prepared single-strand cDNA. We checked the RT-PCR products on 1.5% agarose gel and verified that each product had a single band. We carried out an identical reaction without reverse transcriptase in order to verify the absence of genomic DNA. The relative ratio was calculated for each sample in quadruplicate.

Luciferase reporter gene assay

For promoter reporter constructs, DNA fragments of respective genes were isolated by genomic PCR and subsequently subcloned into pGL3-basic vector (Promega, Madison, WI). The primers for XBP1 and Grp78 were: for XBP1, 5'-TTCCACGCGTCGACAGAAGCAGAAC-TTTAG-3'; 5'-TTCCAGATCTCCTGAGGTAATTCTC-TGTTAG-3'; for Grp78, 5'-TTCCACGCGTCGACAGAA-GCAGAACTTTAG-3'; 5'-TTCCAGATCTCGAGATAG-ACAGCTGCTGAACCA-3'; For transfection, $1X10^4$ HeLa and Huh-7 cells were seeded into 96-well tissue culture plates and transfected with Lipofectamine™ 2000 according to the instructions of the manufacturer; seeding was performed in triplicate. For each well, we added 60 ng of reporter construct or pGL3 basic empty vectors together with 3 ng of *Renilla* luciferase control vector pRL-CMV (Progema, USA). The total amount of transfected DNA in each well was kept constant by the addition of the empty vector pCMV plasmid. To correct for variation in transfection efficiency, reporter firefly luciferase activity was normalized to *Renilla* luciferase activity, which was measured using the DLR (Dual-Luciferase Reporter Assay System) kit (Promega, USA) according to the instructions of the manufacturer. Relative activity was defined as the ratio of firefly luciferase activity to *Renilla* luciferase activity.

Chromatin immunoprecipitation (ChIP) assays

ChIP assays with HeLa cells expressing HCV NS4B and tunimycin (Tu)-treated (Sigma, USA) were carried out as previously described (Weinmann *et al*., 2001; Wells and Farnham, 2002). In brief, cells were crosslinked with 1% formaldehyde for 10 min at room temperature. Formaldehyde was inactivated by the addition of 125 mM Glycine; chromatin extracts containing DNA fragments were immunoprecipitated using XBP1 polyclonal antibody (Santa Cruz Biotechnology, USA). For all ChIP experiments, quantitative PCR analyses were performed in real-time using Rotor-Gene 3000 and SYBR Green Master Mix. The XBP1 and Grp78 primers were noted in the Luciferase reporter gene assay section. Relative occupancy values were calculated by determining the apparent immunoprepitation efficiency (ratios of the amount of immunoprecipitated DNA over that of the input sample); these values were normalized to the level observed at a control, which was defined as 1.0.

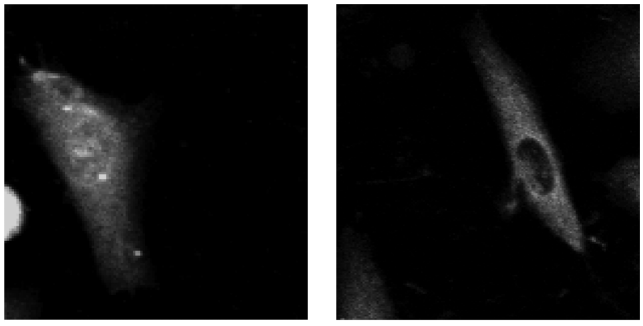
Results

Characterization of NS4B

As a first step in the characterization of NS4B, its cellular localization was determined with a recombinant NS4B with EGFP fused to the C-terminal. Both the original EGFP plasmid and the NS4B-EGFP construct were transfected into the HeLa cell line. When EGFP was expressed alone, it was distributed throughout the entire cell, in both the cytoplasm and the nucleus. However, when fused to NS4B, the pattern of EGFP localization changed. The NS4B-EGFP fusion protein was localized in the cytoplasm (Fig. 1). NS4B Western blot analysis showed that HCV NS4B was expressed in Huh-7 cells and in the stable HeLa cell line (Fig. 2). A HeLa cell line stably expressing HCV NS4B had, therefore, been established.

Detection of XBP1

One role of IRE-1 in the UPR is to convert unspliced



(A) (B)

Fig. 1. NS4B exhibits different kinds of patterns when expressed in cells. HeLa and Huh-7 cells were transfected with pEGFP (A) or pEGFP-NS4B (B). It is shown that NS4B localizes to the cytoplasm.

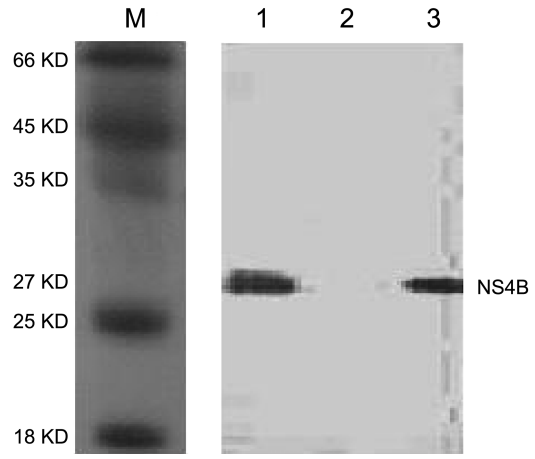


Fig. 2. Expression of NS4B protein was examined by Western blot analysis. Cell lysates from cells transfected by pcDNA3.1(-) NS4B (lanes 1 and 3: HeLa cells and Huh-7 cells, respectively) and HeLa cells transfected by pcDNA3.1(-) (lane 2). It is shown that NS4B can be expressed in HeLa cells and Huh-7 cells.

XBP1 mRNA to a spliced form which is capable of encoding an active XBP1 product. Therefore, to evaluate IRE-1-mediated XBP1 activation, the splicing of XBP1 mRNA was examined by RT-PCR analysis. The total RT-PCR product matched a 3' common region of unspliced and spliced XBP1 transcripts (Fig. 3A). RT-PCR using primers encompassing the spliced region could specifically detect unspliced (442 bp; referred to as u) and spliced (416 bp; referred to as s) transcripts. In HeLa cells containing pcDNA3.1 (–), only XBP1-u was detected, whereas in HeLa cells stably expressing HCV NS4B protein, unspliced and spliced XBP1 could be detected, similar to that of the marked contrast, Tu- and DTT-treated HeLa cells. These findings demonstrated that IRE-1 was activated in response to the cells treated by NS4B, DTT, and Tu (Fig. 3B). We then used Western blot analysis to analyze the expression of XBP1 in the HeLa cell lines. Our results showed that XBP1-s could be expressed in HeLa cells expressing HCV NS4B protein and treated

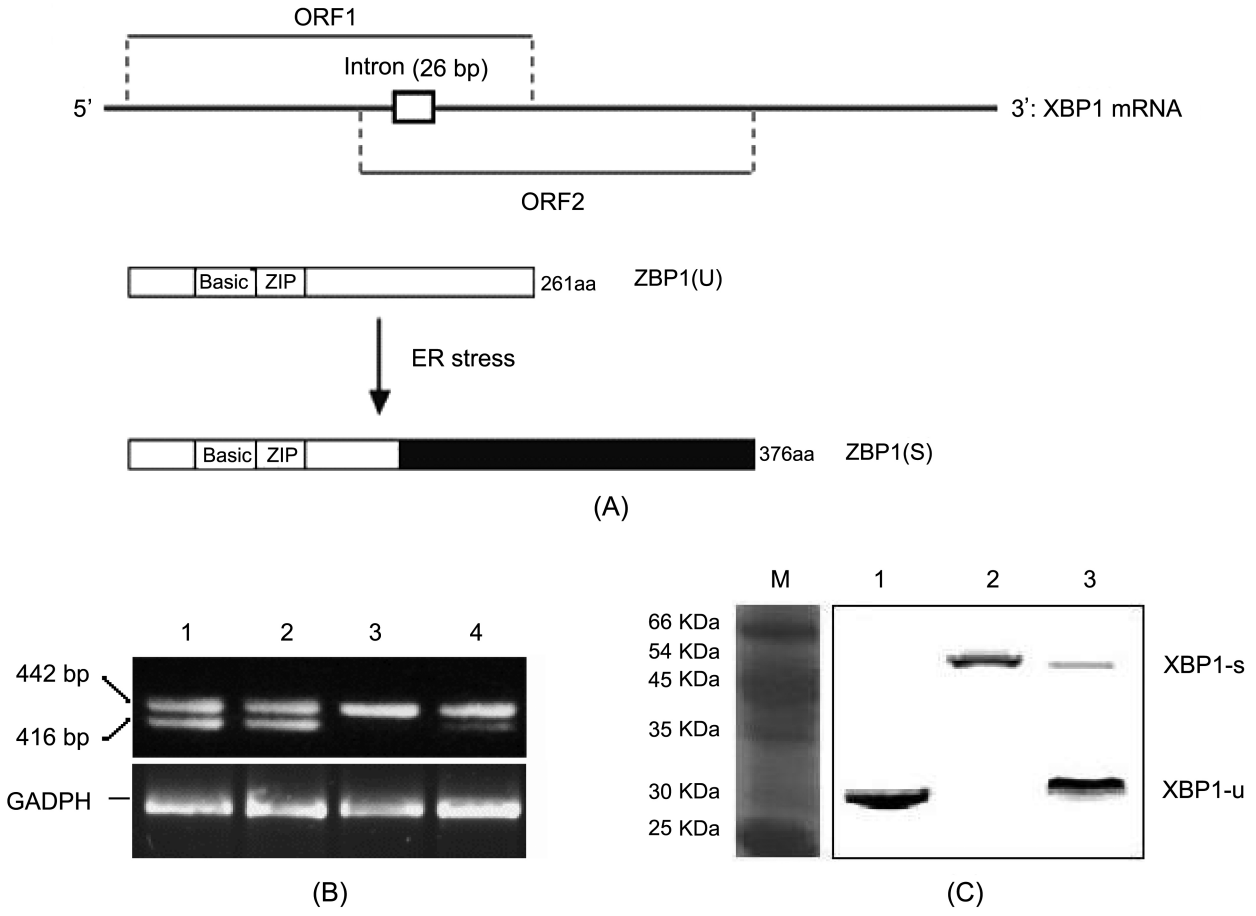


Fig. 3. (A) Schematic illustration of the unspliced and spliced forms of human XBP1 mRNA and protein. The protein products from the two open reading frames and the 26-bp intron from XBP1 mRNA are shown. (B) RT-PCR products from unspliced and spliced XBP1 mRNA are shown. Lane 1, treated with Tu (HeLa cells were treated with 2 μg/ml Tu for 8 hrs); Lane 2, treated with DTT; Lane 3, transfected by pcDNA 3.1(-); Lane 4, transfected by pcDNA3.1(-) NS4B. (C) XBP-1u and XBP1-s proteins in lysates of transfected HeLa cells were analyzed by Western blot using anti-XBP1 antibody. Lane 1, HeLa cells transfected by pcDNA3.1(-); Lane 2, HeLa cells transfected by pcDNA3.1(-) and treated with Tu (HeLa cells were treated with 2 μg/ml Tu); Lane 3, HeLa cells transfected by pcDNA3.1 (-) NS4B. HeLa cells of lane 1 and 3 were treated with 10 μM MG-132 (a proteasome inhibitor) for 6 hrs.

with Tu, but only XBP1-u was detected in the cells transfected by pcDNA3.1 (–) (Fig. 3C). These results indicated that NS4B activated the IRE-1 pathway which leads to the splicing of Xbp-1 RNA.

Detection of the mRNA levels of ATF6, Grp78, caspase-12, and EDEM

The transcripts of ATF6 and Grp78 in HeLa cells stably expressing HCV NS4B were increased at least 1.4-fold higher than those of HeLa cells transfected by pcDNA3.1 (–); this was also verified in Huh-7 cells transfected by pcDNA3.1 (-) NS4B for 36 h (Fig. 4). The transcripts of caspase-12 did not change significantly in HeLa or Huh-7 cells compared with the controls. Since the spliced form of Xbp-1 encodes a transcription factor, we asked whether XBP-1 target genes were transcriptionally activated in cells containing NS4B. Unlike transcription of GRP78, which can be activated by either ATF6 or XBP-1, transcription of EDEM is entirely dependent on XBP-1. Thus, the level of EDEM mRNA in cells with NS4B was measured using real-time RT-PCR. In contrast to treatment with tunicamycin, which resulted in a 2.3-fold increase in the level of EDEM mRNA by 6 h post-treatment, no significant increase in the level of EDEM mRNA was detected in either HeLa cells stably expressing NS4B or Huh-7 cells post-transfected for 36 h. Thus, the transcriptional activity of XBP-1, as indicated by the level of EDEM mRNA, is inhibited in cells with NS4B.

Reporter assay of XBP1 and Grp78 promoter

In order to analyze the cis-acting elements responsible for the constitutive activation of Grp78 and XBP1 in HeLa and Huh-7 cell lines, promoter constructs fused to the luciferase reporter gene were transiently transfected in the HeLa and Huh-7 cell lines. As shown in Fig. 5, the Grp78 and XBP1 promoters showed high activity in the HeLa and Huh-7 cells treated with Tu, and enhanced the reporter expression by approximately 8- to 10-fold. Luc

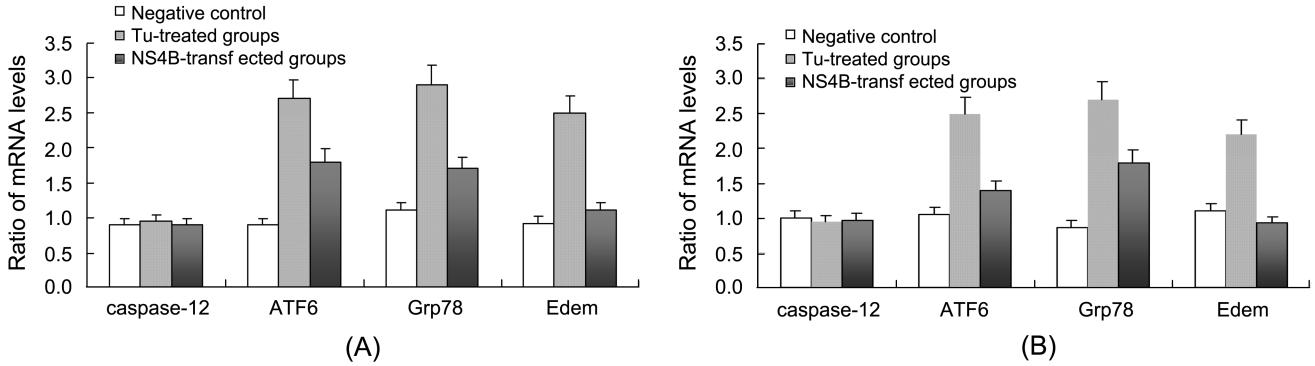


Fig. 4. Effects on mRNA levels of ATF6, Grp78, caspase-12, and EDEM in HeLa and Huh-7 cells transfected by pcDNA3.1 (-) NS4B or pcDNA3.1 (-). The mRNA levels were normalized to that of GADPH, and the ratio of the levels is shown (values include the standard errors of the means). (A) HeLa cells (stable cell lines). (B) Huh-7 cells.

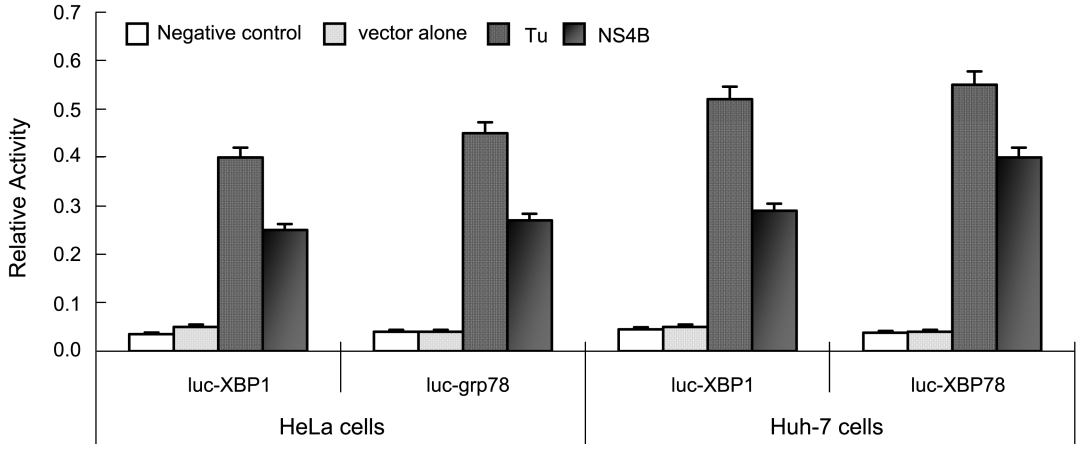


Fig. 5. Luciferase activity detection of luc-XBP1 and luc-Grp78. HeLa and Huh-7 cells were cotransfected with XBP1 and Grp78 luciferase reporter constructs with either pcDNA3.1 (-) or pcDNA3.1 (-) NS4B. Tu-treated cells were considered to be the positive control and untreated-cells were considered to be the negative control for the assay. After 24 hrs, cells were lysed and luciferase activity was measured as a relative value of measured luminescence from firefly luciferase and *Renilla* luciferase from cotransfected pRL-CMV. Values represents the mean and variation of a typical experiment performed in triplicate. The results showed relatively higher luciferase activity in luc-XBP1 and luc-Grp78 promoter in cells with NS4B expression and treated with Tu.

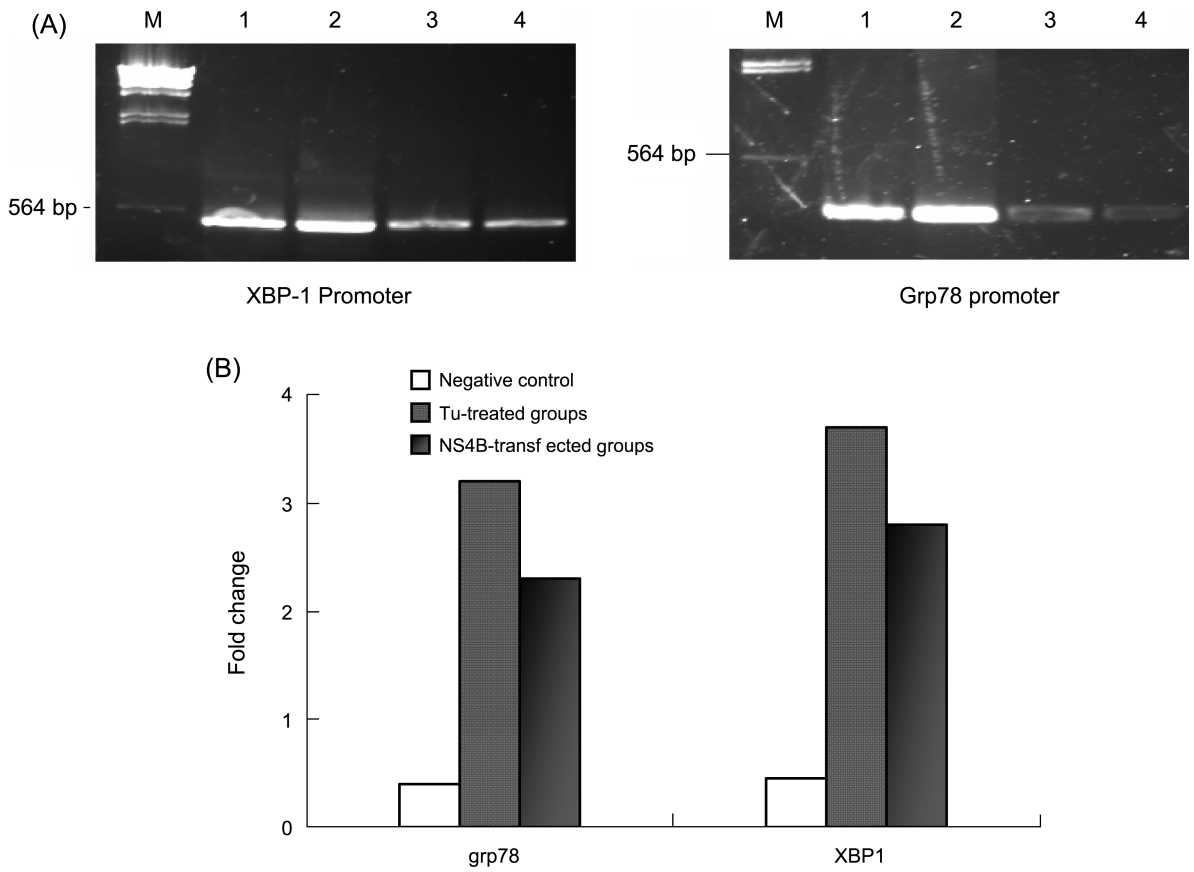


Fig. 6. XBP1 binds directly to XBP1 and Grp78 promoters. (A) The results of PCR in ChIP assay. Lane 1, HeLa cells stably expressing NS4B; lane 2, HeLa cells treated with Tu (2 μg/ml for 8 hrs); lane 3, HeLa cells transfected by pcDNA3.1 (-); lane 4, input sample without XBP1 antibody. (B) Ratios of the amount of immunoprecipitated DNA over that of the input sample, as determined by real-time PCR. HeLa cells stably expressing NS4B and HeLa cells treated with Tu (2 μg/ml for 8 hrs) were harvested. ChIP was performed using XBP1 antibody. Levels of XBP1 binding at XBP1 and Grp78 promoters were determined by ChIP assays.

activity in HeLa and Huh-7 cells expressing HCV NS4B was 5- to 8-fold higher than that of cells without NS4B expression.

ChIP analysis of XBP1 and Grp78 promoter

We then examined whether XBP1 binds directly to the Grp78 promoter. We used XBP1 antibody to immunoprecipitate chromatin from HeLa cells stably expressing HCV NS4B and treated with Tu. The ChIP assays showed that XBP1 could bind the promoter from XBP1, as well as Grp78 in HeLa cells stably expressing NS4B and HeLa cells treated with Tu, but without NS4B expression (Fig. 6). This suggests that XBP1 could bind the promoters of Grp78 and XBP1.

Discussion

It was reported in earlier studies that the HCV NS4B protein tends to localize to the ER, with a small fraction possibly localizing to the Golgi complex when expressed in eukaryotic cells (Hugle *et al.*, 2001; Lundin *et al.*, 2003). Using immunofluorescence, our results confirmed these findings in cells expressing NS4B. The transcriptional activation function of XBP-1 is critical to the UPR since it stimulates the expression of proteins involved in ER stress-induced protein degradation, such as EDEM, as well as chaperones controlled by ATF6 (Lee *et al.*, 2003). Our data demonstrated that NS4B is able to modulate Xbp1mRNA splicing to produce XBP1(s), a transcriptional factor, and target its downstream genes, such as Grp78, and itself, but not caspase-12. However, transcriptional activation of the XBP-1 protein target gene, EDEM, was not detected in cells expressing NS4B. This suggested that either the levels of spliced Xbp-1 mRNA in cells expressing NS4B were too low to produce sufficient XBP-1 protein or the transcriptional activity of XBP-1 was inhibited by NS4B. At this point either or both possibilities could contribute to the inhibition of XBP-1 transcriptional activation by NS4B. In cells containing HCV replicons, the transcriptional activity of XBP-1 was inhibited despite the presence of significant levels of spliced XBP-1 (Tardif *et al.*, 2004). The resulting inhibition of the expression of genes such as EDEM may be beneficial to viral infection, since this would inhibit ER stress-induced

protein degradation, which may otherwise hinder the accumulation of necessary viral proteins. Additionally, XBP-1 splicing may be induced by activated-ATF6 in cells with NS4B. The expression of GRP78 was induced by either spliced XBP-1 or ATF6, which up-regulated Grp78, to benefit viral replication by ensuring the proper folding of viral proteins. Thus, HCV NS4B induces the UPR, through the ATF6 or IRE1-XBP1 pathway, and plays some part in regard to the HCV subreplicons that induce the UPR. However, we did not rule out the other individual or combined roles of proteins in the UPR of HCV subreplicon induction, although NS2 and NS5B expression alone did not induce the UPR (data not shown). The cells containing NS4B are believed to trigger chronic ER stress, since the transcripts of caspase-12 did not increase in cells expressing NS4B. Viruses such as HCV and HBV, which use the ER as an integral part of their replication strategy, must contend with the ER stress response and downstream consequences of ER stress signaling. Noncytopathic viruses, like HCV, which induce ER stress signaling at sublethal levels, are likely to cause alterations in cell physiology which may eventually lead to cellular transformation (Maio *et al*., 2003). It was reported that $IRE1^{-/-}$ and $XBP1^{-/-}$ mice show an embryonic lethal phenotype, and that XBP1 is essential for both the development of hepatocytes and the terminal differentiation of B cells to plasma cells (Reimold *et al*., 2000; 2001). Additionally, the endoplasmic reticulum stress pathway mediated by ATF6 and by the IRE1-XBP1 system seems essential for the transformation-associated expression of the Grp78 gene in HCCs (Shuda *et al*., 2003). It was reported that NS4B protein suppresses translation *in vivo* (Kato *et al*., 2002). It remains unknown whether NS4B has an effect on the PERK pathway.

NS4B reportedly transforms NIH3T3 cells in cooperation with the H-*ras* oncogene (Park *et al*., 2000). It is intriguing to speculate that ATF6 and XBP1 induction may be essential for hepatocyte growth under HCV replicon-induced ER stress. ATF6 and XBP1 induction also maintains cell survival under UPR conditions; these intracellular events suggest a potential function of NS4B in inducing chronic liver disease, including its HCV infection-associated progression to hepatocellular carcinoma. Further study is needed to clarify this issue.

Acknowledgements

We thank Professor Guanxiang Luo (Kentucky University, USA) for HCV-NS4B monoclonal antibody and Fanrong Kong for evaluating the manuscript.

References

Bartenschlager, R. and V. Lohmann, 2000. Replication of hepatitis C virus. *J. Gen. Virol.* 81, 1631-1648.

Egger, D., B. Wolk, R. Gosert, L. Bianchi, H. E. Blum, D. Moradpour, and K. Bienz. 2002. Expression of hepatitis C virus proteins induces distinct membrane alterations including a candidate viral replication complex. *J. Virol.* 76, 5974-5984.

Elazar, M., P. Liu, C.M. Rice, and J.M. Glenn. 2004. An N-terminal amphipathic helix in hepatitis C virus (HCV) NS4B mediates membrane association, correct localization of replication complex proteins, and HCV RNA replication. *J Virol.* 78, 11393-11400.

Farci, P., A. Shimoda, A. Coiana, G.G. Diaz, Peddis, J.C. Melpolder, A. Strazzera, D.Y. Chien, S.J. Munoz, A. Balestrieri, R.H. Purcell, and H.J. Alter. 2000. The outcome of acute hepatitis C predicted by the evolution of the viral quasispecies. *Science* 288, 339-342.

Florese, R.H., M. Nagano-Fujii, Y. Iwanaga, R. Hidajat, and H. Hotta. 2002. Inhibition of protein synthesis by the nonstructural proteins NS4A and NS4B of hepatitis C virus. *Virus Res.* 90, 119-131.

Gosert, R., D. Egger, V. Lohmann, R. Bartenschlager, H. E. Blum, K. Bienz, and D. Moradpour. 2003. Identification of the hepatitis C virus RNA replication complex in huh-7 cells harboring subgenomic replicons. *J. Virol.* 77, 5487-5492.

Hugle, T., F. Fehrmann, E. Bieck, M. Kohara, H.G. Krausslich, C.M. Rice, H.E. Blum, and D. Moradpour. 2001. The hepatitis C virus nonstructural protein 4B is an integral endoplasmic reticulum membrane protein. *Virology* 284, 70-81.

Kato, J., N. Kato, H. Yoshida, S.K. Ono-Nita, Y. Shiratori, and M. Omata. 2002. Hepatitis C virus NS4A and NS4B proteins suppress translation *in vivo*. *J. Med. Virol.* 66, 187-199.

Lee, A.S., N.N. Iwakoshi, and L.H. Glimcher. 2003. XBP1 regulates a subset of endoplasmic reticulum-resident chaperone genes in the unfolded protein response. *Mol. Cell. Biol.* 23, 7448-7459.

Lundin, M., M. Monne, A. Widell, G. Von Heijne, and M.A. Persson. 2003. Topology of the membrane-associated hepatitis C virus protein NS4B. *J. Virol.* 77, 5428–5438.

Maio M., S. Coral, E. Fratta, M. Altomonte, and L. Sigalotti. 2003. Epigenetic targets for immune intervention in human malignancies. *Oncogene* 22, 6484-6488.

Park, J.S., J.M. Yang, and M.K. Min. 2000. Hepatitis C virus nonstructural protein NS4B transforms NIH3T3 cells in cooperation with the Ha-ras oncogene. *Biochem. Biophys. Res. Commun.* 267, 581-587.

Piccininni, S., A. Varaklioti, M. Nardelli, B. Dave, K.D. Raney, and J.E. McCarthy. 2002. Modulation of the hepatitis C virus RNA-dependent RNA polymerase activity by the non-structural (NS) 3 helicase and the NS4B membrane protein. *J. Biol. Chem.* 277, 45670-45679.

Reimold, A.M., A. Etkin, I. Clauss, A. Perkins, D.S. Frand, J. Zhang, H.F. Horton, A. Scott, S.H. Orkin, and M.C. Byrme. 2000. An essential role in liver development for transcription factor XBP-1. *Genes Dev.* 14, 152-157.

Reimold, A.M., N.N. Iwakoshi, J. Manis, P. Vallabhajosyula, E. Szomolanyi-Tsuda, E.M. Gravallese, D. Friend, M.J. Grusby, F. Alt, and L.H. Glimcher. 2001. Plasma cell differentiation requires the transcription factor XBP-1. *Nature* 412, 300-307.

Rosenberg, S., 2001. Recent advances in the molecular biology of hepatitis C virus. *J. Mol. Biol.* 313, 451-464.

Rutkowski, D.T. and R.J. Kaufma. 2004. A trip to the ER: coping with stress. *Trends Cell Biol.* 14(1), 20-28.

Shuda, M., N. Kondoh, N. Imazeki, K. Tanaka, T. Okada, K. Mori, A. Hada, M. Arai, T. Wakatsuki, O. Matsubara, N. Yamamoto, and M. Yamamoto. 2003. Activation of the ATF6, XBP1 and grp78 genes in human hepatocellular carcinoma: a possible involvement of the ER stress pathway in hepatocarcinogenesis *J. Hepatol.* 38(5), 605-614.

Tardif, K.D., K. Mori, and A. Siddiqui. 2002. Hepatitis C virus subgenomic replicons induce endoplasmic reticulum stress activating an intracellular signaling pathway. *J. Virol.* 76, 7453-7459.

Tardif, K.D., K. Mori, R. Kaufman, and A. Siddiqui. 2004. Hepatitis C virus suppresses the IRE1-XBP1 pathway of the unfolded protein response. *J. Biol. Chem.* 279, 17158-17164.

Tong, W.Y., M. Nagano-Fujii, R. Hidajat, L. Deng, Y. Takigawa, and H. Hotta. 2002. Physical interaction between hepatitis C virus NS4B protein and CREB-RP/ATF6 beta. *Biochem, Biophys. Res. Commun.* 299, 366-372.

Weinmann, A.S., M.B. Stephanie, T. Zhang, M.Q. Zhang, and P.J. Farnham. 2001. Use of chromatin immunoprecipitation to clone novel E2F target promoters. *Mol. Cell. Biol.* 21, 6820-6832.

Wells, J. and P.J. Farnham. 2002. Characterizing transcription factor binding sites using formaldehyde crosslinking and immunoprecipitation. *Methods.* 26, 48-56.