Hepatitis C virus NS4B induces unfolded protein response and endoplasmic reticulum overload response-dependent NF-κB activation

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Hepatitis C virus nonstructural protein 4B (NS4B) is an endoplasmic reticulum (ER) membrane associated protein and a potent causative factor of ER stress. Here we reported that unfolded protein response (UPR) can be activated by HCV NS4B through inducing both XBP1 mRNA splicing and ATF6 cleavage in human hepatic cells. Flow cytometric analysis revealed that HCV NS4B stimulates the production of reactive oxygen species (ROS) by perturbing intracellular Ca2+ homeostasis. Luciferase assay showed that HCV NS4B also activates the multifunctional transcription factor, NF-κB, in a dose-dependent manner through Ca2+ signaling and ROS. Further immunoblot analysis showed that HCV NS4B promotes NF-κB translocation into the nucleus via protein-tyrosine kinase (PTK) mediated phosphorylation and subsequent degradation of IκBα. These studies provide an important insight into the implication of NS4B in HCV life cycle and HCV-associated liver disease by affecting host intracellular signal transduction pathways.

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Introduction

Hepatitis C virus (HCV), a member of the Flaviridae family, is a major cause of chronic hepatitis, which can lead to cirrhosis and finally hepatocellular carcinoma (Choo et al., 1989; Farci et al., 2000). Its single-stranded RNA genome is a 9.6 kb positive sense molecule, which carries a large open reading frame (ORF) surrounded by a 5′ and 3′ non-coding region (NCR). The single long ORF encodes a polyprotein precursor of ~3010 amino acids, which can be cleaved to produce the mature structural (core, E1 and E2) and nonstructural (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins (Grakoui et al., 1993). HCV NS4B is a hydrophobic protein with an apparent molecular weight of 27 kDa (Hugle et al., 2001). Structural studies showed that NS4B is an integral membrane protein harboring at least four predicted transmembrane domains and can reorganize intracellular membranes into intense foci that favor viral replication (Lundin et al., 2003; Egger et al., 2002). HCV NS4B has also been demonstrated to influence intracellular signaling pathways to regulate gene expression (Park et al., 2000; Zheng et al., 2005). Park et al. (2000) reported that NS4B can transform NIH3T3 cells by inducing Ras-mediated pathway. We have reported that HCV NS4B activates unfolded protein response (UPR) by triggering XBP1 mRNA splicing in nonhepatic HeLa stable transfectants (Zheng et al., 2005).

UPR is one adaptive strategy employed by cells to alleviate the ER stress caused by various conditions such as the presence of unfolded protein, protein accumulation, and depletion of cholesterol (Pahl, 1999a). Under stress conditions, UPR can be evoked by activating the transcription factor 6 (ATF6), IRE1-XBP1 (X-box binding protein) and PERL-like ER kinase (PERK) pathways, which lead to the following events: (1) transcription of ER chaperon proteins and folding enzymes, (2) ER-related protein degradation, (3) translational attenuation (Harding et al., 1999; Shen et al., 2002; Lee et al., 2003). In response to ER stress, cells can also activate another related signal transduction pathway, ER overload response (EOR) (Pahl, 1999a). EOR can be easily elicited by overexpression or accumulation of ER membrane localized proteins. Ca2+ is released from ER lumen and taken up by mitochondria, which triggers local oxidative stress and production of radical oxygen species (ROS). As a result of changes in
intracellular Ca\(^{2+}\) levels and subsequent production of ROS, nuclear factor-kB (NF-κB) is activated (Meyer et al., 1992; Pahl and Baeuerle, 1995; Gong et al., 2001).

Nuclear factor-kB (NF-κB) is a sequence specific transcription factor that regulates the expression of many cellular and viral genes and plays important roles in immune responses, cell growth and survival (Karin and Lin, 2002; Santoro et al., 2003). In mammals, NF-kB family contains five members: NF-kB1 (p105 and p50), NF-kB2 (p100 and p52), RelA (p65), RelB, and c-Rel (Ghosh et al., 1998). Among these members, heterodimer p50/p65 is the most abundant form and commonly referred as NF-κB. In resting cells, NF-κB is sequestered in the cytoplasm by binding to inhibitory proteins of the IκB family, including IκBα, IκBβ, IκBε (Karin and Ben-Neriah, 2000). After exposure to a variety of pathological stimuli such as bacterial and viral infections, ultraviolet and inflammatory cytokines, IκBα is phosphorylated by IκB kinases (IKKα and IKKβ) and followed by degradation by 26S proteasome. The released active form of NF-κB then translocates into the nucleus and initiates transcription of genes participating in regulation of the host immune and inflammatory response such as cyclooxygenase 2 (COX2), nitric oxide synthase (iNOS) and pro-inflammatory cytokines (Karin and Ben-Neriah, 2000; Pahl, 1999b). An alternative mechanism for NF-κB activation involves phosphorylation of IκBα at Tyr23, Tyr305, and PEST (Pro-Glu-Ser-Thr) sequences under oxidative stress. Tyrosine-phosphorylated IκBα can be degraded and results in the dissociation of NF-κB from its inhibitory complex (Schoonbroodt et al., 2000; Livolsi et al., 2001; Warsi et al., 2003).

HCV replication has been shown to cause ER stress and its gene products such as Core, E2, and NS5A, have also been demonstrated to induce UPR or EOR (Benali-Furet et al., 2005; Chan and Egan, 2005; Gong et al., 2001; Tardif et al., 2002; Sir et al., 2008). In this paper, we reported that another HCV gene product, NS4B, can activate both UPR and EOR pathways in response to ER stress. By transiently or stably expressing NS4B in different human hepatic cell lines, we observed the truncated active form of ATF6 (p50ATF6) as well as previously reported spliced XBP1 mRNA. We further demonstrated that NS4B elicits the EOR pathway by altering the intracellular Ca\(^{2+}\) homeostasis and stimulating the production of radical oxygen species (ROS), which leads to activation of NF-κB. Finally, we showed that NS4B activates NF-κB via protein-tyrosine kinase (PTK)-mediated tyrosine phosphorylation and subsequent degradation of IκBα. These altered intracellular events by HCV NS4B may ultimately address its role in HCV life cycle and liver pathogenesis associated with HCV infection.

Results

HCV NS4B induces both XBP1 mRNA splicing and ATF6 cleavage in human hepatic cells

Under ER stress conditions, XBP1 mRNA can be spliced by endonuclease IRE1 \(α\) (27). Our previous results indicated that NS4B activates XBP1 mRNA splicing in HeLa stable transfectants of nonhepatic origin (Zheng et al., 2005). Since HCV has a narrow host organ range and its replication was primarily detected in human liver cells stably transfected with NS4B-expressing empty vector (Fig. 1A, compare lanes 1 and 7 with lanes 3 and 9). As a positive control, the same XBP1(s) fragment was detected in cells transiently transfected with tunicamycin, a compound causes ER stress by inhibiting protein N-glycosylation (Kaufman, 1999) (Fig. 1A, lanes 5, 6, 11 and 12). Further immunoblot analysis using specific antibody against XBP1 demonstrated that the spliced XBP1 protein (XBP1(S)) was only present in NS4B-expressing cells (Fig. 1B, upper panel, lanes 1, 2, 7 and 8) and tunicamycin treated cells (Fig. 1B, upper panel, lanes 7, 8, 12, and 17), suggesting that spliced XBP1 mRNA is translated into XBP1 protein in cells expressing NS4B. Intriguingly, we detected a higher ratio of spliced XBP1 to unspliced XBP1 in hepatic cell lines than nonhepatic HeLa cells especially at the protein level (compare Fig. 3C in Zheng et al., 2005 with Fig. 1B), suggesting that HCV NS4B exerts pronounced effects in hepatic originated cells.

We then investigated whether NS4B could activate ATF6 proteolysis pathway, another branch pathway of UPR. ATF6 is constitutively expressed as a 90 kDa proteins (p90ATF6), which is proteolytically cleaved into a 50 kDa active transcription factor (p50ATF6) upon ER stress (Shen et al., 2002; Yoshida et al., 2001a, 2001b). Immunoblot

![Fig. 1. HCV NS4B induces both XBP1 mRNA splicing and ATF6 cleavage. A. RT-PCR analysis of XBP1 spliced and unspliced mRNA. Total RNA was extracted from NS4B-expressing, empty vector transfected and tunicamycin treated cells and subjected to RT-PCR using XBP1-specific primers that generate the 416-bp spliced XBP1 mRNA designated XBP1(s) and 442-bp unspliced XBP1 mRNA designated XBP1(u) (upper panel), GAPDH was amplified as an internal loading control (lower panel). The PCR products were subjected to 2% agarose gel electrophoresis. The bands corresponding to XBP1(u), XBP1(s), and GAPDH are indicated by arrowheads. B. Immunoblot analysis of XBP1 expression and ATF6 cleavage using antibodies against XBP1 (upper panel) and ATF6 (middle panel). The actin protein band in the same immunoblot was used as an internal loading control (lower panel). Samples analyzed in (A) and (B) are: lanes 1 and 7, Hep3B and Huh7 cells stably transfected with pcDNA3.1 (−) (−); lanes 2 and 8, Hep3B and Huh7 cells transiently transfected with pcDNA3.1 (−) NS4B; lanes 3 and 9, Hep3B and Huh7 cells stably transfected with pcDNA3.1 (−); lanes 4 and 10, Hep3B and Huh7 cells transiently transfected with pcDNA3.1 (−) NS4B; lanes 5 and 11, Hep3B and Huh7 cells stably transfected with pcDNA3.1 (−) and treated with tunicamycin (2.5 μg/ml) for 6 h; lanes 6 and 12, Hep3B and Huh7 cells transiently transfected with pcDNA3.1 (−) and treated with tunicamycin (2.5 μg/ml) for 6 h.}
analysis using antibody against the first 192 residues of ATF6 revealed the presence of the truncated form p50ATF6 in the Hep3B and Huh7 cells stably or transiently transfected with NS4B expression plasmid, but not in empty vector transfected cells (Fig. 1B, middle panel, compare lanes 1, 2, 7 and 8 with lanes 3, 4, 9 and 10), indicating that ATF6 is processed into its mature form in response to ER stress caused by transient or stable expression of NS4B. The same experiment was carried out using Hep3B cells stably expressing NS2 or NS5B. However, neither of them induced the production of p50ATF6 (data not shown), which confirmed that activated ATF6 cleavage is specifically induced by NS4B.

**HCV NS4B induces Ca2+ signaling and ROS production**

In addition to UPR, ER stress has also been reported to trigger ER overload response (EOR) pathway (Pahl, 1999a). To examine the function of NS4B in EOR pathway, we measured the level of reactive oxygen species (ROS) by flow cytometric analysis (Gong et al., 2001). Transfected cells were stained with dihydroethidium (DHE), which can be oxidized to ethidium by superoxide radicals and intercalate with DNA in the nucleus, where it emits fluorescence at 605 nm. As described in Fig. 2A, more ROS production was detected in cells transiently transfected by pcDNA3.1(−) NS4B than cells transfected by pcDNA3.1(−). What is more, ROS production is stimulated by NS4B in a dose-dependent manner as increased ROS level was observed in the presence of increasing amount of pcDNA3.1(−) NS4B. In the Hep3B and Huh7 cells stably expressing NS4B, there is a significant increase (2.3-fold and 2.8-fold respectively) in the level of ROS (Fig. 2B), which suggests that NS4B is involved in ROS production. To evaluate the requirement of intracellular calcium signaling in NS4B-stimulated ROS production, similar experiment was performed in the presence of Ca2+ chelator, BAPTA-AM and TMB-8 (Pahl and Baeuerle, 1996). As expected, NS4B-induced ROS production was remarkably reduced in chelators treated Hep3B and Huh7 stable transfectants (Fig. 2B), demonstrating that NS4B is capable of stimulating ROS production through Ca2+ signaling in human hepatic cells.

**HCV NS4B induces NF-κB activation**

Since EOR pathway has been shown to activate NF-κB pathway (Pahl and Baeuerle, 1995), we hypothesized that NS4B protein could activate the NF-κB pathway in human hepatic cells. To test this hypothesis, luciferase assay was performed using NF-κB-inducible reporter plasmid, pNF-κB-Luc, a luciferase expression vector under the transcriptional
control of 5 repeats of NF-kB cis-enhancer element (Yoshida et al., 2001b). As described in Fig. 3A, luciferase activity was significantly increased by transfecting cells with increasing amount of NS4B expression plasmid, pcDNA3.1 (−) NS4B. NS4B stably expressing cells were also analyzed by luciferase assay. As described in transient transfectants, luciferase activity was increased dramatically (4.2-fold for Hep3B cells and 6.1-fold for Huh7 cells) in NS4B stably expressing cells, comparable with the cells treated with thapsigargin, which has been shown to activate NF-kB (Pahl, 1999b) (Fig. 3B). These results suggest a direct involvement of NS4B in NF-kB activation in human hepatic cells.

HCV NS4B-induced NF-kB activation depends on both Ca2+ signaling and ROS production

In order to investigate whether NS4B-induced NF-kB activation was dependent on Ca2+ signaling, Hep3B and Huh7 cells stably expressing NS4B were treated with Ca2+ chelators, BAPTA-AM for 2 h or TMB-8 for 4 h. Luciferase assay showed that NS-kB regulated expression was significantly reduced after exposure to Ca2+ chelators (Fig. 4). To investigate whether ROS accumulation is required for NS4B-induced NF-kB activation, antioxidant reagents, N-acetyl L-cysteine (NAC) and pyrrolidine dithiocarbamate (PDTC), were employed in this study. As shown in Fig. 4, NS4B-induced NF-kB activation was completely abolished by treating cells with NAC or PDTC. Thus, NS4B activates NF-kB by disturbing Ca2+ homeostasis and subsequent accumulation of ROS.

To further explore the molecular mechanism of NS4B-induced NF-kB activation, we examined the total quantity of NF-kB in transfected cells by immunoblot with antibodies specific for its subunits, p50 and p65. Interestingly, neither p50 nor p65 was affected by constitutive expression of NS4B in Hep3B and Huh7 cells, arguing that NS4B has little or no effect on the expression and stability of NF-kB (Fig. 5A).

Previous studies have shown that activated NF-kB heterodimer (p50/p65) dissociates from the inhibitory complex and translocates into the nucleus where it activates transcription of various target genes (Karin Ben-Neriah, 2000). To investigate the localization of NF-kB, cytoplasmic and nuclear fractions were prepared and analyzed by immunoblot using antibody recognizing IκBα (Fig. 5B). Under oxidative stress, NF-kB can be activated by phosphorylation of IκBα at Tyr42, Tyr305 and PEST (Pro-Glu-Ser-Thr) sequences (Schoonbroodt et al., 2000; Livolsi et al., 2001; Waris et al., 2003). The phosphorylation status of IκBα was analyzed by immunoblot using antibody recognizing IκBα phosphorylated at Tyr-42 (phospho-IκBα (Tyr-42)). We detected the presence of tyrosine-phosphorylated IκBα in NS4B-expressing cells (Fig. 6B, compare lanes 4 and 9 with lanes 1 and 6), and nuclear fractions were prepared and analyzed by immunoblot using antibodies specific for p50 and p65. As controls, nucleus-specific antibody against YY1 and cytoplasmic-specific antibody against GAPDH were used to preclude cross contamination. Our results showed that in Hep3B and Huh7 cells stably expressing NS4B, a large portion of p50 and p65 was detected in the nucleus and a small portion in the cytoplasm (Fig. 5B). In empty vector transfected cells, almost all the p50 and p65 proteins appeared in the cytoplasm. These results document that NS4B activates NF-kB by facilitating p50/p65 heterodimer movement into the nucleus. Similar results were also observed in Hep3B, Huh7 and Hep2G cells transiently expressing NS4B (data not shown).

HCV NS4B induces PTK-mediated tyrosine phosphorylation and subsequent degradation of IκBα

Under oxidative stress, NF-kB can be activated by phosphorylation of IκBα at Tyr42, Tyr305 and PEST (Pro-Glu-Ser-Thr) sequences (Schoonbroodt et al., 2000; Livolsi et al., 2001; Waris et al., 2003). The phosphorylation status of IκBα was analyzed by immunoblot using antibody recognizing IκBα phosphorylated at Tyr-42 (phospho-IκBα (Tyr-42)). We detected the presence of tyrosine-phosphorylated IκBα in Hep3B and Huh7 cells either stably or transiently transfected with NS4B expression plasmid (Fig. 5A, lanes 2, 5 and 6). However, we failed to detect tyrosine-phosphorylated IκBα in Hep3B and Huh7 cells transfected with empty vector (Fig. 5A, lanes 3, 4, 7 and 8). To evaluate whether phosphorylation of IκBα was carried out by protein-tyrosine kinase (PTK), two known PTK-specific inhibitors, piceatannol and herbimycin, were used in the same analysis (34). These two reagents substantially reduced the level of tyrosine-phosphorylated IκBα in NS4B-expressing cells (Fig. 6A, compare lanes 3, 7 and 8 with lanes 1 and 6). In contrast, IKK inhibitor (BAY11-7085) had no effect on the total level of tyrosine-phosphorylated IκBα (Fig. 6B, compare lanes 4 and 9 with lanes 1 and 6).
immunoblot using antibody against IκBα. A. Immunoblot analysis of IκBα in NS4B-expressing and empty vector transfected cells using anti-phospho-IκBα (Tyr-42) antibody (upper panel) and anti-IκBα antibody (middle panel). Actin protein bands act as internal loading controls (lower panel). Lanes 1 and 5, Hep3B and Huh7 stably transfected with pcDNA3.1 (−) NS4B; lanes 2 and 6, Hep3B and Huh7 cells transiently transfected with pcDNA3.1 (−) NS4B; lanes 3 and 7, Hep3B and Huh7 stably transfected with pcDNA3.1 (−). B. Immunoblot analysis of tyrosine-phosphorylated IκBα in Hep3B and Huh7 stable transfectants in the presence of PTKs inhibitors. Stably transfected Hep3B and Huh7 cells were treated with piceatannol (150 μM) for 0.5 h, herbimycin A (2 μM) for 12 h, or BAY 11–7085 (10 μM) for 1 h before harvesting the cells for immunoblot analysis with anti-phospho-IκBα (Tyr-42) antibody (upper panel) and anti-actin antibody (lower panel). Results are representative of three independent experiments.

suggesting that PTKs, but not IKKs, are responsible for NS4B-induced phosphorylation of IκBα.

We next examined whether IκBα was degraded after being phosphorylated by PTKs. The total amount of IκBα was analyzed by immunoblot using antibody against IκBα. As shown in Fig. 6A, lower level of IκBα was present in NS4B-expressing cells compared with empty vector transfected cells, suggesting that NS4B induces degradation of IκBα via its tyrosine phosphorylation. Taken these observations together, it is reasonable to conclude that NS4B promotes the degradation by PTK-mediated tyrosine phosphorylation of IκBα.

**HCV NS4B induces NF-κB activation through PTK-mediated tyrosine phosphorylation of IκBα**

To determine whether PTK-mediated phosphorylation of IκBα is required for NS4B-induced NF-κB activation, the above two PTK-specific inhibitors were employed in luciferase assay using NF-κB-inducible reporter plasmid. As shown in Fig. 7, PTK inhibitors (piceatannol and herbimycin) effectively reduced the NS4B-induced NF-κB activity. However, little effect was observed by treating transfected cells IKK inhibitor (BAY11–7085) (Figs. 7A and B). Collectively, these results suggest that HCV NS4B activates NF-κB by PTK-mediated tyrosine phosphorylation of IκBα followed by degradation of IκBα, while serine IκBα kinases (IKK) are not required for the whole process.

**Discussion**

HCV replicates from a ribonucleoprotein (RNP) complex that is associated with the ER membrane (Hijikata et al., 1993; Moradpour et al., 1998). Its replication activities cause ER stress and induce UPR by activating three different sensors: PERK, ATF6 and IRE1 (Tardif et al., 2002; Sir et al., 2008). Two of HCV structural proteins, Core and E2, have also been shown to induce UPR (Benali-Furet et al., 2005; Chan and Egan, 2005). HCV NS4B, an ER-membrane associated protein, can cause the morphological changes of the ER membrane (Lundin et al., 2003; Egger et al., 2002; Grétton et al., 2005). HCV NS4B has also been reported to interact with transcription factor ATF6α (Tong et al., 2002). It is tempting to speculate that its expression or its association with ER membrane could cause ER stress and elicit relevant signaling transduction pathways. Here, we showed that HCV NS4B through inducing XBP1 mRNA splicing and ATF6 cleavage (Fig. 1). Furthermore, this activation effect was not limited to certain cell types as similar results were observed in three different hepatic cell lines and one nonhepatic cell lines (Fig. 1; Fig. S2; Zheng et al., 2005). Nevertheless, these results argue that hepatic cells are more authentic than nonhepatic cells to study the functions of HCV NS4B as strong XBP1 mRNA splicing was observed in hepatic cells, which is consistent with limited host organs for HCV replication. ATF6 undergoes cleavage to form active transcription factors to initiate the expression of chaperone proteins that restore the folding of proteins in the ER lumen (Shen et al., 2002). Among these chaperone proteins, glucose-regulated protein-78 (GRP78) has been found to be activated by NS4B expression (data not shown), which might help fold NS4B protein to alleviate the damage to the ER membrane. In addition, UPR induced by HCV has been reported to play a positive role in HCV...
RNA replication in a cell culture system supporting efficient HCV propagation as abrogation one of three UPR pathways can suppress HCV replication significantly (Sir et al., 2008). Hence, it is reasonable to propose that the UPR induced by HCV NS4B alone or in cooperation with other HCV proteins contributes to HCV infection by relieving cells from stress conditions and facilitating replication.

Interestingly, HCV NS4B also activates another ER stress response, EOR, which has been reported to be activated either by HCV subgenomic replicon or NS5A alone. Expression of NS5A perturbs the intracellular calcium signaling pathway and elevates the ROS in mitochondria, which ultimately lead to activation of transcription factors such as NF-κB and STAT (Gong et al., 2001). Similar mechanism for activation of NF-κB via calcium signaling and ROS accumulation has also been reported in HCV-expressing cells (Waris et al., 2003; Waris and Siddiqui, 2005). By employing similar technique, our data clearly indicate that expression of NS4B leads to a remarkable increase in the level of reactive oxygen species (ROS) (Fig. 2). This stimulation of ROS generation was also shown to be dependent on the Ca2+ signaling pathway as NS4B-stimulated ROS production was dramatically reduced by treating with Ca2+ chelators (Fig. 2B). As a result of EOR activation, NF-κB was activated in Hep3B and Huh7 cells expressing NS4B (Fig. 4), which is consistent with the results conducted in HeLa cells expressing NS4B (Kato et al., 2000). Waris et al. (2003) reported that HCV NS5A-induced NF-κB activation involves PTK-phosphorylation of IκB at Tyr42 and IκBα and subsequent degradation. Our results indicated that HCV NS4B induces PTK-mediated tyrosine phosphorylation of IκBα, IκBα degradation, NF-κB translocation and ultimate NF-κB activation (Figs. 5, 6 and 7). It remains to be determined whether the phosphorylation of Tyr42 and Tyr305 of IκBα is required for the HCV NS4B-induced NF-κB activation.

ROS is a potent cause of cancer as it can lead to oxidative damage of DNA, protein and lipids, facilitating DNA mutagenesis and tumor growth (Tardif et al., 2002). Accumulation of ROS in cells causes oxidative stress, which plays an important role in malignant diseases and chronic inflammation, and increased level of ROS has been reported in the livers of patients chronically infected with hepatitis C virus (Kaufman, 1999; Siddiqui, 2005). Elevated COX2 is implicated in inflammatory response, such as cyclooxygenase 2 (COX2), TNFα and IL-1β, IL-6, IL-8, IL-12 (Pahl, 1999b). Hepatitis C virus has been reported to stimulate the expression of cyclooxygenase 2 (COX2) via ROS production and subsequent activation of NF-κB (Waris and Siddiqui, 2005). Elevated COX2 is implicated in inflammation, and tumorogenesis including hepatocellular carcinoma. Whether NS4B-induced NF-κB regulates the expression of genes involved in the inflammatory response and tumorigenesis is under further investigation.

Although the activity of NS4B in ER membrane has been reported to provide sites for HCV replication, here we provided compelling evidence that the expression or association of NS4B in ER causes ER stress followed by activating both the UPR and EOR pathways, as have been documented for a number of viral proteins associated with ER membrane (Meyer et al., 1992; Pahl and Baeuerle, 1995). We also provided a plausible mechanism for NF-κB activation by HCV NS4B. This cascade of altered intracellular events by expression of HCV NS4B implicates its potential role in HCV infection and the associated liver disease. Meanwhile, blockade of these two pathways may provide important clues to the development of drugs to prevent HCV infection and treat HCV-associated liver disease.

Materials and methods

Plasmids and antibodies

NF-κB-inducible reporter plasmid (pNF-κB-Luc) was purchased from Stratagene. Empty vector pcDNA3.1 (−) and HCV NS4B expression plasmid (pcDNA3.1 (−) NS4B), were obtained as described previously (Zheng et al., 2005). The polyclonal antibody against ATF6 was prepared according to standard protocol with little modifications (Timani et al., 2004). Human ATF6 cDNA (GenBank accession no. AB015856) corresponding to the amino acids 1–192 was amplified from RNA isolated from HeLa cells by RT-PCR and cloned into prokaryotic expression vector pET-His (Invitrogen). The forward and reverse primers were used: 5′-GGT ACC GAA TTC AGG ATC CCT AGT GTG GAA CTT-3′; 5′-GGT ACC AAG CCT CCC TCC TGC GGA TGG C-3′. The recombinant plasmid was confirmed by DNA sequencing. Recombinant ATF6 (1–192) protein was expressed in BL21 (DE3) and purified by Ni-NTA affinity column (Stratagen). The purity of protein was examined by electrophoresis on 15% SDS-PAGE. The purified ATF6 (1–192) protein was then immunized rabbit to generate anti-ATF6 polyclonal serum. The reactivity and specificity of antibody were examined by immunoblot analysis. All other antibodies and reagents used are described in the Supplemental data.

Cell lines and transfection

Human hepatic cell lines Hep3B, HepG2, and Huh7 were obtained from China Centre for Type Culture Collection (Wuhan, China). Cells were maintained in Dulbecco’s modified Eagle’s medium (Gibco-BRL, USA), supplemented with 10% heat-inactivated fetal bovine serum (Hyclone), 100 units/ml penicillin, and 100 μg/ml streptomycin. After incubating for 12 h, the cells were transfected with 0.5 μg pcDNA3.1 (−) NS4B or pcDNA3.1 (−) using Lipofectamine 2000 reagent kit (Invitrogen) according to the manufacturer’s instructions. To construct Hep3B, HepG2 and Huh7 cell lines stably expressing NS4B, the medium was replaced with DMEM containing 300 μg/ml of G418 at 24 h post-transfection. About 3 weeks later, individual G418-resistant colonies were isolated, expanded and confirmed by genomic PCR using NS4B specific primers and immunoblot analysis using anti-NS4B monoclonal antibody as described in the Supplemental data. For reagent treatment experiments, transfected cells were treated tunicamycin (2.5 μg/ml) for 4 h, thapsigargin (0.4 μM) for 4 h, NAC (30 mM) for 8 h, PDTC (100 μM) for 8 h, TMB-8 (100 μM) for 4 h, BAPTA-AM (50 μM) for 2 h, piceatannol (150 μM) for 0.5 h, herbimycin A (2 μM) for 12 h, BAY 11-7085 (10 μM) for 1 h before harvesting the cells.

Reverse transcription PCR

Total RNA was extracted using Trizol reagent (Invitrogen). The cDNA strand was synthesized using AMV-reverse transcriptase kit (Takara). To detect the spiked and unspliced XBP1 mRNA, XBP1-specific primers were used as described previously (Yoshida et al., 2001a, 2001b). PCR products were electrophoresed on 2% agarose gel. GAPDH was used as an internal control as described previously (Zheng et al., 2005).

Protein extraction and western blot analysis

Total cell extracts were prepared by incubating cells in lysis buffer (0.3% NP40, 1 mM EDTA, 50 mM Tris–Cl, pH 7.9, 2 mM EGTA, 1% Triton X-100, 150 mM NaCl, 25 mM NaF, 1 mM Na3VO4, 10 μg/ml PMSF) for 30 min on ice and subsequent centrifugation at 12,000 rpm for 15 min. Protein samples were separated by SDS-PAGE and transferred to nitrocellulose membranes. The blots were probed with the primary antibodies against HCV NS4B, ATF6, XBP1, p65, p50, phospho-IκBα (Tyr-42), IκBα, YY1, GAPDH and actin proteins. Next, horseradish peroxidase-labeled goat anti-rabbit IgG as secondary antibodies were applied onto the blots. The specific proteins were visualized by using the ECL Chemiluminescence Detection Kit (Amersham Biosciences).
Preparation of cytoplasmic and nuclear fractions

Cytoplasmic and nuclear fractions were prepared as described with little modifications (Rosner et al., 2007). Briefly, stably transfected cells were incubated in hypotonic buffer (10 mM HEPES, pH 7.9, 5 mM KCl, 1.5 mM MgCl₂, 1 mM NaF, and 1 mM Na₂VO₃, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 2 mg/ml aprotinin, 2 mg/ml leupeptin, and 375 mg/ml soybean trypsin inhibitor) for 15 min on ice, the cytoplasmic fraction was prepared by centrifugation at 3000 rpm for 5 min and then cleared by centrifugation at 13,000 rpm for 15 min. The cell pellet was washed with the hypotonic buffer three times and suspended in high-salt buffer (hypotonic buffer supplemented with 420 mM NaCl and 25% glycerol (vol/vol)). The suspended cell pellet was incubated for 30 min on ice with occasional vortexing, and the nuclear fraction was collected after centrifugation at 13,000 rpm for 10 min. Protein samples were resolved on SDS-PAGE and analyzed by immunoblot.

Luciferase assay

Approximately 3 × 10⁵ cells were plated on a 24-well tissue culture plates 24 h before transfection. To examine the effect of stable expression of NS4B on NF-κB, stably transfected cells were co-transfected with pNF-KB-Luc (0.1 μg/well) and pRL-CMV (0.001 μg/well) (used for normalization). The transfecants were then treated with indicated reagents or untreated before harvesting at 48 h post-transfection. Luciferase activity was measured by the dual-luciferase assay (Promega) according to the protocol recommended by the manufacturer. The firefly luciferase activity was normalized to the Renilla luciferase activity and expressed as relative light units. To examine the dose-dependent effect of NS4B on the NF-κB activation, Hep3B and HuH7 cells were co-transfected with 0–0.4 μg of pcDNA3.1 (−) NS4B, 0.1 μg of pNF-KB-Luc, 0.001 μg of pRL-CMV and 0–0.4 μg pcDNA3.1 (−) adjusted to a total of 0.4 μg.

Flow cytometric analysis of intracellular ROS levels

Stably or 48 h post-transiently transfected cells were incubated with 4 μM dihydroethidium (DHE) for 45 min at 37 °C. Cells were then harvested and washed two times with PBS buffer. The ROS levels were analyzed using an XL00W42322 flow cytometer with an excitation emission at 605 nm. Each experiment was performed independently for three times and data were represented as the mean ± S.D.

Statistical analysis

The method of relative quantification was used for luciferase assay and flow cytometric analysis of cellular ROS levels. Statistical analysis was performed using the Statistical Package Social Sciences (SPSS) program version 11.5 by one-way analysis of variance (ANOVA) and significant differences among groups were determined by Least Significant Difference (LSD). The accepted level of statistical significance was P<0.05.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2009.06.039.

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