Hepatitis C virus (HCV) is the main cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (HCC). HCV is a positive-sense ssRNA virus in the family Flaviviridae, genus Hepacivirus (Ray et al., 2013). Persistent HCV infection causes not only intrahepatic diseases but also extrahepatic manifestations, such as type 2 diabetes. Clinical and experimental studies suggest that HCV is an additional risk factor for the development of diabetes (Mason and experimental studies suggest that HCV is an additional risk factor for the development of diabetes (Mason et al., 1999; Mehta et al., 2003; Negro, 2011; Negro & Alaei, 2012). Moreover, type 2 diabetes increases the risk for the development of HCC in HCV-infected patients (Arase et al., 2009; Shoji et al., 2011). HCV NS5A protein interacts with the transcription factor HNF-1α (Fig. 1a). Human hepatoma cell line Huh-7.5 cells were expressed with both NS5A-Myc-His6 and FLAG-HNF-1α (Bliget et al., 2003), and coimmunoprecipitation analyses were performed. When HNF-1α was deleted from the C terminus (Fig. 1a, rows a–g), NS5A-Myc-His6 was coimmunoprecipitated with most of the C-terminal deletion mutants of HNF-1α (Fig. 1b, right upper panel, lanes 3–8), except FLAG-HNF-1α (aa 1–90) (Fig. 1b, right upper panel, lane 2).

We have sought to identify novel mechanisms of HCV-induced diabetes (Deng et al., 2011; Kasai et al., 2009; Matsui et al., 2012; Shoji et al., 2011). Cellular glucose uptake is conducted by facilitative glucose carriers, i.e. glucose transporters (GLUTs). Glucose uptake in normal hepatocyte is conducted by glucose transporter 2 (GLUT2). We previously reported that HCV suppresses the cell surface expression of GLUT2 in human hepatoma cell lines, as well as in liver tissues of HCV-infected patients (Kasai et al., 2009; Shoji et al., 2011). HCV NS5A protein interacts with the transcription factor hepatocyte nuclear factor 1α (HNF-1α) and induces its lysosomal degradation, resulting in suppression of GLUT2 promoter activity (Matsui et al., 2012). However, the underlying molecular mechanism is still largely unknown.

We aimed to identify the determinants necessary for the degradation of HNF-1α induced by NS5A. To determine the NS5A-binding domain on HNF-1α, we constructed several plasmids for FLAG-tagged HNF-1α deletion mutants (Fig. 1a). Human hepatoma cell line Huh-7.5 cells were expressed with both NS5A-Myc-His6 and FLAG-HNF-1α (Bliget et al., 2003), and coimmunoprecipitation analyses were performed. When HNF-1α was deleted from the C terminus (Fig. 1a, rows a–g), NS5A-Myc-His6 was coimmunoprecipitated with most of the C-terminal deletion mutants of HNF-1α (Fig. 1b, right upper panel, lanes 3–8), except FLAG-HNF-1α (aa 1–90) (Fig. 1b, right upper panel, lane 2).

We further examined the interaction using N-terminal deletion mutants of HNF-1α (Fig. 1a, rows h–l). FLAG-HNF-1α (aa 91–631) was coimmunoprecipitated with NS5A-Myc-His6 (Fig. 1c, upper panel, lane 7). However, other N-terminal deletion mutants were not coimmunoprecipitated with NS5A-Myc-His6 (Fig. 1c, upper panel, lanes 8–10), suggesting that the region from aa 91 to 181 of HNF-1α is important for the interaction with NS5A. Here, FLAG-HNF-1α (aa 91–181) was indeed coimmunoprecipitated with NS5A-Myc-His6 (Fig. 1d, right upper panel, lane 3), indicating that the HNF-1α POU specific (POU S) domain is important for its interaction with NS5A (Fig. 1a).

We previously reported that the region from aa 1 to 126 of NS5A is important for its interaction with HNF-1α (Matsui et al., 2012). FLAG-HNF-1α (91–181) was still largely unknown. We aimed to identify the determinants necessary for the degradation of HNF-1α induced by NS5A. To determine the NS5A-binding domain on HNF-1α, we constructed several plasmids for FLAG-tagged HNF-1α deletion mutants (Fig. 1a). Human hepatoma cell line Huh-7.5 cells were expressed with both NS5A-Myc-His6 and FLAG-HNF-1α (Bliget et al., 2003), and coimmunoprecipitation analyses were performed. When HNF-1α was deleted from the C terminus (Fig. 1a, rows a–g), NS5A-Myc-His6 was coimmunoprecipitated with most of the C-terminal deletion mutants of HNF-1α (Fig. 1b, right upper panel, lanes 3–8), except FLAG-HNF-1α (aa 1–90) (Fig. 1b, right upper panel, lane 2).

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Fig. 1. Mapping of the NS5A-binding domain on the HNF-1α protein. (a) Schematic representation of the HNF-1α protein. HNF-1α consists of three domains: the dimerization domain (Dim; aa 1–32), the POU domain (aa 91–276) and the transactivation domain (aa 281–631). The POU domain functions as a DNA-binding domain, consisting of the POU S domain (aa 91–181) and the POU homeo (POU H) domain (aa 203–280). The HNF-1α deletion mutants (a–l) contain the amino acid residues of HNF-1α as indicated. Each HNF-1α deletion mutant has a FLAG-tag in the N terminus. Lattice region, FLAG-tag sequence; filled boxes, proteins that interacted with NS5A protein; open boxes, proteins that did not. (b–d) Huh-7.5 cells were plated at 1.2 × 10^6 cells dish^-1 (10 cm diameter) and cultured for 12 h. Cells were transfected with the NS5A-Myc-His6 expression plasmid together with each FLAG-HNF-1α mutant plasmid as indicated. (−) indicates vector control. At 48 h post-transfection, cells were harvested and cell lysates were immunoprecipitated (IP) with anti-c-Myc mAb. Input samples and immunoprecipitated samples were analysed by immunoblotting (IB) with anti-FLAG mAb (upper panels) or anti-NS5A mAb (lower panels).
coimmunoprecipitated with NS5A (1–126)-Myc-His<sub>6</sub> (Fig. 1e, upper panel, lane 10), indicating that the region from aa 1 to 126 of NS5A interacts with the HNF-1<sub>α</sub> POU<sub>5</sub> domain.

To determine the minimum HNF-1<sub>α</sub>-binding domain on NS5A, we performed coimmunoprecipitation analyses using several NS5A deletion mutants (Fig. 2a). HA-NS5A (1–83) was not coimmunoprecipitated with FLAG-HNF-1<sub>α</sub> (Fig. 2b, upper panel, lane 7), whereas HA-NS5A (84–447) was coimmunoprecipitated with FLAG-HNF-1<sub>α</sub> (Fig. 2c, upper panel, lane 6). HA-NS5A (1–83), HA-NS5A (1–100) and HA-NS5A (1–120) were not coimmunoprecipitated with FLAG-HNF-1<sub>α</sub> (Fig. 2b, upper panel, lanes 8 and 9). These results suggest that the region from aa 121 to 126 of NS5A is essential for the specific interaction with HNF-1<sub>α</sub> (Fig. 2a).

**Fig. 2.** NS5A consists of three domains (domain I, II, and III) with domains separated by low-complexity sequences (LCS I and II). Amphipathic helix, AH1. The minimum HNF-1<sub>α</sub>-binding domain on the HCV NS5A protein. (a) Schematic representation of the HCV NS5A protein. Each NS5A deletion mutant contains an HA-tag in the N-terminal region. The grey region of each represents the HA-tag sequence. Filled boxes, proteins that interacted with the HNF-1<sub>α</sub> protein; open boxes, the proteins that did not. (b) Huh-7.5 cells were plated at 1.2 × 10<sup>6</sup> cells/dish (10 cm dia.) and cultured for 12 h. Cells were transfected with pCAG-FLAG-HNF-1<sub>α</sub> together with each NS5A mutant plasmid as indicated. (−) indicates vector control. At 48 h post-transfection, cells were harvested and cell lysates were immunoprecipitated (IP) with anti-FLAG beads. Input samples and immunoprecipitated samples were analysed by immunoblotting (IB) with anti-c-Myc mAb (upper panel) or anti-FLAG mAb (lower panel).
To identify the specific amino acid residues of NS5A required for interaction with HNF-1z, we generated substitution mutants of HA-NS5A (1–126) (Fig. 3a). The immunoprecipitation analysis revealed that the mutant NS5A (1–126) V121A failed to interact with FLAG-HNF-1z (Fig. 3b, bottom panel, lane 3). However, other substitution mutants of NS5A maintained the interaction (Fig. 3b, bottom panel, lanes 4–8). Okamoto et al. (2008) reported that the mutant NS5A V121A lost the interaction with FK506-binding protein 8 (FKBP8) and that the NS5A–FKBP8 interaction...
is crucial for HCV replication. They also reported that the mutant NS5A V121I interacted with FKBP8 and supported HCV replication. To determine the role of the V121I mutation on NS5A in the interaction with HNF-1α, we performed an immunoprecipitation analysis. We found that NS5A (1–126) V121I-Myc-His6, was coimmunoprecipitated with FLAG-HNF-1α (Fig. 3c, lower panel, lane 4).

To examine the subcellular colocalization of NS5A and HNF-1α, we performed an immunofluorescence study. HNF-1α was localized primarily in the nucleus, but also to a certain extent in the cytoplasm, as is consistent with the findings of Dong et al. (2015). Our immunofluorescence study revealed that WT NS5A (1–126), but not NS5A V121A (1–126), was colocalized with HNF-1α in the perinuclear region (Fig. 3d). This result is well correlated with the results of the coimmunoprecipitation analysis shown in Fig. 3(b).

We examined whether the V121A mutation disrupts the ability of NS5A to induce the degradation of HNF-1α. Increasing amounts of NS5A (1–213) decreased the levels of endogenous HNF-1α protein (Fig. 3e, first panel). However, the levels of endogenous HNF-1α protein remained unchanged in the cells expressing either HA-NS5A V121A (1–213) or HA-NS5A V121A (214–447) (Fig. 3e, second and fourth panels). These results indicate that NS5A Val121 plays a crucial role in the interaction with HNF-1α, as well as in the degradation of HNF-1α. NS5A Val121 is well conserved among various HCV genotypes, with the exception of the genotype 1a strains, which have Ile in place of Val (Okamoto et al., 2008). Interestingly, increasing amounts of HA-NS5A V121I (1–213) decreased the levels of endogenous HNF-1α protein (Fig. 3e, third panel). This result strongly suggests that the interaction between NS5A and HNF-1α plays a crucial role in the degradation of HNF-1α.

Daclatasvir is a first-in-class HCV NS5A replication complex inhibitor (Gao, 2013; Pawlotsky, 2013). Daclatasvir inhibits HCV replication and reduces NS5A levels. We therefore hypothesized that daclatasvir may prevent the NS5A-induced degradation of HNF-1α. Huh-7.5 cells were infected with HCVcc J6/JFH1 (Lindenbach et al., 2005) with or without 1 μM daclatasvir (Fig. 3e, lanes 4, 6). The level of endogenous HNF-1α was remarkably reduced in HCV J6/JFH1-infected cells (Fig. 3e, middle panel, lanes 2, 4). After the daclatasvir treatment, the HCV RNA level was remarkably reduced in the cells (Fig. 3g), and the level of endogenous HNF-1α was recovered (Fig. 3f, middle panel, lane 6). We previously reported that HNF-1α protein expression level recovered after treatment of the HCV-infected cells with IFN-α (Matsui et al., 2012). Treatment of HCV-infected cells with IFN-α or daclatasvir resulted in the recovery of HNF-1α protein levels. These findings suggest that the recovery of HNF-1α is not due to a direct effect of IFN-α or daclatasvir, but due to inhibition of HCV replication. The present results suggest that daclatasvir inhibits HCV RNA replication and reduces the levels of NS5A, thereby inhibiting the NS5A-induced degradation of HNF-1α.

Our results also indicate that HNF-1α and FKBP8 share overlapping binding motifs on NS5A. The structural analyses of NS5A domain I revealed that Val121 resides in the centre of the β-sheet (Love et al., 2009; Tellinghuisen et al., 2005). These findings suggest that NS5A uses this region in domain I for interactions with the different host factors FKBP8 and HNF-1α, facilitating different functions such as viral replication and viral pathogenesis.

Although the molecular mechanism for NS5A-dependent lysosomal degradation of HNF-1α remains unclear, identification of a single crucial residue on NS5A may contribute to gaining a better understanding of the mechanism. NS5A has an N-terminal amphipathic helix serving as a membrane anchor to ER-derived membrane. On the other hand, FKBP8 is localized predominantly to the outer membrane of mitochondria as well as to ER membrane (Shirane & Nakayama, 2003). It is possible that NS5A serves as a scaffold to bring HNF-1α to FKBP8, or some other host factors involved in lysosomal degradation machinery. Further investigation will be required to elucidate the mechanism of NS5A-dependent lysosomal degradation of HNF-1α.

In conclusion, we obtained evidence suggesting that NS5A Val121 is crucial for viral pathogenesis. Small compounds aimed at NS5A Val121 may lead to the development of new therapeutic agents for HCV.

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