A single-amino-acid mutation in hepatitis C virus NS5A disrupts physical and functional interaction with the transcription factor HNF-1α

Chieko Matsui,¹ Imelda Rosalyn Sianipar,¹,² Nanae Minami,¹ Lin Deng,¹ Hak Hotta¹ and Ikuo Shoji¹

¹Division of Microbiology, Center for Infectious Diseases, Kobe University Graduate School of Medicine, Kobe, Hyogo, Japan
²Department of Physiology, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia

Hepatitis C virus (HCV) infection often causes extrahepatic manifestations, such as type 2 diabetes. We previously reported that HCV infection induces the lysosomal degradation of the transcription factor HNF-1α via an interaction with viral NS5A, thereby suppressing GLUT2 gene expression. However, the molecular mechanism of NS5A-induced degradation of HNF-1α is largely unknown. We aimed to identify the determinants necessary for the degradation of HNF-1α induced by NS5A. Coimmunoprecipitation analysis revealed that the POU specific (POU S) domain spanning from aa 91 to 181 of HNF-1α is responsible for the interaction of NS5A. We also found that the region from aa 121 to 126 of NS5A, which is known as the binding motif of the HCV replication factor FKBP8, is important for the degradation of HNF-1α. A NS5A V121A mutation disrupted the NS5A–HNF-1α interaction as well as the degradation of HNF-1α. Our findings suggest that NS5A Val121 is crucial for viral pathogenesis.
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 POUS domain (aa 91–276) and the transactivation domain (aa 281–631). The POUS domain functions as a DNA-binding domain, consisting of the POUS S domain (aa 91–181) and the POUS homeo (POUS 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 × 10⁴ 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-1α POU<sub>S</sub> domain.

To determine the minimum HNF-1α-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-1α (Fig. 2b, upper panel, lane 7), whereas HA-NS5A (84–447) was coimmunoprecipitated with FLAG-HNF-1α (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-1α (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-1α (Fig. 2a).

![Diagram of NS5A domains and HNF-1α-binding domains](image)

**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-1α-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-1α protein; open boxes, the proteins that did not. (b) Huh-7.5 cells were plated at 1.2 x 10<sup>6</sup> cells/dish (10 cm dia.) and cultured for 12 h. Cells were transfected with pCAG-FLAG-HNF-1α 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-HA rabbit polyclonal antibody (pAb) (upper panel), or anti-HNF-1α goat pAb (lower panel).
To identify the specific amino acid residues of NS5A required for interaction with HNF-1α, 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-1α (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

![Image](https://example.com/image.png)

**Fig. 3.** A single-amino-acid mutation of NS5A domain I is essential for the interaction with HNF-1α. (a) Schematic representation of the HCV NS5A protein. The minimum HNF-1α-binding domain (aa 121–126) is indicated by the open box. Substitution of NS5A (aa 121–126) for Ala is described below. The results of the binding with HNF-1α (Fig. 2b, c) are summarized to the right. (b) Huh-7.5 cells were transfected with pCAG-FLAG-HNF-1α together with the plasmids for NS5A mutants with Ala substituted at the amino acid residues spanning from aa 121 to 126. (--) indicates vector control. At 48 h post-transfection, cells were harvested and cell lysates were immunoprecipitated (IP) with anti-FLAG mAb. Immunoprecipitated samples were subjected to immunoblotting (IB) with anti-HNF-1α pAb (third panel) or anti-c-Myc mAb (fourth panel). The protein expression of NS5A mutants or FLAG-HNF-1α was confirmed using the same cell lysate by immunoblotting with either anti-c-Myc mAb (second panel) or anti-HNF-1α pAb (first panel). (c) The plasmid pCAG-FLAG-HNF-1α was transfected into Huh-7.5 cells together with the substitution mutants of NS5A (aa 1–126) V121A or V121I. (--) indicates vector control. Bound proteins were immunoblotted with either anti-HNF-1α pAb (third panel) or anti-c-Myc mAb (fourth panel). Input samples were immunoblotted with either anti-c-Myc mAb (second panel) or anti-HNF-1α pAb (first panel). (d) Huh-7.5 cells were plated at 1 x 10^5 cells per well (24-well plate) and cultured for 12 h. Cells were transfected with pCAG-FLAG-HNF-1α together with either pEF1A-NS5A (1–126)-Myc-His6 or pEF1A-NS5A V121A (1–126)-Myc-His6. At 48 h post-transfection, cells were stained with anti-c-Myc mAb followed by Alexa Fluor 594-conjugated goat anti-mouse IgG, and with anti-FLAG pAb followed by Alexa Fluor 488-conjugated goat anti-rabbit IgG. The stained cells were examined using a BZ-9000 (Keyence) microscope. (e) Huh-7.5 cells were plated at 2 x 10^5 cells per well (6-well plate) and cultured for 12 h. Cells were transfected with increasing amounts of pCAG-FLAG-HNF-1α and pCAG-NS5A (1–126) for Ala is described below. The results of the binding with HNF-1α (Fig. 2b, c) are summarized to the right. (f) Huh-7.5 cells were plated at 1.0 x 10^5 cells per well (12-well plate) and were infected with HCV J6/JFH1 at an m.o.i. of 2. Cells were cultured and harvested at 6 days, and total RNA was extracted. The NS5A inhibitor DCV or DMSO was administered to the cells for 6 days. The levels of HCV mRNA were determined by quantitative RT-PCR. The amounts of HCV RNA were normalized to GAPDH mRNA expression levels. **P<0.01 vs the control (HCV-infected cells treated with DMSO).
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) V121A was colocalized with 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 corre-
lated 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). How-
ever, the levels of endogenous HNF-1α protein remained
unchanged in the cells expressing either HA-NS5A
V121A (1–213) or HA-NS5A (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 com-
plex 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 recov-
ered (Fig. 3f, middle panel, lane 6). We previously reported
that HNF-1α protein expression level recovered after treat-
ment 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 ana-
lyses 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 func-
tions such as viral replication and viral pathogenesis.

Although the molecular mechanism for NS5A-dependent
lysosomal degradation of HNF-1α remains unclear, identi-
fication 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 mem-
brane anchor to ER-derived membrane. On the other
hand, FKBp8 is localized predominantly to the outer mem-
brane 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.

Acknowledgements
We are grateful to C. M. Rice (Rockefeller University, New York, USA)
for providing Hu7-5 cells and plF-J6/JFH1, and R. Bartenschlager
(University of Heidelberg, Heidelberg, Germany) for providing an
HCV subgenomic RNA replicon (pFKB3/2884Gh). We also thank
K. Hachida for secretarial work. This work was supported by grants-
in-aid for research on hepatitis from the Ministry of Health, Labour
and Welfare, the Ministry of Education, Culture, Sports, Science,
and Technology (MEXT), and Agency for Medical Research and
Development (AMED), Japan. This work was also supported in part
by the Japan Initiative for Global Research Network on Infectious
Diseases program of MEXT, Japan. This study was also carried out as
part of the Global Center of Excellence program of the Kobe
University Graduate School of Medicine and the Science and
Technology Research Partnership for Sustainable Development
program of the Japan Science and Technology Agency and the Japan
International Cooperation Agency. C.M. was supported by a Grant-
Aid for JSPS Fellows (26-1405).

References
Arase, Y., Kobayashi, M., Suzuki, F., Suzuki, Y., Kawamura, Y.,
Akuta, N., Kobayashi, M., Szezaki, H., Saito, S. & other authors
(2013). Effect of type 2 diabetes on risk for malignancies includes
hepatocellular carcinoma in chronic hepatitis C. Hepatology
57, 964–973.


