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Reduction of hepatitis C virus NS5A phosphorylation through its interaction with amphiphysin II ☆

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Abstract

Hepatitis C virus non-structural protein 5A (NS5A) is a pleiotropic protein with key roles in viral RNA replication, modulation of cellular-signaling pathways and interferon (IFN) responses. To search for possible host factors involved in mediating these functions of NS5A, we adopted an affinity purification approach coupled with mass spectrometry to examine protein–protein interactions, and found that human amphiphysin II (also referred to as Bin1) specifically interacts with NS5A in mammalian cells. Pull-down assays showed that the Src homology 3 (SH3) domain of amphiphysin II is required for NS5A interaction and that c-Src also interacts with NS5A in cells. IFN-α treatment reduced the interaction of NS5A with c-Src, but not amphiphysin II, suggesting that the latter is independent of the IFN-signaling pathway. NS5A is a phosphoprotein and its phosphorylation status is considered to have an effect on viral RNA replication. In vitro kinase assays demonstrated that its interaction with amphiphysin II inhibits phosphorylation of NS5A. These results suggest that amphiphysin II participates in the HCV life cycle by modulating the phosphorylation of NS5A.

Keywords: Proteome; NS5A; HCV; Amphiphysin II; Phosphorylation

Chronic infection with hepatitis C virus (HCV) carries with it a substantial risk of severe liver conditions such as chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. The interactions between cellular proteins and HCV gene products may provide clues for novel approaches to interfere with viral propagation and the accompanying pathogenesis. NS5A is a non-structural HCV protein thought to confer resistance to IFN therapy in HCV infection [1,2]. The NS5A protein in certain HCV isolates associates with IFN-induced double-stranded RNA-activated protein kinase (PKR) and inhibits PKR activity [3,4]. In addition, PKR-independent repression of IFN activity by NS5A, such as via the induction of IL-8 transcription, has been reported [5]. Several investigators have examined the role of NS5A in IFN resistance in vivo and in vitro, and have suggested the possibility that the number of mutations in the ISDR of NS5A is related to IFN responsiveness [1–4]. Thus, NS5A could be an important protein for the development of therapies against HCV infection.

The NS5A protein has been reported to interact with Grb2, p53, Cdk1, Pitx1, TRAF-2, karyopherin β3, and the SNARE-like protein, hVAP-33 (the human homologue of the 33-kDa vesicle-associated membrane protein-associated protein) [6–13]. To assess the effect of NS5A on cell function, it is important to identify proteins that interact

☆ *Abbreviations:* NS5A, hepatitis C virus non-structural protein 5A; ISDR, interferon sensitivity determining region; SH3, Src homology region 3; PAGE, polyacrylamide gel electrophoresis; LC–MS/MS, liquid chromatography–mass spectrometry/mass spectrometry.

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with NS5A. In the present study, we investigated NS5A-protein binding by a protein–protein interaction assay using cultured cells. Using LC–MS/MS analysis, we identified amphiphysin II as an NS5A-interacting protein in HeLa cells transfected with NS5A. Amphiphysin II has been reported to interact with c-myc and thereby possess anti-oncogenic function [14]. Zech et al. [15] previously identified amphiphysin II as an NS5A-interacting protein using an in vitro GST pull-down assay and demonstrated that IFN-α treatment abrogated this interaction by reducing NS5A levels in a HuH7 replicon system. However, the effect of this interaction on cellular function was not explored. Here, we determined the consequences of NS5A–amphiphysin II interactions for NS5A phosphorylation in vitro. NS5A is known to be the only phosphoprotein among the HCV non-structural proteins [16,17] and its hyperphosphorylation inhibits HCVRNA replication [18,19]. We demonstrated that its interaction with amphiphysin II reduces NS5A phosphorylation, which may help explain the mechanisms by which host defenses interact with the HCV during its life cycle.

Materials and methods

Cell lines and reagents. HeLa S3, HuH7, and 293T cells were cultured in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Human IFN-α was purchased from Toray Co. Ltd.

Plasmid construction and stable transfectant production. Wild type NS5A cDNA was constructed from NIHJ1 (mutant NS5AcDNA) by modifying the ISDR [20,21]. Wild type and mutant NS5AcDNA were inserted into the *Hin*dIII–*Kpn*I sites of a p3×FLAG-CMV-14 expression vector (Sigma). The wild type NS5A expression vector (designated p5AFLAG) was transfected into HeLa S3 cells using Fugene6 (Roche Biochemicals) and after 2-weeks' culture with G418 (500 μg/ml), drug-resistant HeLa cells were pooled as stably transfected cells. Wild type HA-amphiphysin II cDNA and HA-amphiphysin II Δ10 cDNA (amphiphysin II lacking exon 10) were constructed from the amphiphysin II cDNA and subcloned into the *Bam*HI–*Xba*I sites of the pcDNA3.1(+) vector (Invitrogen) [22]. HA-amphiphysin II ΔSH3 cDNA was subcloned into the *Bam*HI–*Xho*I site of the pcDNA3.1(+) vector. The amphiphysin II mutants amphiphysin II Δ10, amphiphysin II ΔSH3, and amphiphysin II ΔN lacked the amino acids 250–266, 377–451, and 1–47, respectively [22].

Analysis by LC–MS/MS. HeLa cells stably transfected with NS5A-flag expression vector (p5AFLAG) were cultured in a 1 L spinner flask for 5 days. Cell extracts from HeLa cells were incubated with anti-flag M2-agarose and washed, and bound products were then eluted with flag peptide (0.2 mg/ml). The eluted fraction was electrophoresed on SDS–10% PAGE and stained with Silver Quest (Invitrogen). Individual protein bands were excised from the silver-stained gel and destained with a solution of 30 mM potassium ferricyanide/100 mM sodium thiosulfate (1:1) (v/v). Trypsin-digested sample solutions were analyzed by LC–MS/MS.

Preparation of cell extracts, M2-agarose, and HA-agarose pull-down assay, and Western blot analysis. 293T cells were transfected with full length amphiphysin II pcDNA3.1, amphiphysin II Δ10 pcDNA3.1, amphiphysin II ΔSH3 pcDNA3.1 or amphiphysin II ΔN pcDNA3.1 together with p5AFLAG. The transient transfection of 293T cells was carried out with Fugene 6 (Roche Biochemicals). The cells were harvested, washed with PBS, and lysed in lysis buffer M containing 50 mM Tris–HCl (pH 8.0), 100 mM NaCl, 0.1% NP-40, 1 mM EDTA, 1 mM dithiothreitol, and protein inhibitor cocktail (Sigma). Total cell lysates were incubated with M2-agarose (Sigma) or HA-agarose (Sigma) for 2 h on a rotator at 4 °C. After washing the agarose with lysis buffer M three times, the bound proteins were eluted with 0.2 mg/ml flag peptide (Sigma) or 1 mg/ml HA peptide (Sigma), and subjected to electrophoresis on SDS–10% PAGE, and analyzed by Western blotting with anti-flag (Sigma), anti-IRF-3 (Santa Cruz), anti-nucleolin (Santa Cruz), anti-stat1(UBI), anti-amphiphysin II(UBI), anti-c-Src (Santa Cruz), and anti-NS5A (Austral Biologicals) antibodies. The proteins were visualized by Chemiluminescence (Perkin Elmer).

In vitro kinase assays. Lysate from p5AFLAG and HA-amphiphysin II pcDNA3.1 cotransfected cells was incubated with anti-flag M2-agarose, and then the washed precipitate was reacted in kinase buffer (20 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, and 1 mM Na_3VO_4) and 2 μCi, 10 μM [γ-^{32}P]ATP (Amersham) for 30 min at 37 °C. The reaction was terminated by the addition of an SDS sample buffer and boiling for 5 min at 95 °C, followed by SDS–10% PAGE and autoradiography.

Results

NS5A associates with amphiphysin II in HeLa cells

HeLa cells were stably transfected with p5AFLAG encoding NS5A conjugated with flag in the C-terminal domain. Expression of NS5A protein was confirmed by an anti-flag M2-agarose pull-down assay (Fig. 1A). Western blotting using anti-flag and anti-NS5A antibodies confirmed the production of flag-tagged NS5A protein in HeLa cells (Fig. 1A). To isolate proteins that associate with NS5A protein, cell lysate from a large-scale HeLa cell culture was incubated with anti-flag M2-agarose, and then the M2-agarose precipitate was eluted with flag peptide. The eluted fraction was electrophoresed and stained with silver. We observed that three bands a, b, and c increased compared to the level in the control (empty vector transfected) (Fig. 1B, lanes 1 and 2). These bands were cut out of the gel and digested with trypsin, and then the peptides were identified by LC–MS/MS. LC–MS/MS analysis identified band a as NS5A and band b as muscle type amphiphysin II [22]. Band c included β-tubulin, which was later identified as a non-specific protein. We focused on band b as an NS5A interaction protein. The indicated peptide coverage identified by LC–MS/MS for band b was approximately 42% (Fig. 1C). Western blot analysis using the amphiphysin II antibody confirmed that anti-flag M2-agarose precipitated amphiphysin II from HeLa cells transfected with p5AFLAG, but not control cells (Fig. 1D), further suggesting that amphiphysin II was specifically recruited to NS5A. We observed two bands by Western blotting which seemed to be phosphorylated and non-phosphorylated forms of amphiphysin II (Fig. 1D, top panel).

Several investigators have suggested that ISDR mutations in NS5A may be related to IFN responsiveness [1–4]. To examine whether the ISDR is important for interaction with amphiphysin II, an NS5A mutant NIHJ1-flag plasmid (with the ISDR modified as described previously) [20,21] was transfected together with HA-amphiphysin II pcDNA3.1 into 293T cells. Cell lysates were incubated with M2-agarose or HA-agarose, and then resin-bound products were eluted with flag peptide or HA peptide. As shown in Fig. 2, both wild type and mutant NS5A-bound