Host proteins associated with Hepatitis C virus encoded NS4A

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Abstract Virus-associated cancers account for more than 12 % of all the cancers. Hepatitis C virus (HCV) infects nearly 3 % of the population worldwide and has emerged as a major causative agent of liver disease with a big impact on public health. The HCV non-structural protein NS4A is a 54-amino-acid polypeptide that serves as an essential co-factor for the NS3 serine protease. We report here on a proteomic study to identify cellular proteins associated with NS4A. The results of this study show an association of three host cellular proteins with NS4A including two novel NS4A interacting partners. Our data provide evidence for complex involving NS4A with previously unreported cellular proteins including housekeeping protein GAPDH, and PI3P-5 K which is involved in cellular protein trafficking to nucleus. These novel associations add to the diversity of NS4A functions in relation to the virus infection and subsequent pathogenesis.

Keywords HCV · NS4A · Proteomics

Virus-associated cancers account for more than 12 % of all the cancers [3]. Out of these, Hepatitis B Virus (HBV) and Hepatitis C Virus (HCV) account for approximately 5 % of all cancers, and are second only to HPV associated cancers (5.2 %) [3]. Hepatocellular carcinoma is the most common primary malignant tumor of the liver. It is the fifth most common cancer in men and the eighth most common in

women, accounting for 0.52–0.63 million new cases worldwide in 2008 [11]. HCV infects nearly 3 % of the population worldwide and has emerged as a major causative agent of liver disease, resulting in acute and chronic infections that can lead to fibrosis, cirrhosis and hepatocellular carcinoma. With an estimated 130–170 million infected individuals on a worldwide basis, HCV has a major impact on public health.

HCV is a small, enveloped RNA virus belonging to the genus *Hepacivirus* of the family *Flaviviridae*. The HCV genome consists of a single-stranded positive-sense RNA of approximately 9.6 kb which contains an open reading frame (ORF) encoding a polyprotein precursor of approximately 3,000 residues, and is flanked by untranslated regions (UTRs) at both ends. The polyprotein precursor is cleaved into at least 10 different proteins: the structural proteins Core, E1, E2 and p7; and the non-structural proteins NS2, NS3, NS4A, NS4B, NS5A and NS5B. The non-structural protein NS4A is a 54-amino-acid polypeptide that serves as an essential co-factor for the NS3 protease [15]. A trans-membrane helix in the hydrophobic N-terminus anchors the NS3–NS4A complex to intracellular membranes, where proteolytic processing and RNA replication takes place [17]. NS4A also associates with other HCV proteins on the endoplasmic reticulum (ER) derived membranous webs where viral replication complex is assembled [14]. NS4A is localized on the ER as well as on mitochondria. Thus, in addition to its essential role in HCV replication it is also involved in viral pathogenesis by affecting cellular functions. NS4A expression in Huh7 cells has also been reported to lead to altered intracellular distribution of mitochondria and its damage [10]. The role of NS4A is very important in HCV replication. The central region consisting of amino acids 21–34 of NS4A is hydrophobic and required for the activation of NS3 serine protease activity [16]. The acidic

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C-terminus plays an important role in HCV replication by regulating NS5A phosphorylation. Its expression is also needed for NS5A hyperphosphorylation [15].

During processing of the HCV polyprotein, the protease domains of NS2 and NS3 cleave the NS3–NS4A junction, after which NS4A is complexed to the amino-terminal serine protease domain of NS3 [1]. NS4A protein has been shown to inhibit HeLa cells proliferation when expressed by induction under the control of doxycycline [5]. NS4A has additional roles that may be important for the viral life cycle. It has been reported to interact with cellular factors such as Creatine kinase B [4] and elongation factor 1A [8]. NS4A overexpression (even in the absence of NS3 expression) is reported to cause inhibition of cellular translation damage to mitochondria, although it could not be confirmed whether these NS3- independent functions of NS4A are present during *in vivo* HCV infection [10]. The NS3-4A protease has also been found to breakdown host's TLR-mediated innate immune response by preventing activation of IRF-3 and NFκB [9]. In case of TLR-3-dependent cascade, NS3-4A cleaves the Toll-IL-1-receptor domain-containing adapter that induces IFNβ (TRIF) and in-turn IRF-3 [9]. And in TLR-3-independent cascade, NS3-4A cleaves Cardif, which is an adapter that interacts with RIG-1 resulting in IRF-3 and NFκB activation [9]. The NS4A/B precursor inhibits the transport of MHC class I molecules to the cell surface [7]. In addition, the co-variation of sequence of NS4A with multiple viral genes, suggests that it may have an essential role for various processes [2].

Therefore, in order to further elucidate the role of NS4A in HCV pathogenesis, we attempted to identify the cellular interacting partners of NS4A. This will allow further investigations to identify the cellular signaling pathways it may regulate which could be critical for disease pathogenesis. We performed GST pull-down study using recombinant GST-NS4A protein as bait. Cellular protein extract from a hepatic cell line Huh7.5 was used as the pool of prey proteins. Huh7.5 cell line was cultured in DMEM medium supplemented with 10 % fetal bovine serum (FBS), 2 mM L-glutamine and 50 U/ml of penicillin–streptomycin–amphotericin B. The cells were grown at 37 °C in a humidified chamber supplemented with 5 % CO₂. The HCV NS4A gene was amplified by PCR from pJFH1 construct and cloned into pGEX-2TK vector within *Bam*HI and *Eco*RI restriction sites. The clone was confirmed by sequencing.

GST-NS4A was prepared by methods described earlier [6]. In brief, the pGEX-2TK-NS4A construct was transformed in *E. Coli* BL-21 cells. Single colonies were picked and grown overnight in 3 ml of Luria–Bertani (LB) medium. The overnight saturated culture was seeded into 200 ml of LB medium at a dilution of 1:100 and allowed to shake at 37 °C until mid-exponential growth phase. The

Fig. 1 The cellular proteins interacting with HCV-NS4A were resolved on SDS-PAGE after pull-down assay with GST-NS4A. Lane 1 shows the crude protein extract from Huh7.5 cells. Lane 2 is Pre-clear containing proteins that interact with GST beads alone. Lane 3 and Lane 4 are reference protein ladders. Lane 5 shows the control lane loaded with proteins that interact with GST-2TK. Lane 6 shows the proteins that interact with GST-NS4A

cells were then induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 4 h with continuous shaking at 37 °C. Cells were harvested and lysed by sonication. The proteins were solubilized in the presence of protease inhibitors. Solubilized proteins were incubated with Glutathione S-transferase (GST)-Sepharose beads overnight at 4 °C with rotation and then collected by centrifugation and washed three times in NETN buffer with protease inhibitors. GST fusion protein bound to beads was used for binding assays and stored in NETN buffer with protease inhibitors at 4 °C. For preparation of Huh7.5 cell extract, fifty million cells were harvested, washed with cold PBS, resuspended in cell lysis buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 1 % Tergitol NP-40 and protease inhibitors), incubated on ice for 1 h, vortexing at every 15 min. Cellular debris was removed by high-speed centrifugation and supernatant containing cellular proteins was collected and stored at –20 °C. A sample was taken to determine its protein concentration by Bradford assay.

For identification of cellular interacting partners of NS4A, we performed GST-Pull down assay using GST-NS4A as bait. The Huh7.5 cellular extract was first pre-cleared with GST only by incubating with 20 μl beads at 4 °C for 1 h with rotation. The supernatant was collected and split equally into two separate tubes. Half of supernatant was incubated with GST-2TK beads and other half with GST-NS4A beads at 4 °C overnight with rotation. The beads were then collected from both tubes and washed with NETN buffer. The beads were boiled at 95 °C in SDS-PAGE loading buffer and resolved on 12 % SDS-PAGE gel with appropriate controls (Fig. 1).

Table 1 List of the HCV NS4A interacting cellular proteins

S. No.	UniProt entry name	Protein name	GenPept Accession No.	Reported Mol Wt (kDa)
1	KCRB_HUMAN	Creatine kinase B-type	CAA33389	38.5
2	G3P_HUMAN	Glyceraldehyde-3-phosphate dehydrogenase	CAA25833	42
3	FYV1_HUMAN	1-phosphatidylinositol 3-phosphate 5-kinase	Q9Y2I7	227

Three visible protein bands between 23 and 120 kDa size that were unique to the GST-2TK-NS4A pull-down lanes were excised out of the Coomassie-stained gel and in-gel digestion conducted with mass spectrometry grade Trypsin (Promega Corp., Cat # V5280) (Fig. 1). The excised gel pieces were then washed with 100 µl of 25 mM ammonium bicarbonate in 50 % acetonitrile. They were then dehydrated in acetonitrile followed by solvent removal using a vacuum centrifuge. They were rehydrated in 100 µl of digestion buffer containing 25 mM ammonium bicarbonate, 5 mM calcium chloride (50 µl), and 20 ng/ml of trypsin and enzymatic cleavage was allowed to continue overnight at 37 °C. Peptides were extracted into 20 mM ammonium bicarbonate (100 µl) followed by two separate extractions into 100 µl of water/acetonitrile/formic acid (80:20:1, v/v/v). The volume of these extractions was reduced to 20–30 µl by speed drying in vacuum. The digests were analyzed directly by MALDI-TOF/TOF for sequence information. The analysis of mass spectrometry data showed that NS4A protein may interact with 3 host cellular proteins viz. Creatine Kinase B (KCRB), Glyceraldehyde 3-phosphate kinase (G3P or GAPDH) and 1-phosphatidylinositol 3-phosphate 5-kinase (FYV1 or PI3P-5 K) (Table 1).

KCRB has previously been reported to have a role in HCV genome replication through interaction with NS4A. KCRB is a key enzyme of ATP-generation which functions to regulate ATP in subcellular compartments of non-muscle cells and also plays important role in efficient HCV genome replication and infectious virus propagation [4]. KCRB interacts with central (21–39) amino acids of HCV NS4A protein and forms a complex with NS3-4A, which has different catalytic activities [4]. KCRB up regulates both unwinding of DNA and RNA mediated by NS3-4A in vitro, and replicase activity in permeabilized cells replicating HCV [4]. Helicase activity of NS3-4A unwinds RNA–RNA substrates in 3′–5′ direction. The NS3-4A helicase possibly translocates along the nucleic acid substrate by conformational change using ATP during RNA replication. It has been reported that both NS3 and NS3-4A helicase activity unwound dsRNA substrate most efficiently in the presence of KCRB and its substrate [4]. The KCRB-NS4A is a potential target for the development of a

new type of antiviral therapeutic strategy. Our report confirms the interaction of KCRB with NS4A.

We further report the interaction of cellular house-keeping protein GAPDH with NS4A. GAPDH is a key enzyme of glycolysis which interacts with other proteins including actin, tubulin, RNA including viral RNAs, and exhibits RNA helix-destabilizing activity [12]. GAPDH has been reported to interact with the poly U/UC tract in the 3′UTR of the Hepatitis C virus but the functional relevance of the interaction has not been determined yet [12]. Till date there has not been any report of GAPDH interaction with any of the HCV proteins. We here report that GAPDH can interact with NS4A which could have a role in modulation of GAPDH and/or NS4A functions. Our study also identified PI3P-5 K as interacting partner of NS4A. PI3P-5 K is known to regulate both the synthesis and turnover of phosphatidylinositol 3,5-bisphosphate [13]. It catalyzes the phosphorylation of phosphatidylinositol 3-phosphate on the fifth hydroxyl of the myo-inositol ring, to form phosphatidylinositol 3, 5 bisphosphate. It is required for endocytic-vacuolar pathway and nuclear migration and plays a role in the biogenesis of endosome carrier vesicles (ECV)/multivesicular bodies (MVB) transport intermediates from early endosomes. It also mediates protein trafficking to the nucleus and also plays role in signal transduction [13]. Interaction of NS4A with PI3P-5 K could possibly have implications in regulation of protein trafficking to nucleus in HCV infected cells.

The application of MALDI-TOF based peptide analysis to identify NS4A-associated proteins from cellular extract was able to identify three NS4A interacting protein and has expanded the number of proteins which interact with HCV-NS4A. Two novel interacting partners were identified that have potentially important roles in HCV pathogenesis, specifically in nuclear trafficking and routine housekeeping. NS4A therefore forms complexes with proteins belonging to different functional classes. This expanded view of cellular interacting proteins suggests new cellular functional areas that may be involved in HCV biology. Further examination of the identified proteins to NS4A function or HCV pathogenesis, by overexpression or by specific knock down of individual proteins using siRNA, may identify drug targets for HCV-associated diseases.

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