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Subproteomic study of hepatitis C virus replicon reveals Ras-GTPase-activating protein binding protein 1 as potential HCV RC component ☆

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Abstract

Hepatitis C virus (HCV) RNA synthesis takes place on a detergent resistant membrane (DRM) structure. To identify potential cellular proteins related to HCV replication complexes (RC), we purified DRMs from HCV subgenomic replicon cells and its parental Huh7 cells. The proteins of DRM fractions were separated by two-dimensional gel electrophoresis and identified by mass spectrometry. Comparing with parental Huh7 cells, 60 proteins were up-regulated while 14 proteins were down-regulated in HCV replicon cells. Ras-GTPase-activating protein binding protein 1 (G3BP1), one of the elevated proteins, was found to be associated with HCV NS5B and knockdown of G3BP1 by siRNA in HCV replicon cells significantly reduced HCV replication, which may indicate it a potential component of HCV RC. These results suggest that HCV viral gene and proteins may regulate the presence of host cellular proteins in DRM, ensure appropriate concentrations of replication components, and hence control the rates or efficiencies of HCV replication.

Keywords: HCV subgenomic replicon; Detergent resistant membrane; Two-dimensional gel electrophoresis; Mass spectrometry; Replication complex

Hepatitis C virus (HCV) is a member of the family Flaviviridae and possesses a single-stranded, 9.6 kb-positive-sense RNA genome, encoding a single polyprotein which is cleaved co- and post-translationally into at least 10 individual polypeptides with the following gene order: 5′-C-E1-E2-p7-NS2-NS3-NS4A- NS4B-NS5A-NS5B-3′ [1]. Research on HCV replication had been hampered until the development of HCV replicon system. Despite substantial efforts in research, the mechanisms of replication of HCV infection are still not well defined.

Formation of a membrane-associated replication complex composed of viral proteins, replicating RNA, and modified cellular membranes is the general strategy of most of the plus-strand RNA viruses [2–7]. HCV replication is proposed to take similar strategy, residing in endoplasmic reticulum membrane or Golgi apparatus [8,9], inducing a membranous web, where all HCV proteins were found to be associated, and forming a membrane-associated multiprotein complex [10,11]. This kind of membranous web functions as a place for synthesis of the viral RNA and protects viral RNA from accessing to nuclease and protease [12]. Recently, it is reported that HCV replication complex (RC) assembles on detergent resistant membrane (DRM) through interactions between viral nonstructural proteins

☆ *Abbreviations:* HCV, hepatitis C virus; CHCA, α-cyano-4-hydroxycinnamic acid; MALDI-TOF MS, matrix assisted laser desorption/ionization-time of flight mass spectrometry; NS protein, nonstructure protein; 2DE, two-dimensional gel electrophoresis; DRM, detergent resistant membrane.

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and host proteins [13–15]. However, the nature of the hepatitis C virus RNA replication complex associated with DRM has not been well characterized.

In this study, we used a subproteomics approach in combination with the subcellular fractionation and membrane flotation assay to analyze the components of HCV RC associated with DRM from HCV replicon cells. Compared with control liver cells, cellular proteins in DRMs regulated by HCV RC were identified. We identified Ras-GTPase-activating protein binding protein 1 (G3BP1) as one of the proteins enriched in DRMs. Results showed that G3BP1 was associated with HCV NS5B in vivo and knockdown of G3BP1 by siRNA in HCV replicon cells significantly reduced HCV replication, which suggest it a potential component of HCV RC.

Materials and methods

Cell lines and antibodies. The BB7 HCV subgenomic replicon plasmid (genotype 1b) was kindly gifted by Professor Charles Rice (The Rockefeller University, New York). Huh-7 cells were transfected with HCV subgenomic replicon RNA and maintained as described [16]. For DRM depletion, replicon cells were serum starved for 2 h and treated with 10 mM mβCD (Aldrich) for 1 h at 37 °C. Polyclonal antibody against HCV NS3 and NS5B (prepared in our laboratory), monoclonal antibody against HCV NS5A (Virogen), monoclonal antibody against β-actin (Sigma), monoclonal antibody against caveolin-2 (BD Biosciences) and monoclonal antibody against G3BP1 (BD Biosciences).

Membrane flotation assay. Subcellular fractionation was carried out as described [17]. Briefly, cell pellets from approximate 1.5×10^8 cells were re-suspended in 5 ml hypotonic buffer (10 mM Tris–HCl, pH 7.8, 10 mM NaCl) with EDTA-free protease inhibitor cocktail (Roche), allowed to swell 15 min on ice, and disrupted with 50 strokes in Dounce homogenizer. The cell lysate was centrifuged at 900*g* for 5 min at 4 °C. The supernatants were further centrifuged at 15,000*g* for 20 min at 4 °C, resulting in supernatant (S15) and pellet (P15). The P15 was re-suspended with 950 μl hypotonic buffer with EDTA-free protease inhibitor cocktail and treated with 50 μl of 20% NP-40 (final concentration, 1%) on ice for 30 min, then totally 1 ml re-suspended P15 fraction was mixed with 3 ml 72% (w/w) sucrose in low-salt buffer (LSB, 50 mM Tris–HCl [pH 7.5], 25 mM KCl, and 5 mM $MgCl_2$) as described [14] and overlaid with 4 ml of 55% (w/w) sucrose in LSB, followed by 1.5 ml of 10% (w/w) sucrose in LSB. The sucrose gradient was centrifuged at 38,000 rpm in a Beckman SW41 Ti rotor for 16 h at 4 °C. One-milliliter fractions were taken from the top of the gradient. For Western blot analysis, 100 μl samples were taken from each fraction and mixed with 2× SDS loading buffer. The remained gradient was added 4 ml ice-cold methanol and centrifuged at 10,000*g* for 10 min. The pellet was re-suspended with 2DE lysis buffer [8 M urea, 2 M thiourea, 4% (w/v) CHAPS, 65 mM DTT, 0.5 mM PMSF, 0.5% (v/v) IPG buffer, pH 3–10 NL, and 0.25% (w/v) SDS].

2DE, gel staining, and tryptic digestion of 2DE gel spots. Samples (350 μg) were diluted to 340 μl with a rehydration solution containing 8 M urea, 0.5% (w/v) CHAPS, 0.5% (v/v) IPG buffer, pH 3–10 NL, 0.2% (w/v) DTT and loaded on 18 cm IPG strips (Amersham Biosciences) with a pH 3–10 NL. The first dimensional IEF was performed according to the manufacturer's protocol. The second dimension electrophoresis was carried out on 12.5% polyacrylamide gels (20 cm × 20 cm × 1.0 mm) at 40 mA/gel constant. The 2D gels were subjected to Coomassie brilliant blue staining or silver staining. Silver staining of 2D gels was performed as described [18]. Gels were scanned using GS-800 calibrated deensitometer (Bio-Rad) before the analysis by PDQUEST (version 7.0, Bio-Rad). The silver-stained protein spots were digested as previously described [19].

MALDI-TOF MS and database search. Each sample was re-suspended with 0.7 μl matrix solution (CHCA in acetonitrile/water (1:1 v/v) acidified with 0.1% TFA). The mixture was immediately spotted on the MALDI target and allowed to dry and to crystallize. The analyses were performed on a 4700 Proteomics Analyzer (TOF/TOF™) (Applied Biosystems, USA) equipped with a 355 nm Nd:YAG laser. The proteins were identified by peptide mass fingerprinting (PMF) and tandem mass spectra (MS/MS) using the program MASCOT v. 1.9 (Matrix Science, London, United Kingdom) against SWISS-PROT database with GPS explorer software (Applied Biosystems, USA). Only protein identifications with score greater than $p < 0.05$ were considered to be positive.

Confocal microscopic analysis. Cells were fixed with 3.5% paraformaldehyde and permeabilized with 0.2% Triton X-100 in PBS containing 1% fetal calf serum. Samples were then incubated overnight with monoclonal antibody at 4 °C. After being washed thrice with PBS containing 1% fetal calf serum, samples were incubated at 37 °C for 1 h with a FITC-conjugated secondary antibody. Coverslips were finally mounted on glass plates and cells were observed by confocal laser scanning microscope (Leica, TCS-NT, Heidelberg, Germany). Only the merged images were presented.

RT-PCR and quantitative R-PCR. Cells were harvested with Trizol reagent (Invitrogen) according to the manufacturser's protocol. After treated with DNaseI, each RNA sample was reverse-transcribed with random primers (dN_6) by Superscript II (Invitrogen). Quantitative real-time PCR (LightCycler, Roche) was performed using the CYBR I reagents (Roche) according to the manufacturer's protocol. Cellular GAPDH (glyceraldehyde 3-phosphate dehydrogenase) mRNA from same cell lysate was used as an internal control for determining cell number and metabolic status. The primers specific for the HCV 5′NTR were 5′-CCCT GTGAGGAACTA/TCTGTCTTCACGC-3′ (sense) and 5′-TCCAG AGCATCTGGCAC GTA/GGTACTCG-3′ (antisense). Forty cycles of PCR were performed with cycling conditions of 15 s at 95 °C, 20 s at 55 °C, and 25 s at 72 °C. The real-time PCR signals were analyzed using LightCycler 3 software.

Immunoprecipitation. Immunoprecipitation was as previously described [20], briefly, HEK293T cells were transfected with plasmids pCMV-G3BP1 and pCDNA3.0HA-NS5A, NS5B by FuGENE 6 transfection reagent. Cell lysate was immunoprecipitated with antibody against myc and precipitated complexes were analyzed on 8% SDS–PAGE, followed by immunoblotting with antibodies against myc and HA.

RNA interference. Duplex siRNA was obtained from GeneChem (Shanghai, P.R.C). The siRNA sequence targeting human G3BP1 was 5′-CUG CCA CAC CAA GAU UCG CdTdT (sense) and dTdTG ACG GUG UGG UUC UAA GCG-5′ (antisense) as described previously [21]. A nonsensing duplex was used as control. HCV replicon cells were plated on 24-well plate with antibiotic free DMEM overnight and transfected with siRNA by oligofectamine (Invitrogen) according to the manufacturer's protocol. The final concentration of siRNA duplex was 200 nM. Six hours after transfection, the medium was switched to DMEM supplemented with antibiotics.

Results

Purification of detergent resistant membranes from HCV subgenomic replicon cells

To explore potential cellular proteins related to HCV replication complexes (RC), we focused on identification of the components of HCV RC-containing DRMs. We first isolated the P15 fraction which is reported to contain the HCV RC activity in vitro [17,22,23]. Western blot analysis (Fig. 1A) indicated that the HCV nonstructural proteins (NS5A and NS5B) were mainly present in the P15 fractions of HCV subgenomic replicon cells, while no HCV proteins were detected in S15 fraction.

The P15 fractions were then treated with nonionic detergent (1% NP-40) and applied to membrane flotation assay

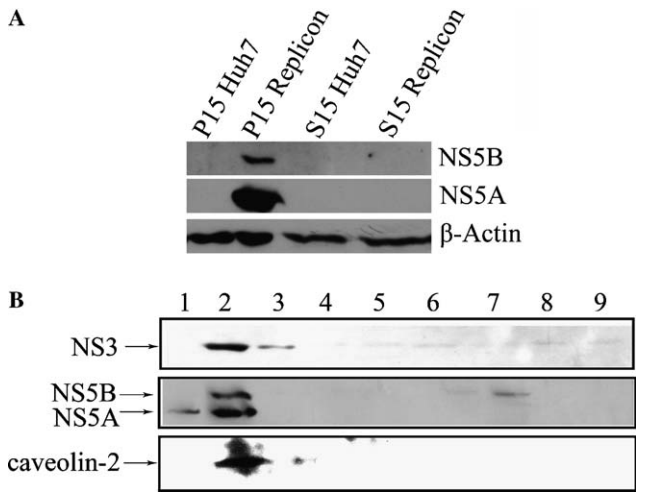

Fig. 1. Purification of detergent resistant membranes from HCV subgenomic replicon cells. (A) Subcellular fractions (P15, S15) were isolated from Huh7 and replicon cells by differential centrifugation. HCV nonstructure proteins were detected by immunoblotting with monoclonal antibody against NS5A and polyclonal antibody against NS5B. (B) P15 fractions were treated with 1% NP-40 and fractionated by discontinuous sucrose gradient centrifugation. Equal volumes of the sucrose fractions were precipitated with methanol and the precipitated proteins were analyzed on a 10% polyacrylamide gel, followed by immunoblotting with monoclonal antibodies against NS5A, monoclonal antibodies against caveolin-2, and polyclonal antibodies against NS5B and NS3. Fractions are numbered from 1 to 9 in order from top to bottom (light to heavy).

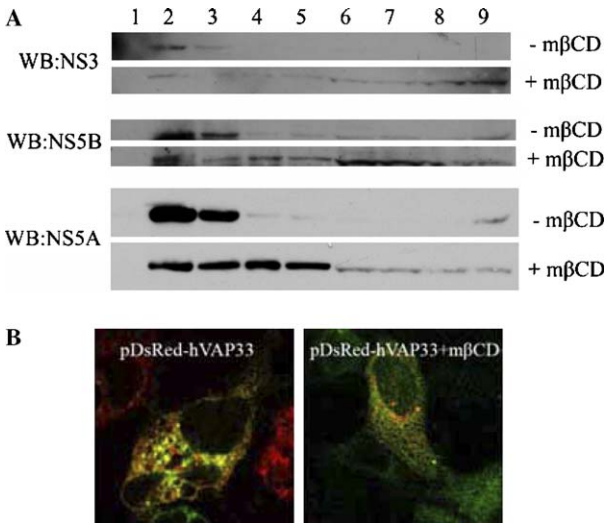

Fig. 2. HCV replication complex was sensitive to cholesterol-disrupting drug. (A) After serum starvation for 2 h, replicon cells were treated or untreated with 10 mM cholesterol-disrupting drug methyl-β-cyclodextrin (mβCD) for 60 min at 37 °C. Detergent resistant membranes were isolated from cells and DRM derived proteins were analyzed on 10% SDS–PAGE, followed by immunoblotting with antibodies against NS3, NS5B, and NS5A (B). Replicon cells were transfected with plasmids pDsRed-hVAP33. At 48 h post-transfection, the cells were treated or untreated with mβCD as above and fixed with 3.5% paraformaldehyde, and then immunostained against NS5A. Coverslips were mounted and observed by confocal laser scanning microscope. Only the merged images were presented.

for DRM isolation. As shown in Fig. 1B, NS3, NS5A, and NS5B were found almost completely in the second fraction (F2). As it was co-fractionated with DRM marker caveolin-2, the second fraction could be considered as DRM fraction.

To further verify the association of HCV RC with DRM, HCV subgenomic replicon cells were treated with cholesterol depletion reagent mβCD [24] to disrupt DRM. After cholesterol depletion, enrichment of HCV NS3, NS5A, and NS5B in the DRM was remarkably reduced (Fig. 2A). Meanwhile, co-localization of HCV NS5A and a known HCV RC component hVAP33 [13] was impaired after mβCD treatment (Fig. 2B). Altogether, these results demonstrated that HCV RC is associated with DRM.

Identification of the components of HCV RC-containing DRMs

It is proposed that during the HCV RC assembling, it includes or excludes some cellular proteins in DRM to control its replication. So, we compared the DRM components from HCV replicon cells and its parental Huh7 cells. The proteins derived from the DRM were separated on pH 3–10 NL IPG strips followed by 12.5% SDS–polyacrylamide gels. The proteins were in-gel digested with trypsin and extracted as peptides and identified by MALDI-TOFMS and database searching. Sixty proteins were up-regulated while 14 proteins were down-regulated in HCV replicon cells (Supplemental table). Identified proteins such as actin, eIF3-P44 have been reported to interact with HCV 5′ IRES by proteomics approach [25]. It is interesting to observe that most of the identified cellular proteins have not been reported to be involved in HCV replication.

G3BP1 was involved in HCV replication

Ras-GTPase-activating protein binding protein G3BP1 is one of the identified cellular proteins which has not been found to be associated with HCV. To investigate the role of G3BP1 in HCV replication, we first verified its DRM association and elevation in replicon cells. Results showed that G3BP1 was enriched in fraction 2 and its abundance was higher in replicon cells than control cells (Fig. 3A and B). Second, we investigated the interaction between G3BP1 and HCV NS proteins. Results showed that G3BP1 was specifically associated with HCV NS5B in HEK293T cells (Fig. 4A). After siRNA against G3BP1 was further introduced into the HCV replicon cells and the cellular expression of G3BP1 was knocked down, the expression of HCV NS5A as well as HCV replication were significantly reduced ($p = 0.027$) (Fig. 4B and C).

Discussion

Recently, it is reported that HCV replication complexes assemble on detergent resistant membrane domain (DRM) through interactions between viral nonstructural proteins and host proteins [13–15]. To determine the potential HCV RC components, we used a proteomics approach in

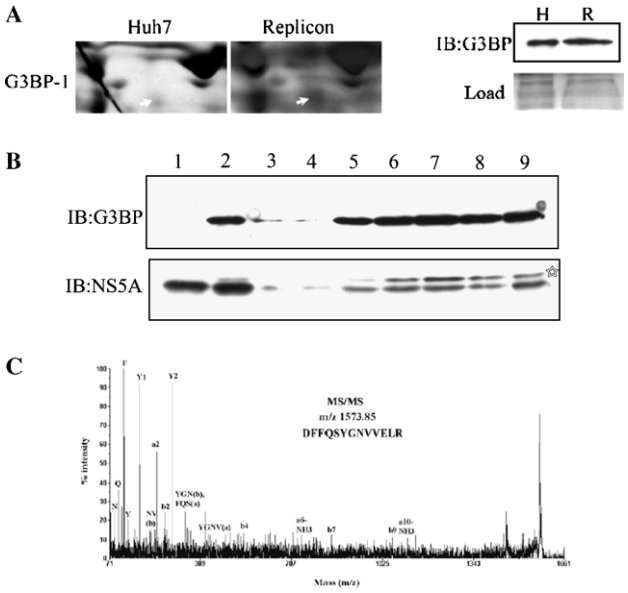

Fig. 3. G3BP1 was enriched in DRM in HCV replicon cells. (A) Close-up sections comparing the intensities of the G3BP1 in Huh7 cells and HCV replicon cells (left panel); equal amounts of DRM fractions (5 μg) from HCV replicon cells and Huh7 cells were analyzed on a 10% polyacrylamide gel, followed by immunoblotting with monoclonal antibodies against G3BP1 (right panel). (B) Replicon cells were scraped with hypotonic buffer and broken by 25 gauge syringe. After centrifugation at 900*g* for 5 min, the cell lysates were treated with 1% NP-40 and fractionated by sucrose gradient centrifugation. Proteins from each sucrose gradient fraction were precipitated and analyzed on 10% SDS–PAGE, followed by immunoblotting with antibodies against G3BP1 and then reprobed with antibodies against NS5A. The star indicated the band of G3BP1. (C) MALDI-MS/MS sequencing result of the peptide 1573.85. The peptide sequence was identified as DFFQSYGNVVELR, which led to the identification of G3BP1. The asterisks (*) indicate the matched peptides.

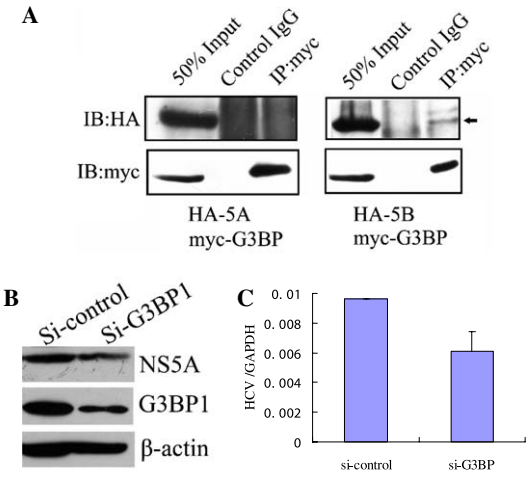

Fig. 4. G3BP1 was involved in HCV replication. (A) HEK293T cells were transfected with plasmids pCMV-G3BP1 and pCDNA3.0HA-NS5A, NS5B. Cell lysate was immunoprecipitated with antibody against myc and precipitated complexes were analyzed on 8% SDS–PAGE, followed by immunoblotting with antibodies against myc and HA. (B) Replicon cells were seeded in 24-well plate and transfected with 200 nM siRNA duplex targeting G3BP1 and scrambled siRNA duplex. At 48 h post-transfection, cells were lysed with SDS–PAGE loading buffer. Proteins were analyzed on 10% SDS–PAGE, followed by immunoblotting with antibodies against NS5A, G3BP1, and β-actin. (C) Or cells were harvested with Trizol reagent. Real-time PCR amplifications were performed with primers specific for HCV 5′NTR. The real-time PCR signals were analyzed using LightCycler 3 software. Each experiment was repeated three times. The results were applied to *t*-test analysis.

combination with the subcellular fractionation and membrane flotation assay to compare the components of DRM from HCV replicon cells with its control cells to identify cellular proteins which are essential for HCV replication.

In the present study, we found that several identified proteins which are different in the presence or absence of HCV replication have been reported to interact with HCV gene and viral proteins. For example, actin, eIF3-P44 have been reported to interact with HCV 5′ IRES by proteomics approach [25]. α-Actinin, a protein previously reported to interact with HCV NS5B [20], was verified to be enriched in DRM of HCV replicating cells (data not shown). Besides, some proteins known to interact with α-actinin, such as annexinA, prohibitin [26], were also identified in this study. All the above results support the application of subcellular fractionation and membrane flotation assay in identification of the components of HCV replication complex residing on DRMs.

It is more interesting to observe that several new cellular proteins including G3BP1 were found to be altered in replicon cells comparing with control cells (Fig. 3A). G3BP1, an effector of Ras and a novel phosphorylation dependent RNase, have been implicated in Ras signalling, NF-κB signalling, the ubiquitin proteosome pathway, and RNA processing [27–29]. Recent experimental evidence also suggests that it is involved in viral replication [30]. In this study, it was shown to be associated with HCV NS5B (Fig. 4A) and furthermore, knockdown of G3BP1 by RNAi in HCV replicon cells reduced HCV replication (Fig. 4B and C). To identify potential cellular proteins related to HCV replication complexes (RC), another study in this laboratory applied biotinylated 3′(+)UTR and its reverse complementary 5′(−)UTR in RNA-pull-down assay to examine host proteins interacting with the HCV 3′UTR. It was interesting to find that G3BP1 could also be identified in the 5′(−)UTR captured complex [31]. All of the above results indicated that G3BP1 may be a component of HCV replication complex. It is possible that G3BP1 may directly bind to HCV NS5B or HCV RNA to modulate the components of HCV replication complex to control HCV replication.

In summary, our study successfully analyzed the altered proteome of DRM in the presence or absence of HCV replication in order to identify the potential HCV RC components, and found that G3BP1 is involved in HCV replication and may be a component of HCV replication complex. The further dissection of the HCV RC and extensive analysis of the RNA-viral protein-G3BP1 protein interaction will be necessary and may facilitate the understanding of HCV replication and pathogenesis as well as the development of novel antiviral drugs.

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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.bbrc.2006.09.027.

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