Antiviral Research 107 (2014) 50–55

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Short Communication

Hepatitis C virus NS5A competes with PI4KB for binding to ACBD3 in a genotype-dependent manner

Zhi Hong [1], Xiaojie Yang [1], Guangbo Yang, Leiliang Zhang *

MOH Key Laboratory of Systems Biology of Pathogens, Institute of Pathogen Biology, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100176, China

ARTICLE INFO

Article history:
Received 16 January 2014
Revised 18 April 2014
Accepted 22 April 2014
Available online 30 April 2014

Keywords:
ACBD3
HCV
NS5A
PI4P
PI4KB

ABSTRACT

Although genotype-dependency of PI4KB involved in HCV replication has been reported, the mechanism underlying that is unknown. In this study, we found that NS5A and PI4KB competed for association of acyl-coenzyme A binding domain containing protein 3 (ACBD3), which inhibited HCV replication. ACBD3 bind to GT1b NS5A with a higher affinity than to GT2a NS5A, which was consistent with higher co-localization between PI4KB and phosphatidylinositol 4-phosphate (PI4P) in GT1b HCV-infected cells than that in GT2a HCV-infected cells. These results suggested that NS5A could rob the preexisting ACBD3/PI4KB complex to form NS5A/ACBD3 complex and PI4KB could relocate to the viral RNA replication sites to facilitate HCV replication. Our findings not only revealed the anti-HCV function of ACBD3, but also shed mechanistic light on how ACBD3 was manipulated by NS5A from different GT of HCV.

Upregulation of intracellular phosphatidylinositol 4-phosphate (PI4P) level has been demonstrated to be an essential step for hepatitis C virus (HCV) replication (Bianco et al., 2012; Hsu et al., 2010; Reiss et al., 2011; Tai and Salloum, 2011; Zhang et al., 2012). Phosphatidylinositol 4-kinase A (PI4KA) is recruited and activated by HCV NS5A protein to synthesize PI4P in the HCV replication area (Berger et al., 2011; Lim and Hwang, 2011; Reiss et al., 2011). PI4P phosphatase Sac1 is dislocated from the site of HCV replication by COPI vesicular transport to maintain high levels of PI4P (Li et al., 2014). Although PI4KB contributes to the induction of PI4P and promotes viral replication for picornaviruses such as poliovirus (Arita et al., 2011; Hsu et al., 2010) and Aichi virus (Sasaki et al., 2012), the involvement of PI4KB in HCV replication is controversial. Some studies reported that PI4KB is required for HCV replication (Borawski et al., 2009; Zhang et al., 2012) and others suggested that it is not (Arita et al., 2011; Berger et al., 2011). This inconsistency might be due to the study of different HCV genotypes. Interestingly, viral replication of genotype 1b (GT1b) HCV were more greatly reduced than that of genotype 2a (GT2a) HCV upon silencing PI4KB (Borawski et al., 2009; Reiss et al., 2011). The mechanism underlying this genotype difference remains elusive.

Recently, acyl-coenzyme A binding domain containing protein 3 (ACBD3) was reported to be a novel PI4KB binding partner (Sasaki et al., 2012). Several studies have demonstrated that picornaviruses co-opted the ACBD3–PI4KB axis for replication but utilize different cellular mechanisms to manipulate the system. ACBD3 is required for Aichi virus replication through recruiting PI4KB to viral replication area (Sasaki et al., 2012). Greninger et al. showed that poliovirus RNA replication is reduced upon silencing ACBD3 (Greninger et al., 2012), while Teoule et al. found that poliovirus RNA replication is significantly increased upon ACBD3 suppression (Teoule et al., 2013). Dorobantu et al. demonstrated that ACBD3 depletion enhanced Coxsackievirus B3 RNA replication (Dorobantu et al., 2014). How ACBD3 regulates HCV replication is unknown.

To elucidate the role of ACBD3 in HCV replication, we treated OR6 cells harboring a full-length GT1b HCV RNA and *Renilla* luciferase reporter with small interfering RNA (siRNA) targeting ACBD3 (ACBD3, 5′- GGAUGCAGAUUCCGUGAUU-3′) or control siRNA and monitored the expression level of viral proteins or *Renilla* luciferase reporter activities. At 72 h after treatment, the protein level of ACBD3, but not actin as a loading control, was significantly decreased (Fig. 1A). Upon ACBD3 depletion, the level of viral proteins NS3 was increased. Likewise, silencing ACBD3 with siRNA reduced the relative *Renilla* luciferase activities normalized with cell viability assessed by the cellular ATP levels (Fig. 1B). Next, viral protein was examined in OR6 cells transfected with plasmid expressing

Abbreviations: HCV, hepatitis C virus; PI4P, phosphatidylinositol 4-phosphate; ACBD3, acyl-coenzyme A binding domain containing protein 3; Co-IP, co-immunoprecipitation; GST, glutathione-S-transferase.

* Corresponding author. Tel.: +86 10 67837355; fax: +86 10 67837132.
E-mail address: armzhang@hotmail.com (L. Zhang).
[1] Co-first authors.

http://dx.doi.org/10.1016/j.antiviral.2014.04.012
0166-3542/

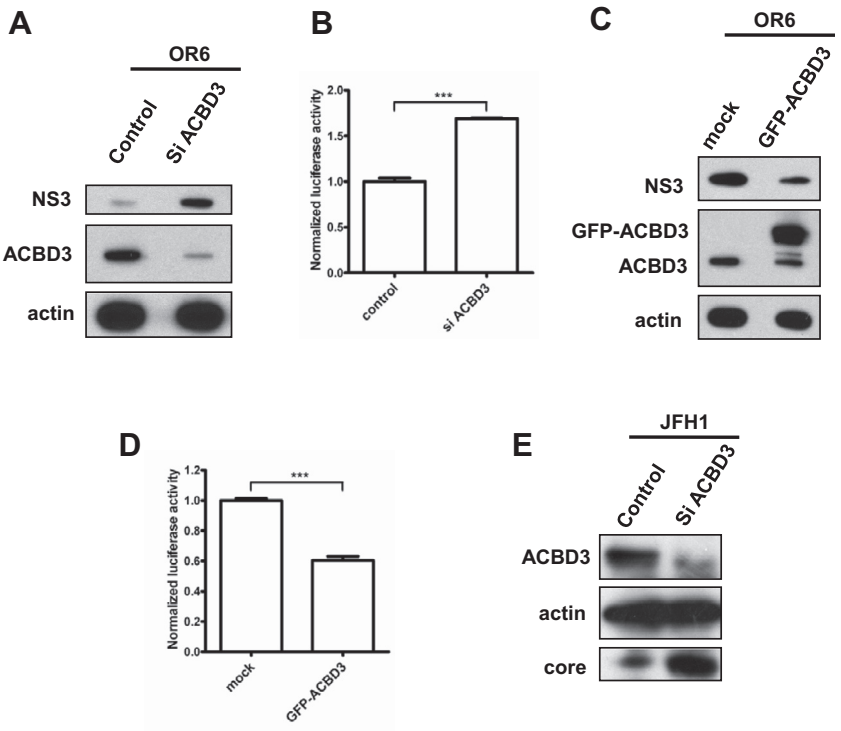

Fig. 1. ACBD3 inhibits HCV replication. (A) OR6 cells were treated with siRNA targeting ACBD3 or control siRNA for 72 h and then cell lysates were analyzed by immunoblotting with indicated antibodies. (B) OR6 cells were treated with siRNA targeting ACBD3 or control siRNA for 72 h and then *Renilla* luciferase activity and cellular ATP levels were measured. Relative luciferase activity was normalized with cellular ATP levels. Data were represented the average ± SD. $^{***}P < 0.0001$. (C) OR6 cells were transfected with GFP-ACBD3 or empty vector for 48 h and then cell lysates were analyzed by immunoblotting with indicated antibodies. (D) OR6 cells were transfected with GFP-ACBD3 or empty vector for 48 h and then *Renilla* luciferase activity and cellular ATP levels were measured. Relative luciferase activity was normalized with cellular ATP levels. Data were represented the average ± SD. $^{***}P < 0.0001$. (E) Huh 7.5.1 cells were treated with siRNA targeting ACBD3 or control siRNA for 72 h and then infected with JFH1 (moi = 1) for another 72 h. Cell lysates were analyzed by immunoblotting with indicated antibodies.

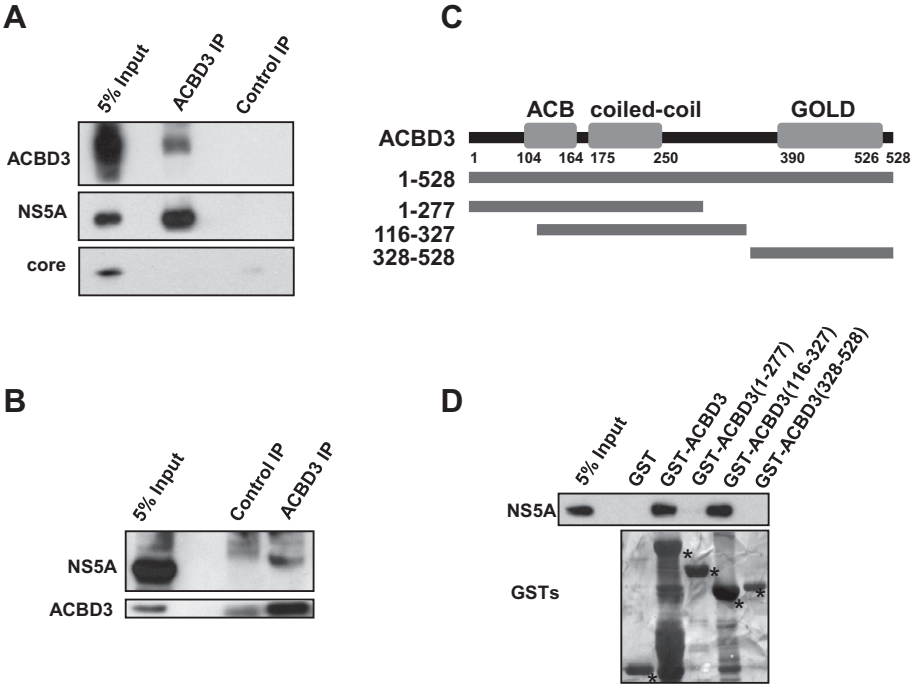

Fig. 2. ACBD3 associates with HCV NS5A protein. (A) Co-immunoprecipitation assays in OR6 cells with mouse anti-ACBD3 and control mouse IgG. Input (5% of total cell lysates) and immunoprecipitated proteins were analyzed by immunoblotting with indicated antibodies. (B) Co-immunoprecipitation assays in JFH1-infected Huh 7.5.1 cells with mouse anti-ACBD3 and control mouse IgG. Input (5% of total cell lysates) and immunoprecipitated proteins were analyzed by immunoblotting with indicated antibodies. (C) Schematic diagram of different constructs of ACBD3 fused to GST. (D) Lysates from 293T cells transfected with construct expressing FLAG-tagged NS5A of JFH1 were pulled down by different domains of ACBD3 fused to GST tag or GST alone. Input represented 5% cell lysates. GST or GST fusion proteins were marked by * in bottom panel.

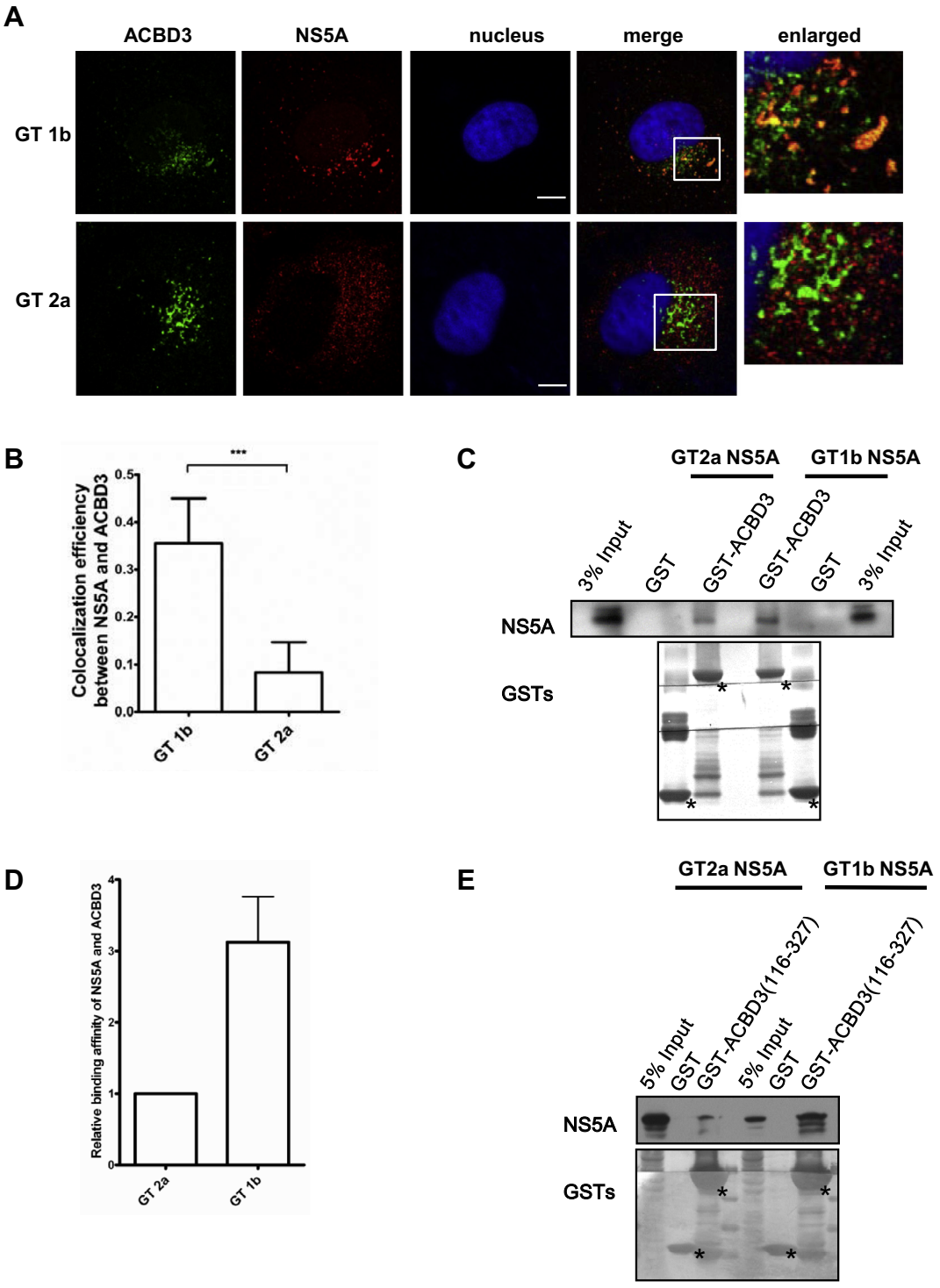

Fig. 3. ACBD3 binds to GT1b NS5A with a higher affinity than to GT2a NS5A. (A) OR6 cells (GT1b) or JFH1-infected Huh7.5.1 cells (GT2a) were stained with indicated antibodies. Scale bar, 10 μm. (B) Correlation coefficients of signals from NS5A and ACBD3 were analyzed using Volocity to represent the colocalization between NS5A and ACBD3 in OR6 cells (GT1b) or JFH1-infected Huh7.5.1 cells (GT2a). Data were collected from at least 20 cells for each condition and represented the average ± SD. $^{***}P < 0.0001$. (C) Lysates from 293T cells transfected with constructs expressing FLAG-tagged NS5A of GT1b or GT2a were pulled down by GST–ACBD3 or GST. Input represented 3% of total cell lysates. GST or GST–ACBD3 was marked by *. (D) The blots of three independent experiments of (C) were quantified using ImageJ. Relative binding affinity of NS5A and ACBD3 was normalized by blot signal of Input and GST fusion protein. (E) Lysates from 293T cells transfected with constructs expressing FLAG-tagged NS5A of GT1b or GT2a were pulled down by GST–ACBD3(116–327) or GST. Input represented 5% of total cell lysates. GST or GST–ACBD3(116–327) was marked by *.

GFP-ACBD3 or mock transfection. At 48 h after transfection with GFP-ACBD3, the level of HCV NS3 protein was reduced (Fig. 1C). Overexpression of GFP-ACBD3 also reduced the relative *Renilla* luciferase activities normalized with cell viability assessed by the cellular ATP levels (Fig. 1D). Furthermore, knockdown of ACBD3 by siRNA significantly increased the protein level of core in JFH1-infected Huh 7.5.1 cells (GT2a HCV) (Fig. 1E). Taken together, these data demonstrate that ACBD3 plays a negative role in HCV replication.

To investigate whether HCV protein(s) could physically associate with ACBD3, we performed ACBD3 immunoprecipitation with mouse anti-ACBD3 antibody. We found that NS5A bind to ACBD3

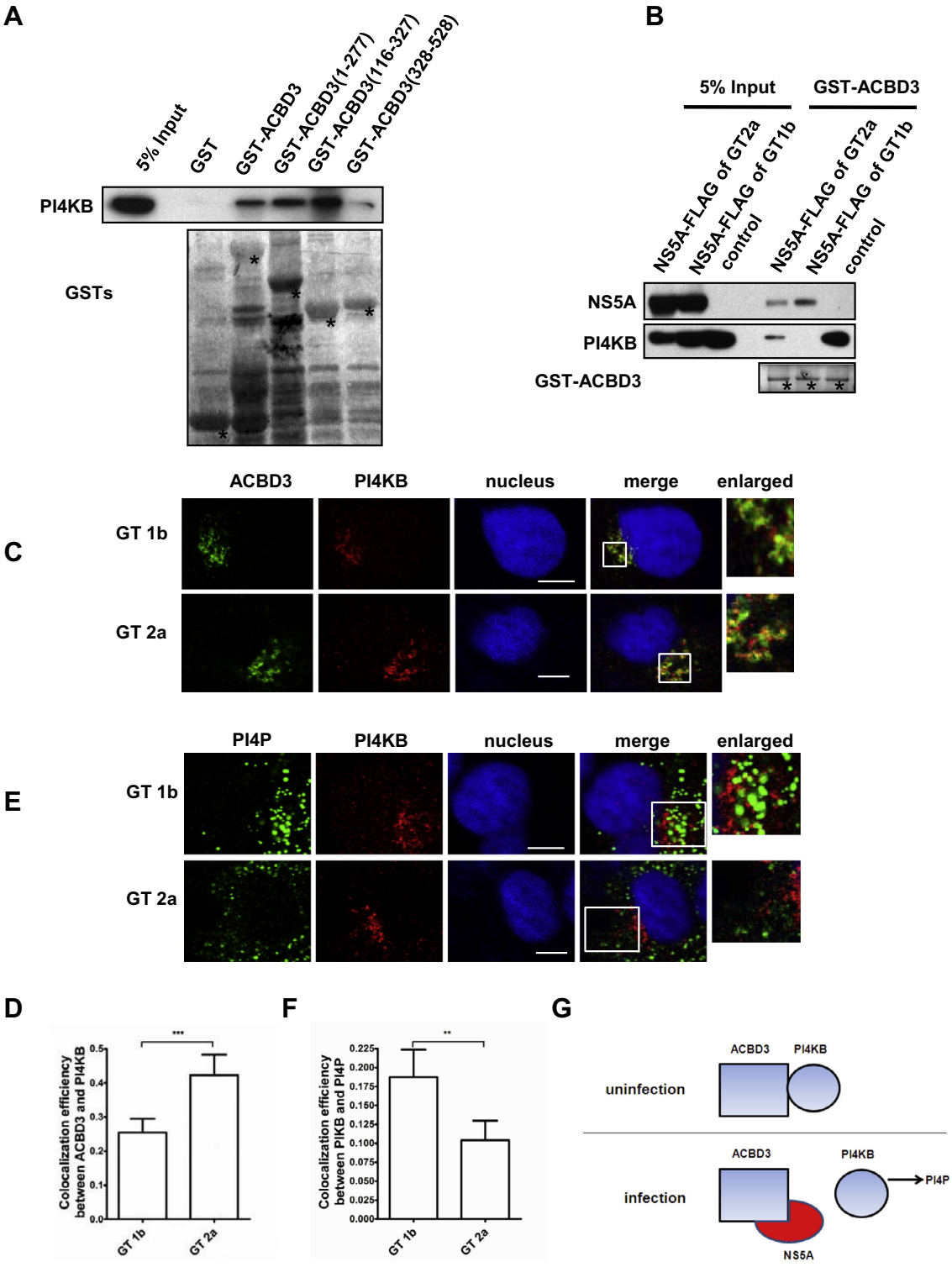

Fig. 4. NS5A competes with PI4KB for association with ACBD3. (A) Lysates from 293T cells transfected with construct expressing Myc-tagged PI4KB were pulled down by different constructs of ACBD3 fused to GST tag or GST alone. Input represented 5% of total cell lysates. GST or GST fusion proteins were marked by *. (B) Lysates from 293T cells transfected with construct expressing Myc-tagged PI4KB plus construct expressing FLAG-tagged NS5A of GT1b or GT2a or empty vector were pulled down by GST–ACBD3. Input represented 5% of total cell lysates. GST or GST fusion proteins were marked by *. (C) OR6 cells (GT1b) or JFH1-infected Huh7.5.1 cells (GT2a) were stained with indicated antibodies. Scale bar, 10 μm. (D) Correlation coefficients of signals from ACBD3 and PI4KB were analyzed using Volocity to represent the colocalization between ACBD3 and PI4KB in OR6 cells (GT1b) or JFH1-infected Huh7.5.1 cells (GT2a). Data were collected from at least 20 cells for each condition and represented the average ± SD. ***$P < 0.0001$. (E) OR6 cells (GT1b) or JFH1-infected Huh7.5.1 cells (GT2a) were stained with indicated antibodies. Scale bar, 10 μm. (F) Correlation coefficients of signals from PI4KB and PI4P were analyzed using Volocity to represent the colocalization between ACBD3 and PI4KB in OR6 cells (GT1b) or JFH1-infected Huh7.5.1 cells (GT2a). Data were collected from at least 20 cells for each condition and represented the average ± SD. **$P < 0.001$. (G) Model for releasing PI4KB to generate PI4P. In uninfected cells, PI4KB binds to ACBD3 through interaction with ACBD3 (top panel). In HCV-infected cells, viral protein NS5A competes with PI4KB for binding to ACBD3. The NS5A/ACBD3 complex is formed as infection progresses, whereas PI4KB was released to produce PI4P (bottom panel).

in OR6 cells, while core didn't associate with ACBD3 (Fig. 2A). We confirmed the interaction of NS5A and endogenous ACBD3 in JFH1-infected Huh 7.5.1 cells (Fig. 2B). To determine the region of ACBD3 required for NS5A association, we performed an *in vitro* binding assay using GST fused to ACBD3, ACBD3(1–277), ACBD3(116–327), ACBD3(328–528), or GST alone (Fig. 2C). GST proteins were prebound to glutathione beads and incubated with Huh 7.5.1 cell lysates expressing FLAG-tagged NS5A from JFH1, and NS5A was detected by immunoblotting with anti-FLAG antibodies. As shown in Fig. 2D, GST–ACBD3 full length and GST–ACBD3(116–327), but not ACBD3(1–277), ACBD3(328–528), or GST alone were able to co-immunoprecipitate with NS5A-FLAG. These data demonstrate that NS5A preferentially recognizes the GST–ACBD3(116–327) fragment.

We further assessed the colocalization of ACBD3 and NS5A in the context of viral infection. Surprisingly, NS5A of GT1b has higher colocalization with ACBD3 than GT2a does (Fig. 3A and B). To compare the binding affinity of ACBD3 to NS5A between GT1b and GT2a, GST–ACBD3 was incubated with lysates from 293T cells transfected with GT1b NS5A-FLAG or GT2a NS5A-FLAG. We found that NS5A from GT1b had higher binding affinity with ACBD3 than that from GT2a (Fig. 3C and D). Because NS5A preferentially recognizes the GST–ACBD3(116–327) fragment, we compared the binding affinity of ACBD3(116–327) to NS5A between GT1b and GT2a, and found that NS5A from GT1b had higher binding affinity with ACBD3(116–327) than that from GT2a (Fig. 3E).

Earlier study suggests that GST–ACBD3(116–327) fragment is the region associated with PI4KB (Sasaki et al., 2012). We used GST pulldown experiments to confirm that ACBD3(116–327) fragment was sufficient to bind PI4KB (Fig. 4A). Thus PI4KB and NS5A share a common binding region on ACBD3. We asked whether NS5A competed with PI4KB for association with ACBD3. Overexpression of NS5A-FLAG diminished the capacity of GST–ACBD3 to capture PI4KB (Fig. 4B). Consistent with our previous studies (Fig. 3C), ACBD3 captured more NS5A and less PI4KB in cells transfected with GT1b NS5A than that in cells transfected with GT2a NS5A. Next, we checked the localization of PI4KB and PI4P in OR6 or JFH1 infected Huh 7.5.1 cells by immunofluorescence staining. As shown in Fig. 4C and D, the colocalization efficiency between PI4KB and PI4P in OR6 cells (GT1b) is higher than that in JFH1 infected Huh 7.5.1 cells (GT2a). To examine the relation between PI4KB and PI4P during HCV infection, we checked the localization between PI4KB and PI4P in OR6 or JFH1 infected Huh 7.5.1 cells by immunofluorescence staining. As shown in Fig. 4E and F, the colocalization efficiency between PI4KB and PI4P in OR6 cells (GT1b) is higher than that in JFH1 infected Huh 7.5.1 cells (GT2a).

Our GST-pulldown assays indicated that the PI4KB-binding region of ACBD3 (aa 116–327) overlapped with the binding domain for the NS5A (Fig. 2D). This implies that PI4KB is at least partly blocked from the HCV RNA replication site through interaction with ACBD3. We propose a model for the release of PI4KB to the HCV RNA replication sites (Fig. 4G). ACBD3 negatively regulates HCV replication by blocking PI4KB from exerting its function. The viral NS5A protein could competitively bind ACBD3, unlocking its inhibitory effect over PI4KB. As infection progresses, the NS5A might rob the preexisting ACBD3/PI4KB complex to form the NS5A/ACBD3 complex, thus releasing PI4KB to viral RNA replication sites to facilitate viral replication.

HCV is classified into 7 divergent genotypes that exhibit extensive sequence variation (Smith et al., 2014). NS5A has been shown to interact with many host proteins to regulate viral replication and cellular signaling pathways. NS5A performs some functions in a genotype-dependent manner. For example, NS5A from GT1 suppresses type I IFN responses more strongly than NS5A from GT3 in conjunction with stronger binding to STAT1 (Kumthip et al., 2012). Multiple genetic variants in NS5A alter the CsA susceptibility of HCV (Ansari and Striker, 2012; Fernandes et al., 2010). In this study, we found that GT1b NS5A binds to ACBD3 with a higher affinity than GT2a NS5A. As a consequence, more PI4KB was released from ACBD3 in GT1b HCV than GT2a HCV, which will explain why PI4KB plays more dependency role in GT1b virus than in GT2a virus. In conclusion, we identified ACBD3 to be a host restriction factor against HCV replication. ACBD3 plays both genotype-dependant and genotype-independent antiviral roles for HCV. The main point of this study is to demonstrate the genotype-dependant role of ACBD3 to HCV. NS5A and PI4KB compete for association of ACBD3 in a genotype-dependent manner. Our findings shed mechanistic light on how ACBD3 is manipulated by NS5A from different genotypes of HCV and will provide a new anti-HCV strategy.

Conflict of interest

The authors declare they have no competing financial interest.

Acknowledgements

This work was supported by grants from National Natural Science Foundation of China (81271832, 81301438), National Science and Technology Major Project of China (2013ZX10004-601), Beijing Natural Science Foundation (7132140), Intramural Research Program of the Institute of Pathogen Biology, CAMS (2012IPB206), Rising Star of PUMC, Program for Changjiang Scholars and Innovative Research Team in University (IRT13007), and the Research Fund for the Doctoral Program of Higher Education of China (20121106120058). We thank Francis Chisari, Takaji Wakita, Yan Zhou and Jun Sasaki for reagents.

References

Ansari, I.U., Striker, R., 2012. Subtype specific differences in NS5A domain II reveals involvement of proline at position 310 in cyclosporine susceptibility of hepatitis C virus. Viruses 4, 3303–3315.

Arita, M., Kojima, H., Nagano, T., Okabe, T., Wakita, T., Shimizu, H., 2011. Phosphatidylinositol 4-kinase III beta is a target of enviroxime-like compounds for antipoliovirus activity. J. Virol. 85, 2364–2372.

Berger, K.L., Kelly, S.M., Jordan, T.X., Tartell, M.A., Randall, G., 2011. Hepatitis C virus stimulates the phosphatidylinositol 4-kinase III alpha-dependent phosphatidylinositol 4-phosphate production that is essential for its replication. J. Virol. 85, 8870–8883.

Bianco, A., Reghellin, V., Donnici, L., Fenu, S., Alvarez, R., Baruffa, C., Peri, F., Pagani, M., Abrignani, S., Neddermann, P., De Francesco, R., 2012. Metabolism of phosphatidylinositol 4-kinase IIIalpha-dependent PI4P Is subverted by HCV and is targeted by a 4-anilino quinazoline with antiviral activity. PLoS Pathog. 8, e1002576.

Borawski, J., Troke, P., Puyang, X., Gibaja, V., Zhao, S., Mickanin, C., Leighton-Davies, J., Wilson, C.J., Myer, V., Cornellataracido, I., Baryza, J., Tallarico, J., Joberty, G., Bantscheff, M., Schirle, M., Bouwmeester, T., Mathy, J.E., Lin, K., Compton, T., Labow, M., Wiedmann, B., Gaither, L.A., 2009. Class III phosphatidylinositol 4-kinase alpha and beta are novel host factor regulators of hepatitis C virus replication. J. Virol. 83, 10058–10074.

Dorobantu, C.M., van der Schaar, H.M., Ford, L.A., Strating, J.R., Ulferts, R., Fang, Y., Belov, G., van Kuppeveld, F.J., 2014. Recruitment of PI4KIIIbeta to coxsackievirus B3 replication organelles is independent of ACBD3, GBF1, and Arf1. J. Virol. 88, 2725–2736.

Fernandes, F., Ansari, I.U., Striker, R., 2010. Cyclosporine inhibits a direct interaction between cyclophilins and hepatitis C NS5A. PLoS ONE 5, e9815.

Greninger, A.L., Knudsen, G.M., Betegon, M., Burlingame, A.L., Derisi, J.L., 2012. The 3A protein from multiple picornaviruses utilizes the golgi adaptor protein ACBD3 to recruit PI4KIIIbeta. J. Virol. 86, 3605–3616.

Hsu, N.Y., Ilnytska, O., Belov, G., Santiana, M., Chen, Y.H., Takvorian, P.M., Pau, C., van der Schaar, H., Kaushik-Basu, N., Balla, T., Cameron, C.E., Ehrenfeld, E., van Kuppeveld, F.J., Altan-Bonnet, N., 2010. Viral reorganization of the secretory pathway generates distinct organelles for RNA replication. Cell 141, 799–811.

Kumthip, K., Chusri, P., Jilg, N., Zhao, L., Fusco, D.N., Zhao, H., Goto, K., Cheng, D., Schaefer, E.A., Zhang, L., Pantip, C., Thongsawat, S., O'Brien, A., Peng, L.F., Maneekarn, N., Chung, R.T., Lin, W., 2012. Hepatitis C virus NS5A disrupts STAT1 phosphorylation and suppresses type I interferon signaling. J. Virol. 86, 8581–8591.

Li, H., Yang, X., Yang, G., Hong, Z., Zhou, L., Yin, P., Xiao, Y., Chen, L., Chung, R.T., Zhang, L., 2014. Hepatitis C virus NS5A hijacks ARFGAP1 to maintain a PI4P-enriched microenvironment. J. Virol. 88, 5956–5966.

Lim, Y.S., Hwang, S.B., 2011. Hepatitis C virus NS5A protein interacts with phosphatidylinositol 4-kinase type IIIalpha and regulates viral propagation. J. Biol. Chem. 286, 11290–11298.

Reiss, S., Rebhan, I., Backes, P., Romero-Brey, I., Erfle, H., Matula, P., Kaderali, L., Poenisch, M., Blankenburg, H., Hiet, M.S., Longerich, T., Diehl, S., Ramirez, F., Balla, T., Rohr, K., Kaul, A., Buhler, S., Pepperkok, R., Lengauer, T., Albrecht, M., Eils, R., Schirmacher, P., Lohmann, V., Bartenschlager, R., 2011. Recruitment and activation of a lipid kinase by hepatitis C virus NS5A is essential for integrity of the membranous replication compartment. Cell Host Microbe 9, 32–45.

Sasaki, J., Ishikawa, K., Arita, M., Taniguchi, K., 2012. ACBD3-mediated recruitment of PI4KB to picornavirus RNA replication sites. EMBO J. 31, 754–766.

Smith, D.B., Bukh, J., Kuiken, C., Muerhoff, A.S., Rice, C.M., Stapleton, J.T., Simmonds, P., 2014. Expanded classification of hepatitis C virus into 7 genotypes and 67 subtypes: updated criteria and genotype assignment web resource. Hepatology 59, 318–327.

Tai, A.W., Salloum, S., 2011. The role of the phosphatidylinositol 4-kinase PI4KA in hepatitis C virus-induced host membrane rearrangement. PLoS ONE 6, e26300.

Teoule, F., Brisac, C., Pelletier, I., Vidalain, P.O., Jegouic, S., Mirabelli, C., Bessaud, M., Combelas, N., Autret, A., Tangy, F., Delpeyroux, F., Blondel, B., 2013. The Golgi protein ACBD3, an interactor for poliovirus protein 3A, modulates poliovirus replication. J. Virol. 87, 11031–11046.

Zhang, L., Hong, Z., Lin, W., Shao, R.X., Goto, K., Hsu, V.W., Chung, R.T., 2012. ARF1 and GBF1 generate a PI4P-enriched environment supportive of hepatitis C virus replication. PLoS ONE 7, e32135.