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RacGTPase-activating protein 1 interacts with hepatitis C virus polymerase NS5B to regulate viral replication

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ABSTRACT

Hepatitis C virus (HCV) is a positive-strand RNA virus responsible for chronic liver disease and hepatocellular carcinoma (HCC). RacGTPase-activating protein 1 (RacGAP1) plays an important role during GTP hydrolysis to GDP in Rac1 and CDC42 protein and has been demonstrated to be upregulated in several cancers, including HCC. However, the molecular mechanism leading to the upregulation of RacGAP1 remains poorly understood. Here, we showed that RacGAP1 levels were enhanced in HCV cell-culture-derived (HCVcc) infection. More importantly, we illustrated that RacGAP1 interacts with the viral protein NS5B in mammalian cells. The small interfering RNA (siRNA)-mediated knockdown of RacGAP1 in human hepatoma cell lines inhibited replication of HCV RNA, protein, and production of infectious particles of HCV genotype 2a strain JFH1. Conversely, these were reversed by the expression of a siRNA-resistant RacGAP1 recombinant protein. In addition, viral protein NS5B polymerase activity was significantly reduced by silencing RacGAP1 and, vice versa, was increased by overexpression of RacGAP1 in a cell-based reporter assay. Our results suggest that RacGAP1 plays a crucial role in HCV replication by affecting viral protein NS5B polymerase activity and holds importance for antiviral drug development.

1. Introduction

The hepatitis C virus (HCV) affects more than 170 million of the global population and causes chronic liver disease. Most people who are infected with HCV but fail to clear virus, will establish a chronic infection, leading to cirrhosis and hepatocellular carcinoma (HCC) [1]. HCV is a positive-sense RNA virus with a genome of 9.6 kb encoding a polyprotein of about 3,000 amino acids. The polyprotein is cleaved by host peptidase and viral protease to form 10 functional viral proteins [2,3]. These mature viral proteins include structural (Core, E1, E2, and p7) and nonstructural (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins. HCV-associated HCC is induced by viral protein-mediated oxidative stress and DNA damage. For instance, expression of core viral protein increases the level of reactive oxygen species in transgenic mice and through inhibition of mitochondrial electron transport in cultured cells [4,5]. When oxidative stress in cells is increased, it may potentially damage DNA causing liver fibrosis. In addition, oxidative stress also leads to an active STAT-3 signaling pathway to contribute to cellular transformation [6].

The HCV replication complex (RC) contains nonstructural proteins and host factors for viral RNA replication. The RC is directly or indirectly associated with membranous webs as the replication site. NS5B is the viral RNA-dependent RNA polymerase (RdRp) and catalyzes the viral RNA synthesis. NS5B polymerase has been targeted for anti-HCV drug development. Drugs developed using this strategy include nucleoside inhibitors (NIs) and nonnucleoside inhibitors (NNIs). NIs block the 3′-hydroxyl group and are incorporated into HCV RNA, terminating the elongation step. NNIs are small molecules binding one of five allosteric pockets in NS5B. These NS5B polymerase inhibitors thereby inactivate NS5B polymerase function [7].

RacGTPase activating protein 1 (RacGAP1) is mainly localized at the nucleus and cytoplasm based on mitotic stages, and belongs to

Abbreviations: CE, cell extract; co-IP, coimmunoprecipitation; DMEM, Dulbecco's modified Eagle's medium; dsRNA, double-stranded RNA; FBS, fetal bovine serum; FFU, focus-forming units; GAP, GTPase activating protein; GFP, green fluorescent protein; HEK, human embryo kidney; Huh7, human hepatoma cell 7; IFN, interferon; MDA5, melanoma differentiation-associated protein 5; MOI, multiplicity of infection; NI, nucleoside inhibitor; NNI, non-nucleoside inhibitor; NS, nonstructural protein; p.i., postinfection; PRR, pathogen recognition receptor; qPCR, quantitative real-time PCR; RC, replication complex; RdRp, RNA-dependent RNA polymerase; RIG-I, retinoic acid-inducible gene 1; SD, standard deviation; siCtrl, control siRNA; TK, thymidine kinase.

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the GTPase-activating proteins (GAPs) family [8]. GAPs activate GTPase activity by stimulating GTPase activity to hydrolyze GTP, while guanine nucleotide-exchange factors activate GTPase for recharging GTP. RacGAP1 negatively controls the function of Rho family GTPases through GTP hydrolysis to regulate cytoskeletal organization [9]. RacGAP1 is upregulated in patients with early recurrence of HCC [10], and other studies using quantitative real-time PCR (qPCR) analysis suggest the RacGAP1 gene is increased in HCV-induced HCC patient samples [11]. Here, we demonstrate that the level of RacGAP1 is upregulated by HCV infection in human hepatoma cells to enhance HCV replication through binding to and affecting NS5B polymerase activity.

2. Materials and methods

2.1. Cell culture

All cell lines were cultured at 37 °C under a humidified atmosphere with 5% CO_2. Human embryo kidney (HEK) 293T and human hepatoma 7 (Huh7) cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS). Huh7-GL cells (bearing pCDNA6-TR/TIGHT JFH1) that stably produced infectious HCV of genotype 2a (JFH1) were cultured in DMEM containing 10% FBS, 1% nonessential amino acids (Gibco BRL, Gaithersburg, MD, USA), and 5 μg/mL blasticidin (Sigma–Aldrich, St. Louis, MO, USA) [12]. The stable cell line Huh7-replicon (AVA5) was obtained from Dr. Charles Rice and maintained in DMEM with 10% FBS and G418 (0.5 mg/mL) [13].

2.2. Plasmids, siRNA, and antibodies

The human RacGAP1 (DNA GenBank accession number XM_006719360) was amplified from the cDNA of the HEK293T cell line and fused to pEGFPN2. The plasmids expressing nonstructural proteins of HCV were amplified from genotype 1b (strain Con1) plasmid by PCR, and were kindly provided by Dr. Charles Rice [13]. The DNA fragment encoding rRacGAP1 was produced by PCR using the primers listed in Supplemental Table S1. The PCR product was inserted into pcDNA3.1-His-myc. The DNA fragments of NS3/4A (S139A) and NS5B (GAA) were amplified by PCR and these mutation sites were made to inactivate functions of NS3 protease and NS5B polymerase. Retinoic acid-inducible gene 1 (RIG-I) and melanoma differentiation-associated protein 5 (MDA5) plasmids were purchased from Addgene. Interferon beta (IFN-β)/Fluc plasmid was kindly provided by Dr. Takashi Fujita [14]. All constructs produced by PCR using specific primers are listed in Supplemental Table S1. The antibodies against GFP and calnexin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibodies against viral protein NS3 and NS5B were purchased from Abcam (Cambridge, UK). The mouse monoclonal and rabbit polyclonal antibodies against GAPDH and RacGAP1 were from Abnova (Taiwan) and GeneTex (Irvine, CA, USA), respectively. The siRNA sequence targeting the RacGAP1 coding region is 5′-GCG AAG GAC TTT GAG GAT TTC CGT A-3′ (Invitrogen).

2.3. Coimmunoprecipitation (co-IP) assays

HEK293T cells were transfected with various plasmids and harvested at 24 h after transfection. Equal amounts (500 μg) of lysates were used for co-IP assays as previously described [15].

2.4. Preparation and titration of cell-culture-derived HCV (HCVcc)

The preparation of cell-culture-derived HCV from Huh7-GL stable cell lines containing full-length sequences of the JFH1 genotype or from JFH1 RNA-transfected Huh7 cells was performed as described previously [12,16]. The viral titer was determined by immunofluorescence staining of HCVcc-infected Huh7.5 cells using a specific antibody against NS5A: the number of HCV foci was counted, and the titer was expressed as focus-forming units per milliliter (FFU/mL) [15].

2.5. HCVcc infection and viral protein and RNA determination

HCV infection was performed as described previously [17]. Briefly, Huh7 cells harboring siRacGAP1 were infected with HCVcc at a multiplicity of infection (MOI) of 0.01 for 4 h. The HCV-infected cells were washed with PBS and then incubated for 3 days with DMEM containing 10% FBS. Total RNA was extracted with TRIzol (Invitrogen) for qPCR determination, and viral protein levels were determined by Western blotting as previously described [15].

2.6. Membrane flotation assay

This assay was modified as previously described [18]. Huh7.5 cells were infected overnight with HCVcc at a MOI of 0.1. The HCV-infected cells were washed with PBS and then incubated for 3 days with DMEM containing 10% FBS. The cells were then treated with trypsin, washed, and resuspended in 3.5 mL PBS containing 0.25 M sucrose plus protease inhibitor cocktail (Roche, Mannheim, Germany). The cells were lysed in a tight-fitting Dounce homogenizer and the resulting cell lysate was then spun at 2500*g* for 10 min. The supernatants with equal amounts of protein (2 mg) in a volume of 2 mL with PBS/sucrose were mixed with 2 mL of 60% iodixanol (Sigma), resulting in a 30% iodixanol concentration. A discontinuous iodixanol gradient (10%, 20%) was layered on top of the lysate/iodixanol mixture, and the gradient was spun at 200,000*g* for 16 h at 4 °C in a SW41T rotor. A total of 20 fractions (500 μL each) were collected from the top and an equal volume of each fraction was subjected to SDS–PAGE and Western blotting.

2.7. Cell-based reporter assays

The method was modified as described previously [19]. Briefly, plasmids expressing NS5B or NS5B(GAA), RIG-I or MDA5, and two luciferases—one was a firefly luciferase driven by an IFN-β promoter and the other a *Renilla* luciferase driven by a thymidine kinase (TK) promoter—were cotransfected into HEK293T cells. At 24 or 48 h posttransfection, the cells were harvested and analyzed for firefly and *Renilla* luciferase activities using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

2.8. Statistical analyses

Statistical graphs were generated using the GraphPad Prism software, and statistical analyses were performed using a two-tailed Student's *t* distribution test with Microsoft Excel. The graphs represent the mean ± standard deviation (SD) from at least three independent experiments. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Expression of RacGAP1 level and distribution were changed by virus infection

According to previous data from a transcriptomic study, the mRNA levels of RacGAP1 are changed in patients with virus-induced HCC [11]. We explored the possibility of whether HCVcc infection can change RacGAP1 expression in hepatoma cell lines.

When the virus infects the Huh7 cell line, a permissive cell line for HCV infection, at 3 days postinfection (p.i.) the RacGAP1 protein level was markedly upregulated compared with the uninfected controls (mock) (Fig. 1A). We explored whether viral infection might recruit RacGAP1 for viral replication using a fractionation approach. RacGAP1 was localized in bottom fractions in mock infected cells, which might represent the nuclear fraction [8]. However, the distribution of RacGAP1 was shifted from these bottom fractions to endoplasmic reticulum (ER) fractions, defined by the ER marker calnexin (fractions 13–17), and colocalized with viral protein NS3 during virus infection (Fig. 1B). Therefore, these results indicated that the RacGAP1 level was upregulated and subcellular localization was changed in order to associate with replication compartments in HCV infection.

3.2. HCV NS5B interacts with RacGAP1 in a replicon and in a HEK293T cell line

To understand the role of RacGAP1 in HCV replication, we investigated whether nonstructural proteins of HCV can interact with RacGAP1 in mammalian cells. We cotransfected green fluorescent protein (GFP)-tagged RacGAP1 (RacGAP1-GFP) with various His-myc-tagged nonstructural proteins of HCV into HEK293T cells, and co-IP was performed with anti-His antibodies (Fig. 2A). Immunoprecipitation of the different nonstructural proteins of HCV led to the detection of the RacGAP1-GFP protein in NS5B-expressing cells (Fig. 2A). To examine further whether endogenous RacGAP1 could interact with NS5B, endogenous RacGAP1 was immunoprecipitated in cells expressing His-myc-NS5B, as distinct from the absence of specific binding of RacGAP1 to vector control (Fig. 2B). This RacGAP1–NS5B interaction was also detected in AVA5 cells carrying a HCV replicon (Fig. 2C). After immunoprecipitation of the RacGAP1 with anti-RacGAP1 antibody, we found that NS5B in AVA5, but not in its parental Huh7 cells, was precipitated, indicating that the interaction was genuine (Fig. 2C). Next, we investigated whether the NS5B polymerase catalytic site would play a role in the interaction with RacGAP1. We constructed a mutation at the NS5B polymerase active site (GDD to GAA) as His-myc NS5B (GAA). Co-IP showed that RacGAP1-GFP was precipitated with both NS5B proteins with similar efficiency (Fig. 2D), suggesting that RacGAP1 might not interact with the catalytic site of NS5B (Fig. 2D). Collectively, our data showed that RacGAP1 specifically interacts with NS5B in mammalian cells expressing NS5B or a replicon and that a NS5B catalytic-null mutation might not be involved in the interaction.

3.3. RacGAP1 is involved in the replication of HCV

It is tempting to speculate that RacGAP1 plays a role in viral replication because of the interaction between RacGAP1 and NS5B. We transfected a siRNA targeted at RacGAP1 in AVA5 and Huh7 cell lines. Huh7 cells were then inoculated with HCVcc 4 h posttransfection. Seventy-two hours after transfection in AVA5 or infection in Huh7, the endogenous RacGAP1 protein and mRNA were efficiently silenced by siRNA (Fig. 3A and B). The synthesis of the viral protein, as indicated by the level of expression of NS3, viral RNA, and viral titers were considerably decreased in siRacGAP1-transfected cells compared with control siRNA (siCtrl)-transfected cells, suggesting RacGAP1 plays an important role in HCV replication (Fig. 3A–C). Conversely, we transiently transfected a nondegradable recombinant RacGAP1 (rRacGAP1) in AVA5 or Huh7 cell lines that had been treated with siRacGAP1. Then we inoculated HCVcc in Huh7 cells and harvested the cells for Western blot and qPCR analysis. The HCV viral RNA and viral protein NS3 were significantly upregulated in the rescued cell (rRacGAP1) compared with the vector control cell line (Fig. 3D and lanes 3 and 4 of Fig. 3E). These results suggest that RacGAP1 is necessary and sufficient for the replication of HCV.

3.4. RacGAP1 is involved in NS5B polymerase activity

Next, we examined whether RacGAP1 may regulate NS5B polymerase activity via a RacGAP1–NS5B interaction. We used a cell-based assay for the detection of RNA synthesis by NS5B polymerase

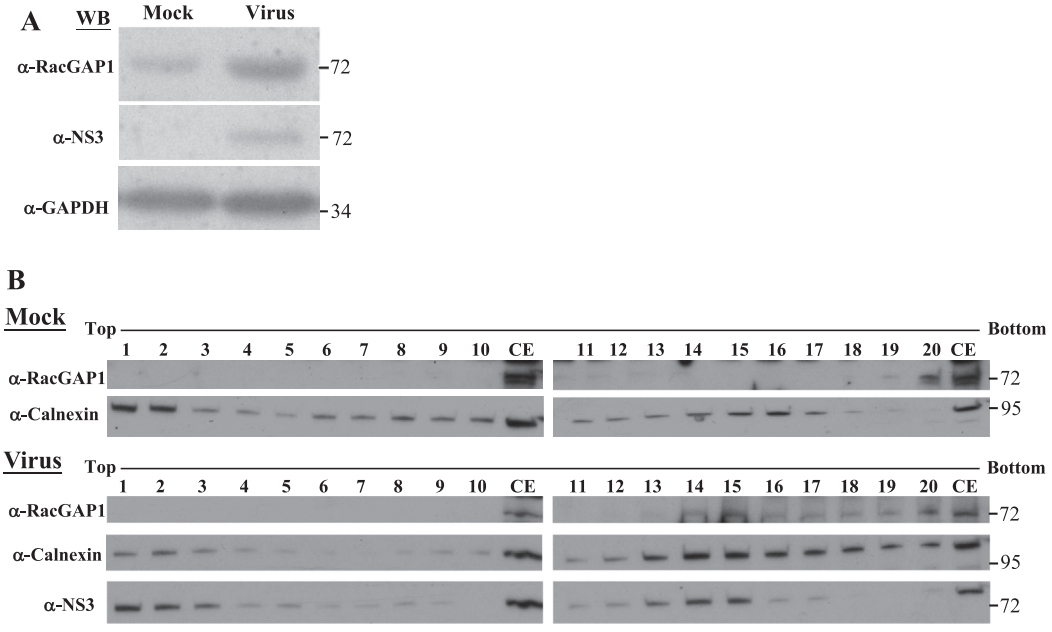

Fig. 1. RacGAP1 level and subcellular distribution during HCV infection. (A) RacGAP1 level was increased in virus infection. Huh7 cells were infected with HCVcc (0.01 MOI) for 4 h and harvested at 72 h p.i. for Western blot analysis ($n = 3$). (B) Membrane flotation assay in an iodixanol gradient in virus-infected cells. The gradient fractions were collected and an equal volume of each fraction was analyzed by Western blotting. Crude extract (CE) was the starting material used as a loading reference ($n = 2$).

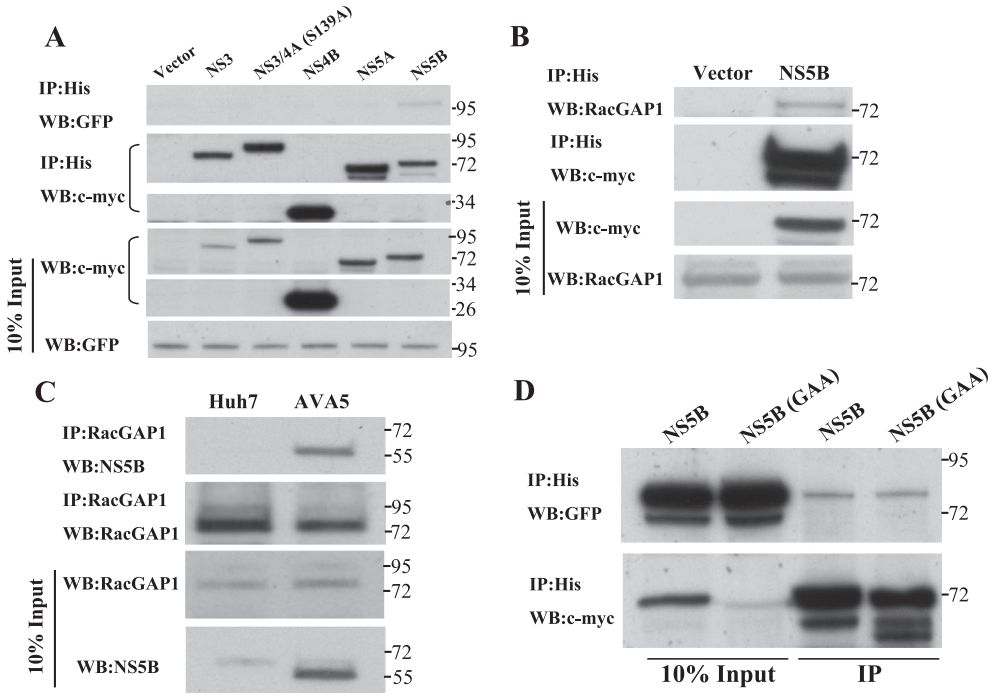

Fig. 2. Interaction of RacGAP1 with viral protein NS5B by co-IP. (A) The RacGAP1-GFP protein and various viral proteins (NS3, NS3/4A (S139A), NS4B, NS5A, and NS5B) with His-tag were exogenously expressed in HEK293T cells and coimmunoprecipitated with anti-His antibodies. Immunoprecipitates were subjected to Western blotting with an anti-GFP or an anti-c-myc antibody. Input, 10% of the cell lysate used in the co-IP reaction ($n = 3$). (B) Endogenous RacGAP1 interacts with exogenous expression of NS5B-His-tagged protein. The NS5B-His plasmid was transfected to HEK293T cells. At 24 h posttransfection, cells were harvested for co-IP using anti-His antibody ($n = 3$). (C) Endogenous RacGAP1 interacts with NS5B in replicon cells. Replicon cells (AVA5) and parental Huh7 cells were harvested for the co-IP assay. Immunoprecipitates with anti-RacGAP1 were subjected to Western blotting ($n = 3$). (D) The polymerase catalytic site of NS5B may not be involved in the RacGAP1–NS5B interaction. RacGAP1-GFP and wild-type or catalytic mutant NS5B with His-tag were cotransfected into HEK293T cells. After 24 h, cells were harvested for co-IP with an anti-His antibody and subjected to Western blotting using an anti-GFP or an anti-c-myc antibody ($n = 3$).

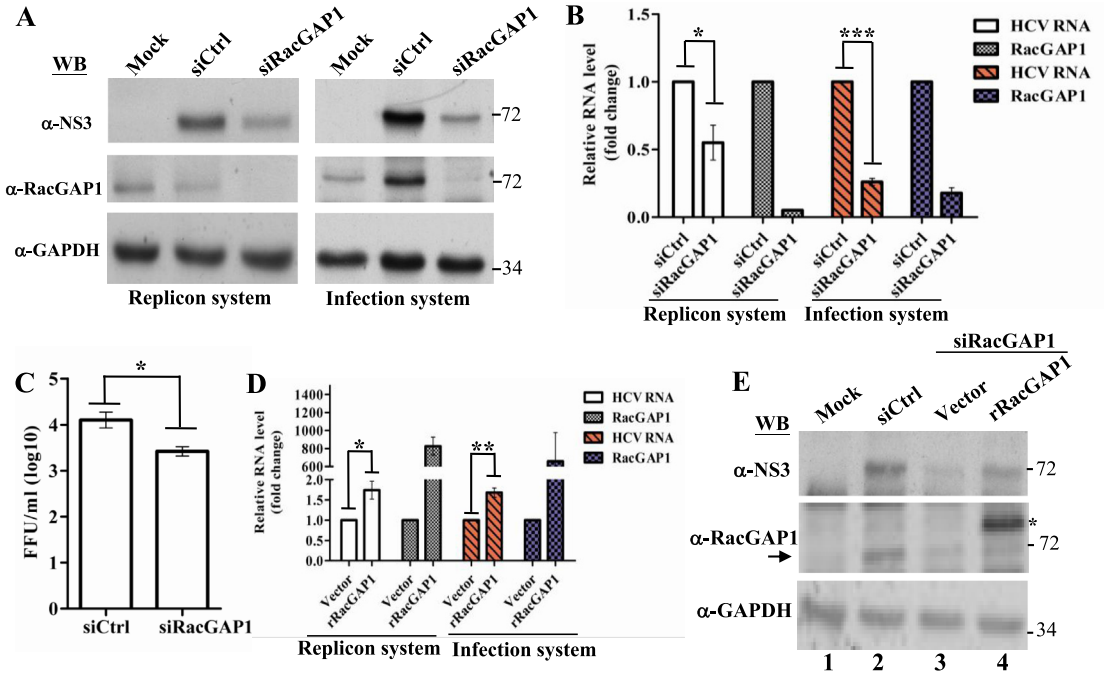

Fig. 3. Regulation of HCV replication by RacGAP1 in replicon-harbored AVA5 and virus-infected Huh7 cells. (A–C) AVA5 (replicon system) and Huh7 (infection system) cells were first transfected with a control siRNA (siCtrl) or a siRNA targeted to the *RacGAP1* (siRacGAP1) at a final concentration of 10 nM. AVA5 cells were harvested at 72 h posttransfection for analysis. At 24 h posttransfection, Huh7 cells were inoculated with HCVcc (0.01 MOI) for 4 h and harvested at 72 h p.i. for analysis. The cell lysates of AVA5 and Huh7 were subjected to Western blotting (A) and qPCR (B). The ratio of RNA to the internal control GAPDH in siRacGAP1-transfected cells was normalized to that of the siCtrl-transfected cells, which was arbitrarily set to 1. The effect of knockdown of *RacGAP1* was verified based on its mRNA levels. (C) The viral titer in the supernatant of siRNA-transfected Huh7 cells was determined and expressed as FFU/mL ($n = 3$). (D and E) Analysis of viral protein expression in rRacGAP1-transfected cells. Cells were transfected with siRacGAP1 for 24 h followed by transfection with vector or rRacGAP1. HCVcc was then inoculated into cells and the cells were harvested at 72 h p.i. for qPCR and Western blot analysis. The ratio of RNA to the internal control GAPDH in siRacGAP1-transfected cells was normalized to that of the vector-transfected cells (vector), which was arbitrarily set to 1. *rRacGAP1 and endogenous RacGAP1 (arrow). rRacGAP1 is larger because of the epitope tag. The data presented in this figure are expressed as the means of the results of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

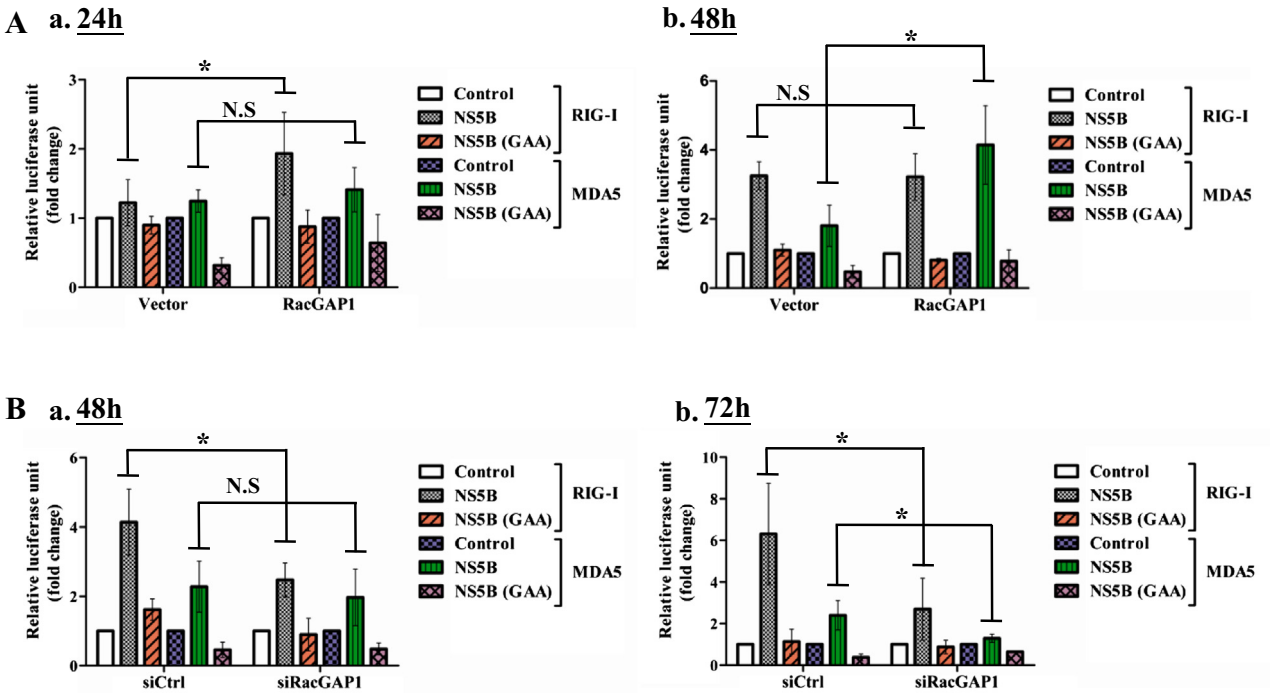

Fig. 4. The involvement of RacGAP1 in the polymerase activity of viral protein NS5B in mammalian cells. (A) Overexpression of RacGAP1 enhances NS5B polymerase activity in a RIG-I- and MDA5-dependent manner. HEK293T cells were cotransfected with NS5B, RIG-I or MDA5 and RacGAP1-His, and reporter plasmids. The transfection efficiency was normalized to RL-TK luciferase activity. After transfection, 24 h (in panel a) and 48 h (in panel b), cells were harvested to assay their luciferase activity. (B) NS5B polymerase activity was decreased in knockdown of RacGAP1 cells. HEK293T cells were transfected with siRacGAP1 or siCtrl. After 24 h after reseeding, transfected cells were cotransfected with RIG-I or MDA5, IFN-β luciferase and NS5B or NS5B(GAA). 48 h (panel a) and 72 h (panel b) after the second transfection, cells were harvested to assay their luciferase activity. The data are the means ± SD of the results of three independent experiments. $^{*}P < 0.05$.

activity [19]. Viral RNA polymerase generated 5′-ppp RNA or dsRNA during virus replication and it was recognized by pathogen recognition receptors (PRRs). Retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5) were PRRs for inducing type I interferons (IFNs; e.g. IFN-α and IFN-β) [19]. NS5B polymerase synthesizes nascent viral RNA that can bind to RIG-I or MDA5 innate immune receptors to activate luciferase reporter driven by an IFN-β promoter. We first cotransfected RacGAP1, RIG-I or MDA5, NS5B, and reporter plasmids into HEK293T cells and harvested the cells for a dual reporter assay at 24 and 48 h posttransfection. The results showed that overexpression of RacGAP1 enhanced NS5B polymerase activity in RIG-I or MDA5 receptors at 24 or 48 h (Fig. 4A). The activation of these receptors has a temporal effect, as RIG-I receptor could bind shorter RNA fragments for the activation of IFN-β promoter and thus was preferentially activated before MDA5 at 24 h (Fig. 4A, panel a). However, MDA5 receptors seemed to bind long RNA fragments in order to activate the IFN-β promoter at 48 h (Fig. 4A, panel b), demonstrating that this activation of RIG-I and MDA5 is RNA-length dependent [20]. The negative control NS5B(GAA) lacking the polymerase activity did not respond to the expression of RacGAP1, indicating the specificity of the cell-based assay. The effect of NS5B(GAA) might not result from reduced expression because the level of NS5B(GAA) was not altered in any sample (Supplemental Fig. S1). By contrast, NS5B polymerase activity was also affected by knockdown of RacGAP1 in HEK293T cells. The data showed IFN-β promoter luciferase activities were decreased in siRacGAP1 cells, which contain RIG-I or MDA5 receptors, at 48 and 72 h (Fig. 4B, panels a and b). Similarly, RIG-I receptors activate IFN-β promoter luciferase activities earlier than MDA5 receptors because these two receptors sense different lengths of viral RNA. These results indicated that the regulation of innate receptors by NS5B polymerase activity was dependent on RacGAP1.

4. Discussion

In the present study, we demonstrated that RacGAP1 was upregulated by virus infection and specifically interacted with NS5B protein in mammalian cells. Our data also indicated that depletion of RacGAP1 significantly decreased HCV RNA and protein level and restored HCV RNA and protein level by expressing siRNA-resistant RacGAP1 protein in siRacGAP1 cells. Mode-of-action examination of the role of RacGAP1 in NS5B polymerase activity using a cell-based assay for RNA synthesis revealed that RacGAP1 regulates NS5B polymerase activity. These results suggest a critical role for RacGAP1 in HCV replication.

4.1. GTPase-activating proteins involve HCV replication by different mechanisms

However, some reports indicated that different small GTPase-activating proteins are also involved in HCV replication. For instance, host RasGTPase-activating protein-binding protein 1 (G3BP1) engages viral replication through binding to NS5B protein [21]. HCV subverts TBC1D20, via binding to the N-terminal domain of NS5A, to mediate the endocytosis and trafficking of viral particles for viral replication [22]. In addition, HCV binds the major receptor CD81 to activate members of the RhoGTPase family, such as Rho, Rac1, and Cdc42, for HCV entry [23]. Therefore, RacGAP1 may activate Rac1 and Cdc42 for virus entry. However, in our study, we found knockdown of RacGAP1 negatively affected virus replication in a replicon system (Fig. 3). Thus, RacGAP1 regulates the HCV replication at replication steps, but not at virus entry or release steps.

4.2. Drug discovery for NS5B allosteric modulators by targeting RacGAP1

Many direct-acting antivirals have been developed to inhibit NS5B polymerase activity. For example, the newly licensed

sofosbuvir (brand name Sovaldi manufactured by GILEAD) is a uridine nucleotide analog of pyrimidine and targets the activity site of NS5B [24]. The crystal structure of the GAP domain of RacGAP1 has been released at a resolution of 1.9 Å [25]. The reports indicate that Arg385 is a key residue for GAP activity and plausibly presents an ideal target for drug discovery against host RacGAP1, which possibly binds to the allosteric site of NS5B. Allosteric modulators or regulators targeting viral or host proteins have become an emerging topic of interest because they offer high selectivity and competitive advantages over most classic drugs, which were designed to target orthosteric (active) sites [26]. This has been applied to rational design for HCV NS5B inhibitors [27]. Thus the inhibitors against RacGAP1 might be combined with allosteric drugs to achieve a more potent inhibition against NS5B to combat HCV.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.10.008.

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