Aggregation of a hepatitis C virus replicase module induced by ablation of p97/VCP

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Abstract

Hijacking host membranes to assemble a membrane-associated viral replicase is a hallmark of almost all positive-strand RNA viruses. However, how the virus co-opts host factors to facilitate this energy-unfavourable process is incompletely understood. In a previous study, using hepatitis C virus (HCV) as a model and employing affinity purification of the viral replicase, we identified a valosin-containing protein (p97/VCP), a member of the ATPases associated with diverse cellular activities (AAA+ ATPase family), as a viral replicase-associated host factor. It is required for viral replication, depending on its ATPase activity. In this study, we used VCP pharmacological inhibitors and short hairpin (sh) RNA-mediated knockdown to ablate VCP function and then dissected the roles of VCP in viral replicase assembly in an HCV subgenomic replicon system and a viral replicase assembly surrogate system. Ablation of VCP specifically resulted in the pronounced formation of an SDS-resistant aggregation of HCV NS5A and the reduction of hyperphosphorylation of NS5A. The NS5A dimerization domain was indispensable for aggregation and the NS5A disordered regions also contributed to a lesser extent. The reduction of the hyperphosphorylation of NS5A coincided with the aggregation of NS5A. We propose that HCV may co-opt VCP to disaggregate an aggregation-prone replicase module to facilitate its replicase assembly.

INTRODUCTION

Positive-strand RNA viruses, the largest class of viruses, include many medically and economically important pathogens. Assembling the viral replicase on modified host intracellular membranes to form a replication complex (RC) is a hallmark of almost all positive-strand RNA virus replication [1]. The formation of the RC represents a load-and-choke point of the viral life cycle [2] and may serve as an attractive anti-viral target. Targeting viral RC formation as a potent anti-viral therapy has been encouraged by the discovery of the hepatitis C virus (HCV) NS5A inhibitors that act on this non-enzymatic key ‘building block’ for viral replicase assembly [3–5].

HCV is a member of the Flaviviridae family. It chronically infects approximately 160 million people worldwide and causes hepatocellular carcinoma (HCC) in a significant proportion of the chronically infected individuals [6]. The 9.6kb positive-sense HCV RNA genome encodes a single polyprotein. The viral polypeptide is co- and post-translationally cleaved into at least 10 individual proteins by the host and viral proteinase to produce the individual structural and non-structural proteins in the following protein order: 5′-C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-3′ (for a review, see [7]). The expression of the HCV non-structural proteins in the endoplasmic reticulum (ER) induces the formation of a double-membrane vesicle (DMV), a structure that is believed to be the viral RC. The DMV is a protrusion from the ER membranes toward the cytosol [8]. The viral replicase is presumably enclosed in the DMV and viral replication takes place within it [8, 9]. It is believed that the HCV non-structural viral proteins, NS3, NS4A, NS4B, NS5A and NS5B, constitute the viral replicase. NS3 has a serine protease domain located in the N-terminal and RNA helicase/NTPase domain in the C-terminal region. NS4A is a protease cofactor for NS3. NS4B is a multi-spanning integral membrane protein and is implicated in DMV formation. NS5A is a multifunctional viral protein with no enzymatic activity. NS5B is an RNA-dependent-RNA polymerase (for a review, see [10]).


DMVs can be induced by overexpression of the HCV polyprotein encompassing NS3 to NS5B, as observed in virally infected cells. Further, NS5A, when overexpressed alone, can also induce DMVs, albeit less efficiently, which suggests that NS5A may play a central role in RC formation [8]. HCV NS5A consists of three domains. Domain I (DI), which is highly structured, is required for viral RNA replication and mediates NS5A dimerization [11, 12]. Domain II (DII) is intrinsically disordered and is involved in genome replication [13, 14]. Domain III (DIII) is natively unfolded and prone to partially folding into an α-helix [15], and is required for virion production [16–18].

Like other viruses that hijack host factors to facilitate the energy-unfavourable RC assembly process [19], HCV NS5A, together with other viral proteins, recruits host factors to locally remodel intracellular membranes for RC assembly [20]. Despite an accumulation of information about how HCV usurps components of the host cellular pathways for RC assembly, insights into how NS5A, together with other viral proteins, hijacks host factors for replicase assembly have only begun to emerge.

In a previous study, using HCV as a model and employing affinity purification of the viral replicase, we identified the viral replicase-associated host factors and uncovered an ATPases associated with diverse cellular activities (AAA +ATPase family member), a valosin-containing protein (VCP), as a pivotal host factor that is required for viral replication, depending on its ATPase activity [21]. VCP is recruited to and co-localizes with HCV non-structural protein clustering sites and inhibition of VCP alters HCV NS5A distribution, as judged by fluorescence microscopy in an HCV replicase assembly surrogate system [21], which suggests that VCP has a role in HCV replicase assembly. The AAA+ proteins use their ATPase activity to catalyse conformational changes in diverse substrate proteins [22]. The molecular mechanisms of VCP participating in HCV replicase assembly are still unknown.

In this study, we inhibited VCP function using VCP pharmacological inhibitors and short hairpin (sh) RNA-mediated knockdown, and then dissected the roles of VCP in viral replicase assembly in a subgenomic replicon system and a non-replicating replicase assembly surrogate system [8] through biochemical experiments. We found that inhibition of VCP specifically resulted in pronounced HCV NS5A aggregation and reduced its hyperphosphorylation.

**RESULTS**

**Pharmacological inhibitors of VCP reduce HCV replication in a subgenomic replicon cell**

We sought to use VCP inhibitors as tools to dissect the roles of VCP in viral replicase assembly. In a previous study, VCP inhibitor EerI treatment inhibited HCV infection dramatically [21]. First, we examined whether EerI reduces viral replication at a post-entry step as the knockdown of VCP does [21]. To bypass the viral entry step, we transfected Huh7.5 cells with RNAs of HCV Jc1G that contained an in-frame fused secreted Gaussia luciferase to monitor viral replication [23]. We then treated the cells with 4 µM EerI and monitored the luciferase reporter activity at various time points. EerI treatment dramatically reduced luciferase reporter activity at 1 and 2 d post-treatment (Fig. 1a), suggesting that EerI reduces viral replication at a post-entry step. We next extended our study to include other commercial VCP inhibitors to examine their effect on HCV infection. We examined NMS-873, the most potent VCP inhibitor to date [24], and DBeQ, a reversible inhibitor of VCP [25]. We infected Huh7.5 cells with Jc1G for 8 h and then treated the cells with various concentrations of NMS-873 and DBeQ before measuring viral replication 1 day later by determining the luciferase reporter activity. NMS-873 and DBeQ treatment both reduced HCV infection (Fig. 1b) without obvious reduction of cell viability (Fig. 1c). Notably, NMS-873 was more potent than DBeQ in inhibition of HCV replication (Fig. 1b), as NMS-873 effectively reduced viral replication at lower concentrations.

We next chose EerI and NMS-873 to examine whether these two VCP inhibitors could reduce the already established viral replication within an HCV subgenomic replicon cell bearing the self-replicating bicistronic viral RNA that encodes the viral polypeptide encompassing NS3 to NS5B [26]. We first took a genotype 2a subgenomic replicon sgJFH1 [21] and treated the cells with 4 µM EerI and 2 µM NMS-873 for up to 2 days. EerI and NMS-873 treatment significantly reduced viral replication in the replicon cells at 1 and 2 d post-treatment, as evidenced by the reduction of the viral NS3 protein levels (Fig. 1d). Then we examined a genotype 1b subgenomic replicon BB7 [27], and EerI and NMS-873 treatment also significantly reduced viral replication in BB7 replicon cells, as in sgJFH1 (Fig. 1e). Thus, pharmacological inhibition of VCP reduces HCV replication at a post-entry step and in HCV subgenomic replicon cells.

**Pharmacological inhibition of VCP results in pronounced HCV NS5A aggregation**

Given that VCP inhibitor treatment reduced the viral replication within a subgenomic replicon cell, we sought to monitor the effects of VCP inhibition on the viral replicase assembly in a replicon cell. In our previous study, VCP inhibitor treatment or knockdown of VCP resulted in aberrant distribution of HCV NS5A and coalescence of NS5A [21]. Some AAA+ proteins, such as Hsp104, participate in protein disaggregation [22]. These data prompted us to ask if HCV replicase components are prone to aggregation, and if VCP is involved in the disaggregation of these viral proteins.

We treated the subgenomic sgJFH1 replicon cells with VCP inhibitors at higher dosages for 4 h to block the VCP activity within a short period of time before significant reduction of the viral proteins due to the reduction of viral replication, and then analysed viral protein aggregation by semi-denaturing detergent agarose gel electrophoresis (SDD-AGE). This method is widely used in prion aggregation research, in
which cell lysates are mixed with 2% SDS containing loading buffer without boiling and then resolved in an agarose gel to visualize the SDS-resistant protein aggregation as high-molecular-weight smears due to its heterogeneity [28]. The sgJHF1 replicon cells were treated with 10 µM EerI, 5 µM NMS-873 and 6 µM DBeQ for 4 h, respectively. At these dosages, the EerI and NMS-873 only reduced the cell viability by less than 20% (Fig. 2a) and did not significantly reduce the viral protein expression level (Fig. 2b), which allows us to assess the viral protein aggregation. We observed that treatment with a higher concentration (10 µM) of DBeQ resulted in dramatic cytotoxicity. In the SDD-AGE experiments, we distinguished the SDS-resistant smears from the monomers by first identifying the monomer peaks and then quantifying the density of the areas according to the ‘monomer’ and the ‘smeared species’ (Fig. 2d, e). Compared with the DMSO treatment, treatment of all the VCP inhibitors resulted in smears of high-molecular-weight NS5A, which is an indication of SDS-resistant protein aggregation (Fig. 2c, top panel). Notably, NMS-873 treated samples exhibited more potent smearing (Fig. 2c, top panel), with a higher ratio of ‘smeared’ to ‘monomer’ (Fig. 2d and 2e Fig. 2d, e), whereas DBeQ-treated samples exhibited much less smearing compared with NMS-873, which was probably due to its lower potency at this dosage, as evidenced by its reduced anti-viral activity (Fig. 1b). After the lysates were boiled, the NS5A smears were reduced (Fig. 2c, top panel; Fig. 2d, e), indicating that the smears were sensitive to boiling and that smearing was unlikely due to the covalent modification of NS5A. It should be noted that in DMSO-treated cells there was a basal level of NS5A aggregation that was also sensitive to boiling (Fig. 2c, top panel; Fig. 2d, e). Another HCV nonstructural protein NS3 barely exhibited smearing (Fig. 2c, lower panel; Fig. 2d, e). Thus, HCV NS5A forms pronounced aggregates when VCP enzymatic activity is inhibited.
Knockdown of VCP results in HCV NS5A aggregation

We next examined whether the knockdown of VCP also results in HCV NS5A aggregation. We used a viral replicase assembly surrogate system wherein HCV NS3-5B polyprotein is expressed to mimic the induction of DMVs as in virally infected cells [8]. In this system, EerI and NMS-873 induced NS5A aggregation as in the replicon cells (data not shown). We knocked down VCP in Huh7 cells using lentiviruses expressing shRNA against VCP (shVCP) and then expressed HCV polyprotein NS3-5B in the shVCP cells as well as in the control cells (shIRR). We analysed the cell
lysates by SDS-PAGE (Fig. 3a) and SDD-AGE (Fig. 3b). To compensate the variation of HCV protein expression levels due to the difference in the transfection efficiency in the shIRR cells and the shVCP cells as evidenced by the difference of the expression levels of the co-transfected GFP (Fig. 3a), we loaded two-fold of lysates from the shVCP cells compared to those from the shIRR cells for SDD-AGE assay (Fig. 3b, top panel). There were pronounced NS5A smears in the shVCP cells compared with the shIRR cells (Fig. 3c, d), whereas no obvious NS3 smears were observed in the same samples (Fig. 3b, middle panel; Fig. 3c, d). In addition, mitochondrial antiviral signalling (MAVS), a cellular protein that is capable of forming aggregation under specific anti-viral stimulation [29], did not form obvious aggregates in the shVCP cells (Fig. 3b, bottom panel; Fig. 3c, d). Thus, like pharmacological inhibition of VCP, knockdown of VCP also specifically results in pronounced HCV NS5A aggregation.

**HCV NS5A dimerization domain is indispensable for aggregation and the NS5A disordered regions contribute to aggregation**

Given that inhibition of VCP specifically results in NS5A, but not NS3, aggregation, we considered whether other HCV non-structural proteins, such as NS5A, form aggregation upon treatment with VCP inhibitors. We expressed N-terminally FLAG-tagged HCV NS3/4A, NS4B, NS5A and NS5B from BB7 (genotype 1b) subgenomic replicon constructs [27] in HEK293T cells (Fig. 4a) and then analysed protein aggregation by SDD-AGE after EerI (10 μM) treatment for 4 h. Upon EerI treatment, only FLAG-tagged NS5A exhibited obvious smearing (Fig. 4b).

We then examined the individual NS5A domains for their roles in the formation of aggregation. HCV NS5A consists of three domains. Fig. 4c shows a schematic of the tested constructs. We expressed the FLAG-tagged DI, domain I plus domain II (DIDII) and full-length (FL) of NS5A from an HCV genotype 2a subgenomic replicon construct sgJFH1 [21] (Fig. 4c, d). Upon EerI treatment, compared with the DMSO-treated counterparts, the FL exhibited obvious smearing (Fig. 4e, diamonds), with a ratio of ‘smeread’ to ‘monomer’ of 3.70 (Fig. 4f). In contrast, although DI alone and DIDII also exhibited smearing (Fig. 4e), they smeared much less than the FL, with lower ratios of ‘smeread’ to ‘monomer’ (Fig. 4f), suggesting the DI alone is capable of smearing, but with much less efficiency.

We then deleted the DI and DII in the context of NS3-5B while retaining the amphipathic helix (AH) that mediates membrane association of NS5A [30] (Fig. 4c, g), and monitored the NS5A variants’ ability to smear by SDD-AGE. Compared with full-length NS5A (WT), the deletion of DI (NS5A.dDI) dramatically reduced the basal level of NS5A aggregation and EerI-induced aggregation (Fig. 5g, i). Deletion of DII also slightly reduced the basal level of NS5A aggregation and the EerI-induced aggregation of the NS5A (Fig. 5h, i), suggesting that DI and DII both contribute to NS5A aggregation. Taking these data together, the NS5A dimerization domain is indispensable for aggregation, while the whole region of NS5A, including the disordered regions, is needed for efficient aggregation.

**VCP inhibition results in reduction of HCV NS5A hyperphosphorylation**

To examine the effect of NS5A aggregation on viral replication, we treated the sgJFH1 replicon cells with EerI at 4 μM and monitored the viral protein expression at various times post-treatment. We observed that at as early as 10 h post-treatment, EerI treatment reduced the NS5A species with a higher molecular mass without affecting the NS5A species with a lower molecular mass (Fig. 5a). NS5A is extensively phosphorylated and there are two species that apparently have different molecular weights when resolved by SDS-PAGE. The species with a higher molecular mass is referred to as the hyperphosphorylated form, whereas the species with a lower molecular mass is referred as the basal phosphorylated form [31]. Thus, EerI treatment reduced the hyperphosphorylation of NS5A. We also examined the hyperphosphorylation of NS5A in the NS5B expression system. We treated the HEK293T cells expressing the NS3-5B with EerI. Treatment with EerI at 10 μM for 8 h, which only slightly reduced cell viability (Fig. 5b), also significantly reduced the hyperphosphorylated form of NS5A without affecting the basal phosphorylated level of NS5A (Fig. 5c). We observed a similar effect from another VCP inhibitor, NMS-873, in the 3B expression system (data not shown). We also examined whether knockdown of VCP reduced the hyperphosphorylation of NS5A. We expressed NS3-5B in VCP-knockdown cells. There was a significant reduction of the NS5A hyperphosphorylation level in the VCP-knockdown cells (shVCP) compared with that in the control cells (shIRR) (Fig. 5d).

As VCP inhibitors induce NS5A aggregation as early as 4 h post-treatment (Fig. 2c), we examined the kinetics of the NS5A aggregation and the reduction of its hyperphosphorylation after VCP inhibitor treatment more carefully. We treated the sgJFH1 cells with EerI and NMS-873 and harvested the cells at various time points after treatment. We resolved the cell lysates by SDS-PAGE to examine the hyperphosphorylation of NS5A, and by SDD-AGE to examine the aggregation of NS5A. EerI and NMS-873 treatment induced obvious NS5A smearing as early as 2 h post-treatment compared with the DMSO-treated cells. Meanwhile, in the EerI- and NMS-873-treated cells, the NS5A hyperphosphorylation levels were reduced at 2 h post-treatment compared with that at 1 h post-treatment and dramatically reduced at 4 h post-treatment (Fig. 5e). These data indicate that the reduction of NS5A hyperphosphorylation coincides with NS5A aggregation (see the Discussion section).

**DISCUSSION**

In this study, we used HCV as a model to dissect the mechanism of a host factor VCP that we identified as an active HCV replication modulator [21] in viral replicase assembly. We
inhibited VCP function using VCP inhibitors as well as shRNA-mediated knockdown and then dissected the biochemical phenotypes of HCV replication. We found that inhibition of VCP specifically resulted in HCV NS5A aggregation (Figs 2 and 3). Of the VCP inhibitors, NMS-873 exhibited the most potent activity in inducing NS5A aggregation (Fig. 2) as well as more potency in the inhibition of viral replication (Fig. 1), which was probably due to the greater potency of this inhibitor compared to the others [24].

Protein aggregation may result from misfolding, assembly defects of the protein complex, irreversible modification-mediated misfolding and aging [32]. The formation of aggregates may also have different degrees of structure, including mostly unstructured, disordered aggregates and highly structured β-sheet-rich amyloid fibrils [32]. HCV NS5A DI is indispensable for aggregation, as deletion of DI nearly abolishes EerI-induced aggregates (Fig. 4h, i). Protein purification of NS5A DI from bacteria reveals the dimeric

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**Fig. 3.** Knockdown of VCP results in pronounced aggregation of HCV NS5A. (a) Huh7 cells were transduced with lentiviruses expressing shRNA against an irrelevant target (IRR) or VCP. After splitting, the cells were reseeded and mock transfected (mock) or co-transfected with plasmids expressing HCV NS3-5B (3-5B) and GFP. Cells were harvested at 2 d post-transfection and the cell lysates were analysed by Western blotting. Migration of size standards (in kilodaltons) is indicated to the left of the gels. (b) Alternatively, the same lysates were mixed with SDD-AGE loading buffer and applied to SDD-AGE analysis. The blots were probed (immunoblot, IB) with indicated antibodies. (c) Relative density profile of each lane in the blot from (b). Representative data from one lane from the duplicated samples are shown. (d) The density of the areas corresponding to the ‘monomer’ bands and the smeared species (‘smeared’) were quantified and the ratio of the ‘monomer’ bands to the smeared species (‘smeared’) was calculated and plotted. Data from the two independent experiments are shown. Mean values ± standard deviations are shown (n=5). Statistical analysis was performed between the shIRR groups and the shVCP groups (***P<0.001, two-tailed, unpaired t-test). The asterisks in (b) and (c) indicate non-specific bands. Samples from two duplicated wells are labelled identically. The high-molecular-weight smears are labelled as ‘smearing’ in (b). Similar results were observed in multiple independent experiments.
Fig. 4. The roles of HCV NS5A domains in aggregation. (a, b) HEK293T cells were transfected with plasmids expressing FLAG-tagged HCV non-structural proteins. One day later, the cells were treated with EerI (10 µM) for 4 h. (a) The cell lysates were analysed by Western blotting. Asterisks indicate the HCV non-structural proteins. (b) Alternatively, the cell lysates were subjected to SDD-AGE and the blot was probed (immunoblot, IB) with indicated antibodies. The diamonds indicate the aggregated species. Similar results were obtained from another independent experiment. (c) Schematic of NS5A mutants. Each domain and the first and last amino acid numbers are indicated. AH, amphipathic helix. (d–f) FLAG-NS5A-FL, FLAG-NS5A-DI and FLAG-NS5A-DIDII were expressed in HEK293T cells, and then the cells were treated with EerI (10 µM) for 4 h and harvested. (d) The cell lysates were analysed by Western blotting. Asterisks indicate FLAG-NS5A mutants. (e) Alternatively, the cell lysates were subjected to SDD-AGE analysis. The diamonds indicate the aggregated species. Similar results were obtained in multiple independent experiments. (f) Relative density profile of NS5A in each lane in the blot from (e) is shown. The density of the areas corresponding to the ‘monomer’ bands and the smeared species (‘smeared’) was quantified and the ratio of the ‘monomer’ bands to the smeared species (‘smeared’) was calculated. (g–i) HEK293T cells expressing the indicated NS3-5B variants were treated with EerI (10 µM) for 4 h. (g) The cell lysates were analysed by Western blotting. (h) Alternatively, the cell lysates were analysed by SDD-AGE. (i) Relative density profile of NS5A in each lane in the blot from (h) is shown. The density of the areas corresponding to the ‘monomer’ bands and the smeared species (‘smeared’) was quantified and ratio of the ‘monomer’ bands to the smeared species (‘smeared’) was calculated. Representative pictures of multiple independent experiments with similar results are shown. The values to the left of the blots in (a), (d) and (g) are the molecular sizes in kilodaltons. The high-molecular-weight smears are labelled as ‘smeared’ in (h).
Fig. 5. VCP inhibition results in the reduction of HCV NS5A hyperphosphorylation. (a) HCV sgJFH1 replicon cells were treated with Eerl (4 µM) (+) or DMSO (–) and harvested at the indicated time points. The cell lysates were subjected to Western blotting analysis using the indicated antibodies. (b) Cell viability after VCP inhibitor treatment. HEK293T cells were treated with 10 µM Eerl for 8 h and then cell viability was assessed. Mean values ± standard deviations are shown (n=3). (c) HEK293T cells were co-transfected with plasmids expressing HCV NS3-5B and GFP (for monitoring transfection efficiency) were treated with Eerl (10 µM) for 8 h and harvested for Western blotting analysis. The protein abundance of NS5A was quantified and the ratio of the hyperphosphorylated form of NS5A (hyper) to the basal phosphorylated form of NS5A (basal) is indicated at the bottom of the blot. Mean values ± standard deviations are shown (n=3). Statistical analysis was performed between the treated groups (+) and the untreated groups (–) as indicated. (***P<0.001, two-tailed, unpaired t-test). (a, b) Representative pictures of multiple independent experiments with similar results are shown. (d) Huh7 cells were transduced with lentiviruses expressing shRNA against an irrelevant target (IRR) or VCP. After splitting, the cells were reseeded and co-transfected with plasmids expressing HCV NS3-5B and GFP. Cells were harvested at 36 h post-transfection and the
cell lysates were analysed by Western blotting. Representative data from two adjacent wells out of three independent experiments are shown. The protein abundance of NS5A was quantified and the ratio of the hyperphosphorylated form of NS5A (hyper) to the basal phosphorylated form of NS5A (basal) is indicated at the bottom of the blot. Mean values ± standard deviations from three independent experiments are shown (n=7). Statistical analysis was performed between the shIRR groups (+) and the shVCP groups (−) as indicated. (**P<0.001, two-tailed, unpaired t-test). (e) HCV sgJFH1 replicon cells were treated with DMSO, EerI (10 µM), NMS-873 (5 µM). The cells were harvested at the indicated time points and the cell lysates were analysed by SDS-PAGE or SDD-AGE and the blots were probed (immunoblot, IB) with the indicated antibodies. The protein abundance of NS5A was quantified and the ratio of the hyperphosphorylated form of NS5A (hyper) to the basal phosphorylated form of NS5A (basal) is indicated at the bottom of the blot. The values to the left of the gels in (a), (c) and (d) are the molecular sizes in kilodaltons. The hyperphosphorylated form of NS5A is labelled as ‘hyper-pi-NS5A’ in all the panels. The high-molecular-weight smears are labelled as ‘smearing’ in (e).

Although limited evidence exists regarding NS5A homodimerization in viral replicating cells, the heterogeneous oligomeric state of the NS5A DI in solution has been observed [11]. Pharmacological inhibitor studies imply the existence of polymers of NS5A within the cells [33]. We proposed that the aggregation of NS5A is most likely initiated by DI-mediated polymer formation and might ensue from the disordered region DII– or DIDII-mediated cross-linking, as DI alone and DIDII could not smear as efficiently as full-length NS5A (Fig. 4e), and the deletion of DII slightly impaired NS5A aggregation (Fig. 4h). Protein aggregation could also be mediated by protein modification. VCP inhibitor-induced NS5A aggregation was sensitive to boiling, suggesting that aggregation is unlikely to be mediated by covalent protein modification. However, we cannot rule out the possibility of protein modifications contributing to NS5A aggregation, and being indicated by the presence of residual smears in the SDD-AGE blots when the samples were boiled (Fig. 2b). It is also possible that protein modification could not be detected by SDD-AGE due to the limitations of this method.

VCP is a candidate for exerting disaggregation activity in animal cells. Loss-of-function mutations of VCP are linked to an accumulation of protein aggregates [34] and VCP associates with polyubiquitylated proteins and is involved in their subsequent solubilization [35]. These data suggest a potential role of VCP in disaggregating protein aggregates. We noted that there are basal levels of NS5A aggregation under normal conditions, as judged by SDD-AGE (Figs 2c, 3b and 4h). We propose that the basal level of NS5A aggregation is also derived from the NS5A polymer, and under normal conditions, VCP disaggregates the aggregation to prevent the NS5A polymer from aberrant aggregation, while VCP inhibition results in pronounced aberrant NS5A aggregation (Fig. 6).

Aberrant NS5A aggregation coincided with reduction of its hyperphosphorylation status (Fig. 5). Phosphorylation

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**Fig. 6.** Proposed model of VCP-mediated HCV NS5A disaggregation. (i) Under normal condition, NS5A forms polymers through dimerization of domain I (DI); VCP, cooperating with its cofactor(s), disaggregates HCV NS5A to prevent the NS5A polymers from aberrant aggregation to facilitate replicase assembly. (ii) Inhibition of VCP ATPase activity results in NS5A aggregation. (iii) Aggregation of NS5A may affect NS5A-mediated protein–protein interactions and reduce NS5A hyperphosphorylation, which in turn will impair viral replicase assembly.
of NS5A at certain sites plays important roles in the viral life cycle and in viral replication complex formation [31, 36]. Numerous phosphorylation sites and the kinases that participate in NS5A phosphorylation and hyperphosphorylation have been identified [31]. Hyperphosphorylation of NS5A may regulate viral protein interaction with host factors to regulate viral replication [37]. However, the mechanism of hyperphosphorylation regulation remains elusive. Adaptive mutation S2204I abolishes NS5A hyperphosphorylation in HCV con1b subgenomic replicon cells [38] and hyperphosphorylation of NS5A only occurs when a polypeptide encompassing NS3-5A polypeptide is expressed, suggesting that the hyperphosphorylation of NS5A may need protein–protein interactions among the viral proteins [39]. In this study, we found that VCP inhibition through both pharmacological inhibitors and shRNA-mediated knockdown resulted in the reduction of the hyperphosphorylation of NS5A (Fig. 5). The formation of NS5A aggregation and the reduction of NS5A hyperphosphorylation are concurrent in the sgJFH1 cells after VCP inhibitor treatment (Fig. 5d). Given that the NS5A from con1b subgenomic replicon cells we used in this study has adaptive mutation S2204I, which abolishes the hyperphosphorylation of NS5A [38] (Fig. 4A), and that this NS5A variant is capable of forming aggregation (Fig. 4b), VCP inhibition probably first results in aberrant NS5A aggregation, and this in turn, by interfering with the accessibility of NS5A, affects NS5A hyperphosphorylation and protein–protein interaction and finally abolishes viral replicase assembly (Fig. 6).

VCP is involved in poliovirus replication [40] and colocalizes with poliovirus non-structural proteins in virally infected cells [40]. Very recently, a human genome-wide RNAi screening also identified VCP as being involved in enterovirus 71 (EV71) replication [41]. It has been reported that VPS4, an AAA+ ATPase family member, participates in brome mosaic virus (BMV) and tomato bushy stunt virus (TBSV) replication complex formation by cooperating with Endosomal Sorting Complexes Required for Transport (ESCRT) complexes [42, 43]. It will be intriguing to examine whether VPS4 acts similarly to VCP to prevent viral replicase modules from aggregating.

VCP barely exhibits substrate specificity. It employs a large number of cofactors for temporal and spatial regulation [44]. VCP may co-opt NS5A-interacting protein(s) to disaggregate NS5A (Fig. 6). In future studies, it will be necessary to identify the VCP cofactor(s) involved in HCV replication.

**METHODS**

**Inhibitors**

EerI was purchased from Tocris Bioscience (cat. no. 3922) and dissolved in DMSO to make 10 mM stock. NMS-873 was purchased from Selleckchem (cat. no. S7285) and dissolved in DMSO to make 5 mM stock. DBeQ was purchased from Selleckchem (cat. no. S7199) and dissolved in DMSO to make 10 mM stock.

**Plasmids**

Plasmid phCMV-NS5A was generated by PCR amplification of the NS5A region from a HCV infectious clone Jc1G (genotype 2a) [23] plasmid and ligation into the BglII/EcoRI sites in phCMV (Genelantis). The plasmids phCMV-3-5B, dDI and phCMV-3-5B.dDII with deleted NS5A domains I and II were generated by fusing PCR-mediated mutagenesis with phCMV-3-5B [21] as a backbone. HCV subgenomic replicon constructs sGFH1 [21] and the BB7 (genotype 1b) harbouring the S2204I adaptive mutation have been described previously [27]. To generate the plasmids pCDNA3×FLAG-NS3/4A, pCDNA3×FLAG-NS4B, pCDNA3×FLAG-NS5A and pCDNA3×FLAG-NS5B were generated by subcloning the viral protein regions from BB7 replicon plasmid and have been described previously [27]. To generate the plasmids pCDNA3×Flag-NS5A-FL, pCDNA3×Flag-NS5A-DI and pCDNA3×Flag-NS5A-DI(DII), the DNA regions encompassing the NS5A (FL), NS5A domain I (DI), and the NS5A domain I and II (DII) regions were PCR-amplified using Jc1G [23] as templates and digested by EcoRI/BamHI and then ligated into the similarly digested pCDNA3×FLAG plasmids [27]. The lentiviral-based shRNA plasmids targeting the VCP coding region and irrelevant target (IRR) have been described previously [21]. All the constructs were proofed by DNA sequencing.

**Cells**

Human embryonic kidney cell line HEK293T (Cell Bank of the Chinese Academy of Sciences, Shanghai, China, http://www.cellbank.org.cn) was routinely maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Biological Industries, cat. no. 04-001-1). Human hepatoma cell line Huh7 (Cell Bank of the Chinese Academy of Sciences, Shanghai, China, http://www.cellbank.org.cn) and Huh7.5 (kindly provided by Charles Rice) were maintained in a similar medium supplemented with 25 mM HEPES (Gibco) and non-essential amino acid (Gibco). HCV subgenomic replicon cells were generated by electroporation of in vitro-transcribed RNAs into Huh7.5 cells and selected in conditioned medium supplemented with 5 µg blastocidin ml⁻¹ (Invitrogen). The surviving cells were pooled and maintained with the conditioned medium supplemented with 0.5 µg blastocidin ml⁻¹.

The cell viability was determined by the Cell Counting kit 8 (CCK-8) (Dojinodo Laboratories, CK04) according to the manufacturer’s protocol.

**Antibodies**

Anti-β-actin antibody was purchased from Sigma (A1978) and used in Western blotting at 1:5000 dilution. Anti-NS5A (9E10) (kindly provided by Charles Rice) was used at 1:2000 dilution in Western blotting. Anti-FLAG antibody (Sigma; F1804) was used in the Western blotting analysis at 1:10 000 dilution. Anti-GFP antibody (Santa Cruz; sc-9996) was used in the Western blotting analysis at 1:2000 dilution. Anti-HCV NS3 antibody (Virogen; 217-A) was used in
SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

Cells were lysed directly with 2× SDS loading buffer [100 mM Tris-Cl (pH 6.8), 4% (w/v) SDS (sodium dodecyl sulfate), 0.2% (w/v) bromophenol blue, 20% (v/v) glycerol, 2-Mercaptoethanol 10%] or cell lysates were mixed with an equal volume of 2× SDS loading buffer. The lysates were then boiled for 5 min. Boiled proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane (GE Healthcare Life Sciences, cat. no. 10401396). The membrane was incubated in blocking buffer (PBS, 0.05% Tween 20, 5% dried milk) and then incubated with primary antibody diluted in blocking buffer. The membrane was washed three times in PBS supplemented with 0.05% Tween 20 and incubated with HRP-conjugated secondary antibody. After three washes, the membrane was visualized by Western Lightning Plus-ECL substrate (PerkinElmer, cat. no. NEL10500). The blots were reprobed by another antibody after being stripped with restore stripping buffer (Thermo, 21059), if necessary. Protein bands were quantified by densitometry with ImageJ, if necessary. Briefly, the relative density of the contents of a rectangle over each lane was plotted. The peaks in the profile plot correspond to the protein bands. For quantification in the SDD-AGE experiment, the monomer peaks were first identified to distinguish the smeared species and the density of the selected areas corresponding to the monomers and the smeared species was quantified.

Statistical analysis

Statistical analysis was performed with GraphPad Prism 6 software. The specific tests are described in the figure legends.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

References


