surface of lipid droplet as well as apolipoprotein AII *in vitro* [8,9]. Interaction of core with lipid droplet was molecularly dissected using core mutants, and as a result the responsive region as well as structure of core was revealed [10,11••,12•]. Moreover, it has been shown that the core has a negative effect on the microsomal triglyceride transfer protein (MTP) activity [13]. Besides the HCV core, the presence of subgenomic replicon that lacks core expression has also been found to disturb MTP activity in the hepatocytes [14]. In this case, NS5A has been observed to interfere with MTP function [14]. As MTP is an essential chaperone for the assembly of very low-density lipoproteins (VLDL), which transfers triglyceride, phospholipids, and cholesterol from the hepatocytes, regulation of MTP is closely related to the accumulation of lipids in the hepatocytes. In fact, MTP deficiency results in large fat droplets in the hepatocytes and scattered accumulation of inflammatory cells [15]. HCV type-3 infected patients show reduced MTP activity and mRNA levels as well as high degree of steatosis [16]. So far, it is not clear whether MTP activity is directly regulated by HCV. Rather, MTP is likely to be controlled by activators (HNF1α, LRH-1, and HNF4α) and repressors (insulin and SREBP), some of which are modulated by HCV [14,17]. Reduced activity of MTP results in decreased secretion of VLDL, which seemingly leads to lipid accumulation. However, it is noteworthy that MTP activity is not completely suppressed by HCV. As will be described later, MTP activity is required for virus egress from infected cells. Collectively, these results suggest that HCV proteins alter lipid metabolism by activating lipid synthesis and modulating secretion of lipoprotein through interaction with cellular proteins, which results in accumulation and storage of lipids in cells expressing those viral proteins. However, the role of these viral proteins in modulating lipid metabolism related to HCV proliferation remains unknown until an *in vitro* HCV infection system was established.

Association of HCV with lipoproteins in the blood of HCV-infected individuals

HCV particles present in circulating serum show properties of heterogenous and lower density than those expected from its putative viral structure and can be captured by antilipoprotein antibody, which partly reflects the binding of a fraction of the virions to VLDL or low-density lipoproteins (LDLs) [18–22]. These low-density HCV-RNA-containing particles (called as lipo-viro-particle: LVP) contain core and apolipoprotein B (ApoB), and are rich in triglyceride with a diameter of >100 nm [19].

Although the physiological meaning of HCV-containing LVP in the circulating blood is not clear, it seems that HCV has either a high affinity to lipoproteins or is assembled with lipoproteins through the mechanism of lipoprotein synthesis. LVP-like structure may self-protect from the host immunological surveillance and/or increase interaction with lipoprotein receptor(s) which may act as a HCV receptor [20].

Importance of the lipid droplet in establishing a microenvironment for HCV assembly

The roles of modulation of lipid metabolism and association of HCV proteins with host factors involved in lipid metabolism for life cycle of HCV were not clear until an infectious *in vitro* HCV replication system was established [23–25].

Subcellular localization of HCV proteins in cells with replication of the infectious HCV RNA revealed association of core with ER membrane as well as lipid droplet. This finding is consistent with the previous reports that analyzed cells expressing on the core complex [11]. NS proteins, such as NS5A and NS5B, were found to be distributed around the core-coated lipid droplet as well as ER membrane. HCV envelope protein, E2, was detectable in the lipid droplet-enriched fraction isolated by density gradient centrifugation. Importantly, the lipid droplet present in the cytoplasm, detected by BODIPY 493/503 staining, was observed to be enriched in HCV genome replicating cells in a core-dependent manner. Furthermore, it was noted that the cells harboring HCV genome, which lacked core-coding region, did not accumulate the lipid droplet, indicating the importance of core function to activate cellular lipid metabolism, as suggested previously [17]. Moreover, the HCV genome encoding a mutant core, unable to associate with the lipid droplet, failed to produce virus. This suggests the importance of the association of core with the lipid droplet for virus assembly and release. The core-coated lipid droplet is surrounded by membranous components that are rich in nonstructural HCV proteins that constitute HCV replication complex. This spatial microenvironment seems to be important not only for virus assembly, but also for infectious virus production, because the HCV genomes carrying NS5A point mutations, which are not associated with the core-coated lipid droplet, reduce or inhibit the production of infectious virus despite minimal interference with the replication of the genome [11,26•]. Moreover, other NS proteins do not associate with the lipid droplet in cells bearing the NS5A-mutated HCV replicon. Thus, NS5A may have a crucial role in recruiting other NS proteins around the core-coated lipid droplet. However, how the HCV assembly takes place from such a microenvironment is still unclear.

Recent data have shown the importance of NS2 for viral morphogenesis. Molecular interactions between NS2 and other HCV proteins, such as envelope proteins (E1/E2), p7 and NS3 have been demonstrated, which suggest bridging between structural and nonstructural viral proteins and involvement in the assembly process [27–29]. NS2 accumulates in the ER-derived membranous

structures and co-localizes with the viral envelope glycoproteins and viral components of the replication complex at close proximity to the HCV core protein and lipid droplets.

Using HCV subgenome replicon cells, we have previously shown that very few HCV nonstructural protein complexes embedded in the membranous structure (referred as 'replication complex'; areas stained with dark blue color (Figure 2) are sufficient to synthesize HCV RNA present in the HCV replicon cells [30]. The level of the HCV proteins in the 'replication complex,' estimated by western blot analysis, has been found to be lower than one-tenth of the total viral proteins in the cells. The majority of the other HCV nonstructural proteins have been observed to remain associated with the ER membrane, possibly as a complex that is not protected by membranous components. As NS2 is dispensable for HCV RNA synthesis, it is likely that NS2 is not necessarily associated with the 'replication complex.' However, NS2 that is associated with envelopes and p7 has also been found to be associated with NS3 in the nonstructural protein complex that is not protected by the membrane. In this study, we refer to this complex as the 'HCV protein complex.'

Involvement of lipoproteins in HCV assembly and its infectivity

Lipoprotein is a biochemical particle consisting of apolipoproteins, triglyceride, cholesterol, and phospholipids, and transfers lipids through the bloodstream to the tissues. Liver, an endogenous source of lipoproteins, synthesizes VLDL using MTP. Lipoprotein lipase (LPL) hydrolyzes VLDL to supply lipids to the tissues, and converts VLDL into intermediate density lipoprotein (IDL). Hepatic triglyceride lipase (HTGL) hydrolyzes IDL to LDL.

ApoB is a main component of VLDL produced in the hepatocytes. Several groups have asserted the requirement of ApoB for HCV production [31•,32•,33•]. Huang *et al.* [31•] demonstrated that ApoB reduction by MTP inhibitor or a siRNA against ApoB led to decreased infectious HCV production. Gastaminza *et al.* [32•] reported similar results. Icard *et al.* [33•] showed that the release of HCV envelopes depended on the secretion of ApoB. Another study [34•] showed negative data for the requirement of ApoB, in which the authors described that apolipoprotein E (ApoE) rather than ApoB is important for HCV production and infectivity. Thus, ApoB requirement for virus production is still unclear.

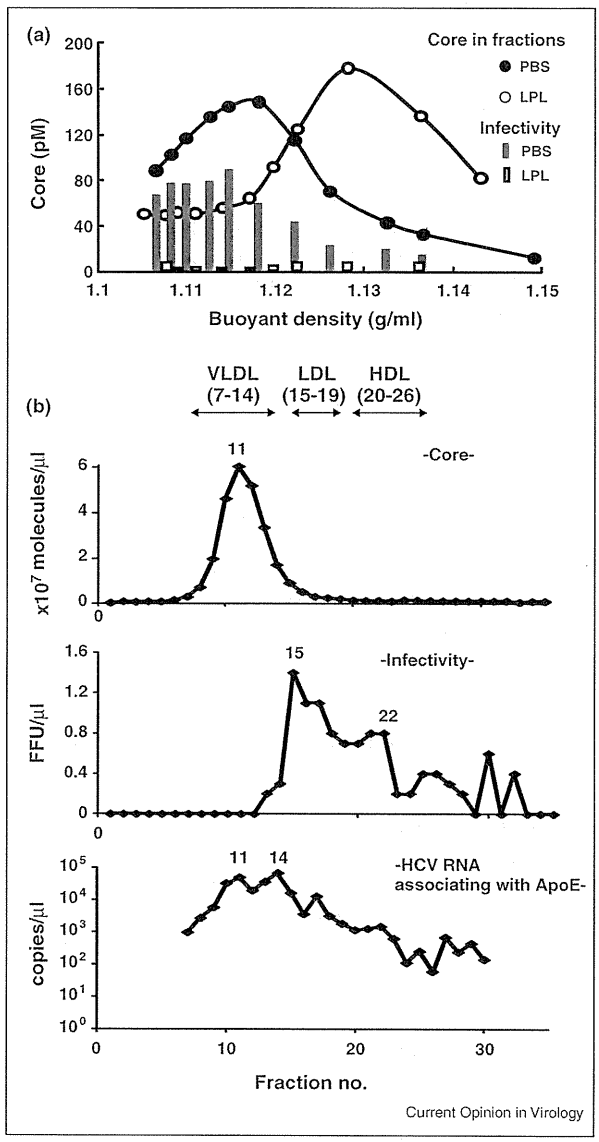
ApoE, a component of lipoproteins, has attracted much attention as an important factor for HCV infectivity, because it has been shown that knockdown of ApoE could reduce HCV infectivity at a higher degree than the knockdown of ApoB, apolipoprotein A1 (ApoA1), and ApoC1 ([35••,36••], and data not shown). ApoE has three major isoforms (ApoE2, ApoE3, and ApoE4) differing by amino acid substitution at one or two sites (residues 130 and 176), which have different effects on lipid and neuronal homeostasis. ApoE3 is the most common isoform with no report of association with disease. ApoE2 has lower affinity for the low-density lipoprotein receptor (LDLR) and is a major risk factor for type III hyperlipoproteinemia, while ApoE4 is the major risk factor for Alzheimer's disease. Since ApoE not only functions as a ligand for LDLR and scavenger receptor class B type 1 (SR-B1), but also is associated with HCV, the effect of ApoE associated with HCV on the interaction of these lipoprotein receptors was examined by measuring the infectivity in naïve cells. Rescue of ApoE with ApoE3 ectopic expression in ApoE-knockdown cells recovered HCV infectivity, whereas ectopic ApoE2 expression did not. This result strongly indicates that ApoE, in particular some isoforms of ApoE, is necessary for HCV infectivity. ApoA1, a component of chylomicron, LDL, and high-density lipoprotein (HDL), is also important for HCV production, but is found to be less effective than ApoE ([37] and unpublished data). Taken together, HCV is seemingly produced as a hybrid of HCV and lipoproteins. While VLDL is a main lipoprotein secreted from normal hepatocytes, HCV may modulate the production of lipoproteins in the host cells to make the original lipoproteins more effective/appropriate for viral replication and persistence.

To further strengthen the involvement of lipoproteins in HCV infectivity, we evaluated HCV as a substrate of LPL [38•]. LPL treatment significantly suppressed HCV infectivity and increased the buoyant density of HCV (Figure 1a; modified from Ref. [38•]). This indicates that LPL hydrolyzes lipoproteins fused with HCV to result in decreased HCV infectivity. In accordance with the change in the buoyant density, the amount of ApoE associated with HCV decreased. As the HCV-bearing supernatant used in this study contained endogenous HTGL from the hepatocytes, these effects might result from HTGL in addition to LPL. Thus, we consider that detachment of ApoE from the HCV particle led to reduced HCV entry.

Relation between HCV particle size and infectivity

Ultracentrifugation of HCV in a gradient of iodixanol has revealed the discrepancy in buoyant density between the major virus peak and the virus with infectivity; the latter showed lower buoyant density than the former. However, the underlying physicochemical differences between noninfectious and infectious viruses still remain elusive. Thus, we tried to characterize the infectious virus from a different perspective. To evaluate the relationship between virus size and infectivity, the virus was analyzed by gel filtration chromatography (Shimizu *et al.*, unpublished

Figure 1



Relationship between physicochemical properties and infectivity of HCV. **(a)** LPL treatment shifts HCV to higher buoyant density and reduces HCV infectivity. The HCV (JFH1)-bearing culture medium was treated with PBS or LPL (500 μg/ml) for 1 hour at 37 °C and ultracentrifuged through iodixanol gradients. Thirty fractions were collected for analyzing the amount of core by ELISA. Culture medium from HuH7.5 cells inoculated with aliquots of each fraction was subjected to Core ELISA for infectivity. **(b)** Size distribution of HCV, infectious HCV, and HCV associated with ApoE. The HCV (JFH1)-bearing culture medium was subjected to gel filtration chromatography. The sample was eluted with 0.05 mol/l Tris-buffered acetate (pH 8.0) containing 0.3 mol/l sodium acetate, 0.05% sodium azide, and 0.005% Brij-35. A total of 50 fractions collected were analyzed for core, infectivity, and HCV RNA associated with ApoE. The representative fractions from 1 to 35 (core and infectivity) and from 7 to 30 (HCV RNA associated with ApoE) are shown. Elution of VLDL, LDL, and HDL was found in fractions 7–14, 15–19, and 20–26, respectively.

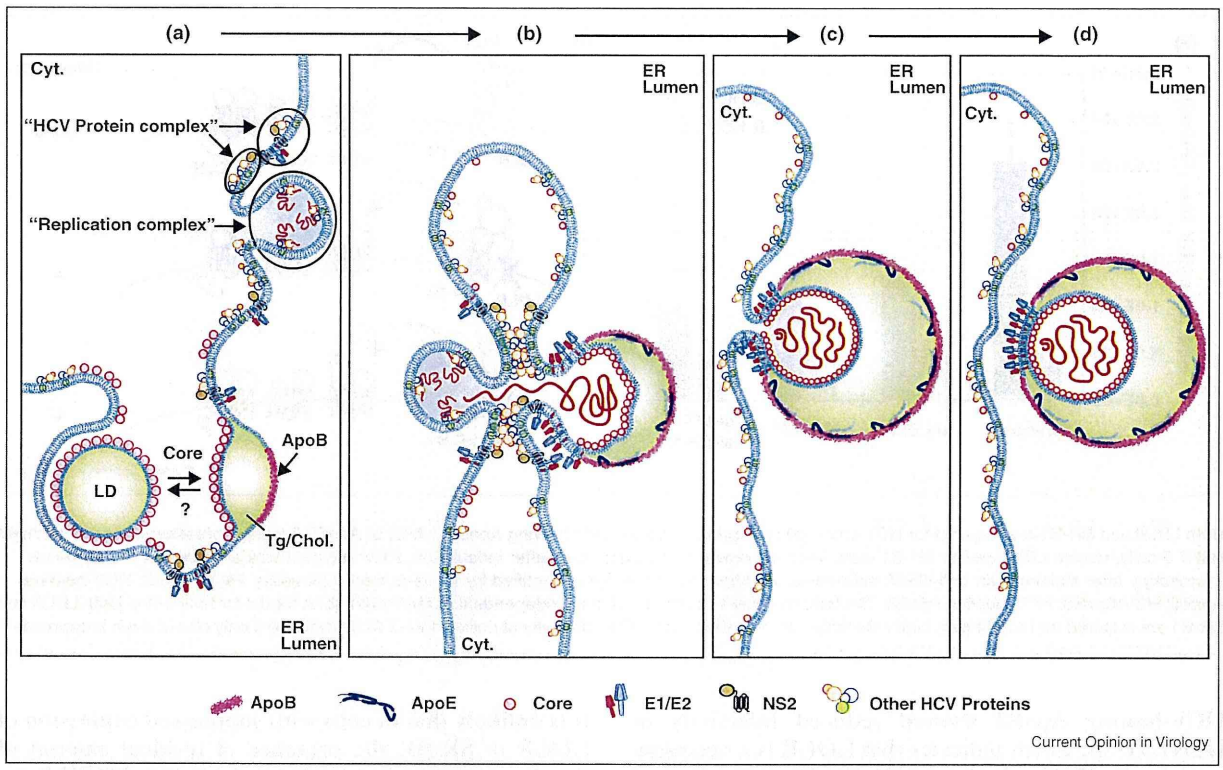
data). HCV released from infected HuH7.5 cells was applied to in-tandem-connected columns (300 mm × 7.8 mm) of TSKgel LipopropakXL resin (Tosoh, Tokyo). The eluent from the column was continuously separated into a total of 50 fractions. Then, each fraction was analyzed for core, HCV RNA, infectivity, ApoE, and E2. The major peak of the core was in fraction (frac.) 11 (Figure 1b). The HCV RNA peak corresponded to the core peak. E2 associated with the viral particle, analyzed by immunoprecipitation (IP) using anti-E2 antibody followed by RT-PCR for HCV RNA, was also in this fraction. This suggests that HCV eluted to frac. 11 retained the virus-like structure. However, importantly, HCV in frac. 11 did not have infectivity. The HCV fraction associated with infectivity showed multiple peaks ranging from frac. 15 to 22. Furthermore, frac. 15 demonstrated highest infectivity among all the fractions, and there was no linear correlation between the virus size and infectivity.

To determine whether the lack of ApoE is implicated in undetectable infectivity of HCV in frac. 11, we quantified HCV RNA associated with ApoE in each fraction by IP using anti-ApoE antibody, followed by RT-PCR. The HCV RNA associated with ApoE was detected from fractions irrespective of HCV infectivity (Figure 1b). This indicates that the association with ApoE is a necessary factor, but not sufficient for HCV infectivity.

Model of HCV budding into ER lumen

By taking these observations on HCV assembly into consideration, we drew the model of HCV assembly process and virus egress to ER lumen (Figure 2). The microenvironment of membranous structure of the core-coated lipid droplet has been found to play important roles in virus assembly [11••]. However, there is no proof to show a direct interaction of the core-coated lipid droplet with other HCV proteins. We presume that the core-coated lipid droplet may be localized at the vicinity of the 'HCV protein complex' enriched ER membrane, and that the triglyceride and cholesterol of the lipid droplet are reversely transferred to the inside of the bilayer of the ER membrane to where the core gets accumulated (Figure 2, stage a) [11••]. Core protein is not only enriched on the lipid droplet, but also visible on the ER by confocal microscopy [11••]. The presence of membranous web structure in the HCV genome replicating cells suggests dynamic alteration of the ER membrane structure [39]. Formation of the membranous web may include production of the 'replication complex' as well as dynamic alteration of ER membranous structure surrounding the core-coated lipid droplet, to make close association of 'replication complex' with core-enriched putative budding site of the viral particle (Figure 2, stage b). HCV protein complexes may gather each other to establish a compartment to constitute the 'replication complex' and putative virus precursor, so that the HCV

Figure 2



A model of virus egress to ER lumen. Stage **(a)**: Accumulation of triglyceride and cholesterol in the inside of the ER bilayer membrane enhances core association with the cytoplasmic monolayer membrane. These lipids may be reversely transferred from the core-coated lipid droplet or may be present in the inside of the ER bilayer membrane *ab origine*. On the membrane of ER, 'replication complex' as well as 'HCV protein complex' exists. Stage **(b)**: Distortion of the ER membrane, which seems to be driven by HCV protein complexes, makes the "Replication complex" location in the vicinity of the core-coated lipid-rich ER membrane, where HCV RNA secreted from the "replication complex" is exported to the putative virus budding site to establish a nascent HCV nucleocapsid. Simultaneously, ApoB and MTP function to synthesize lipoprotein-like structure surrounding the viral particle. Stages **(c)** and **(d)**: Progressing images of Stage B until egress of the virus/lipoprotein complex into ER lumen.

genome synthesized in the 'replication complex' is exported to the putative precursor of the viral particle to make a nascent HCV nucleocapsid. Simultaneously, lipoprotein formation proceeds with the help of MTP and ApoB, and buds into the ER lumen (Figure 2, stages c and d). It is still not clear at which stage of the virus assembly, other apolipoproteins, such as ApoA1 and ApoE, are incorporated into the virus/lipoprotein complex. However, NS5A may play some roles in incorporating ApoE into virus/lipoprotein complex during the process of virus assembly, because ApoE associates with NS5A [40].

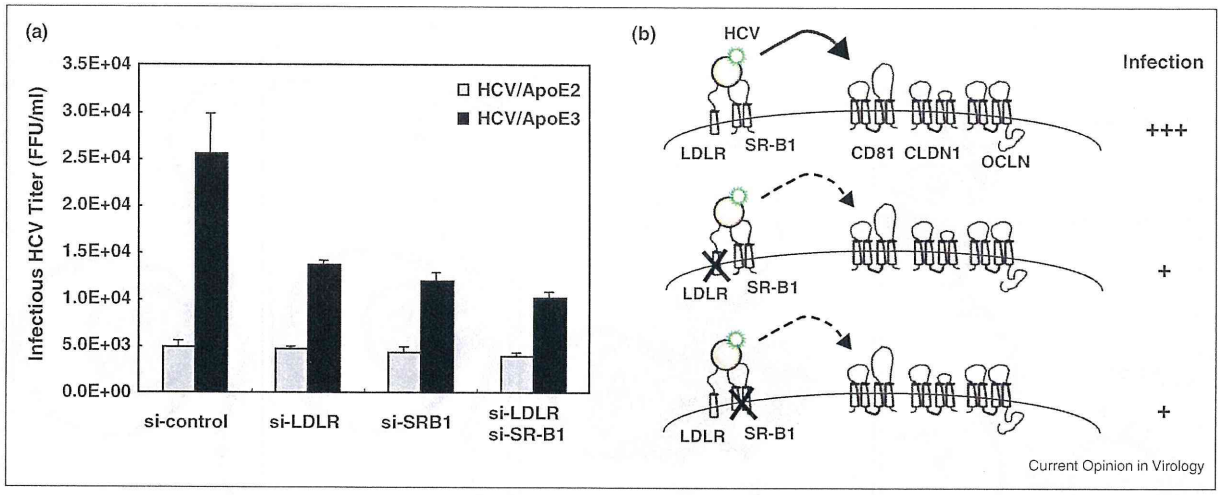
Both LDLR and SR-B1 are likely to be recognized by HCV for adsorption into naïve cells for entry

Several cell-surface molecules, such as CD81, Claudin, Occludin, SR-B1, LDLR, and glycosaminoglycan heparan sulfate (HS) function as receptors for HCV infection [41,42,43•,44••,45]. Suppressed expression of any of these molecules in naïve HuH7.5 cells reduces or inhibits HCV infection [44••,46], indicating the necessity of concerted action by these molecules for entry and full establishment of HCV infection. However, hierarchical order of actions by these molecules in the process of HCV entry remains elusive.

SR-B1 is expressed in many cells, but is mainly expressed in the liver and steroidogenic tissues. SR-B1 recognizes various types of lipoproteins that include HDL, LDL, and VLDL, as well as modified lipoproteins, such as oxidized and acetylated LDL. In addition, SR-B1 is shown to be associated with the soluble recombinant HCV E2 glycoprotein [47]. LDLR, expressed in many tissues including liver, is a cell-surface receptor that recognizes ApoB in LDL and ApoE in IDL and VLDL.

It is still controversial whether LDLR or SR-B1 is used as an entry receptor by HCV [42,44••]. To address this problem, infectivity was analyzed on HCV bearing a different ApoE isoform [36••]. As described earlier,

Figure 3



Both LDLR and SR-B1 are required for HCV entry. **(a)** HCV produced from cells bearing ApoE2 (white) or ApoE3 (black) expression was used to infect HuH7.5 cells, whose LDLR and/or SR-B1 were knocked down. Forty-eight hours after inoculation, HCV infection was analyzed by fluorescence microscopy after staining with anti-NS5A antibodies. Infectious HCV titer was quantified by focus-forming unit assay. HCV/ApoE2: HCV-bearing ApoE2; HCV/ApoE3: HCV-bearing ApoE3. The data represent the means of three independent experiments. **(b)** A model for HCV entry. Both LDLR and SR-B1 are required for HCV to gain high infectivity. On the other hand, HCV infectivity of cells is found to be reduced if only one of them is expressed.

HCV-bearing ApoE2 showed reduced infectivity in HuH7.5 cells, which indicates that LDLR is a necessary receptor for HCV infection [36••]. Further, infectivity of HCV to LDLR or SR-B1 knockdown cells was examined. Infectivity of HCV-bearing ApoE3 was reduced almost to the same level in LDLR or SR-B1 knockdown cells. With the expectation of additive reduction in infectivity in the cells with double knockdown of LDLR and SR-B1, infection in the double knockdown cells was analyzed [36••]. Unexpectedly, cells with suppressed expression of both the receptors did not show additive reduction in infectivity (Figure 3a). The level of infection of HCV-bearing ApoE2 to cells with suppressed expression of LDLR, SR-B1, or LDLR/SR-B1 was almost the same as that of the si-control transduced HuH7.5 cells (Figure 3a).

With regard to LDLR knockdown cells, the result was as expected, because ApoE2 has been found to have low affinity to LDLR. However, with regard to SR-B1 knockdown cells, we expected further reduction in HCV infection than that observed in si-control cells, because there was no significant difference in the association of ApoE2 and ApoE3 with SR-B1 [48]. Infectivity analysis using antibodies against LDLR/SR-B1 showed similar result as that observed in infectivity to LDLR or SR-B1 knockdown cells. From these results, we presume that HCV requires both LDLR and SR-B1 for infection and lack of either of the proteins suppresses HCV infectivity, as shown in Figure 3b. However, it is not known why the interaction of HCV with these two molecules results in high infectivity.

It is unlikely that in cells with suppressed expression of LDLR or SR-B1, the presence of residual amount of these proteins could establish infectivity of HCV-bearing ApoE3, because >90% reduction of these proteins in the knockdown cells has been confirmed [36]. However, this suggests the presence of another receptor through which ApoE-associated HCV could interact. To isolate a new candidate for HCV receptor(s), we focused our attention on lipoprotein receptor(s) in HuH7.5 cells. High expression of LRP1 and LRP8 was observed in this cell line among the candidates. However, knockdown of these genes in the cells did not reduce their susceptibility to HCV infection. Thus, it is likely that an unidentified protein in the HuH7.5 cells could play a role.

Conclusion

Patients with chronic hepatitis C often develop steatohepatitis. However, the reason why HCV-infected individuals cause abnormal lipid metabolism remains elusive. Recently it is suggested that HCV diverts lipid metabolism of host cells to establish its own proliferative machinery. This includes accumulation of lipid droplets to establish the microenvironment for virus assembly as modeled in this paper, and modification of lipid transfer for virus egress. Association of lipoprotein with HCV was biochemically demonstrated. Moreover, lipoprotein component(s) such as ApoE associated with virus is required for viral entry and may determine cell tropism. Further elucidation of the entire mechanism of HCV infection as well as identification of the host factors involved in this

process may contribute to more effective therapies for liver diseases caused by HCV infection.

Acknowledgements

We are grateful to Drs C. Rice and T. Wakita for HuH7.5 and JFH1 cells, respectively, and Dr J. McCubrey, East Carolina University, for comments on this manuscript. This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Health, Labor, and Welfare of Japan and from the Ministry of Education, Culture, Sports, Science, and Technology.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. World Health Organization: **Hepatitis C: global prevalence**. *Wkly Epidemiol Rec* 2000, **75**:18-19.

2. Liang TJ, Jeffers LJ, Reddy KR, de Medina M, Parker IT, Cheinquer H, Idrovo V, Rabassa A, Schiff ER: **Viral pathogenesis of hepatocellular carcinoma in the United States**. *Hepatology* 1993, **18**:1326-1333.

3. Yoon EJ, Hu K-Q: **Hepatitis C virus infection and hepatic steatosis**. *Int J Med Sci* 2006, **3**:53-56.

4. Goodman ZD, Ishak KG: **Histopathology of hepatitis C virus infection**. *Semin Liver Dis* 1995, **15**:70-91.

5. Brunt EM: **Nonalcoholic steatohepatitis: definition and pathology**. *Semin Liver Dis* 2001, **21**:3-16.

6. Moriya K, Fujie H, Shintani Y, Yotsuyanagi H, Tsutsumi T, Ishibashi K, Matsuura Y, Kimura S, Miyamura T, Koike K: **The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice**. *Nat Med* 1998, **4**:1065-1070.

7. Lerat H, Honda M, Beard MR, Loesch K, Sun J, Yang Y, Okuda M, Gosert R, Xiao SY, Weinman SA *et al.*: **Steatosis and liver cancer in transgenic mice expressing the structural and nonstructural proteins of hepatitis C virus**. *Gastroenterology* 2002, **122**:352-365.

8. Barba G, Harper F, Harada T, Kohara M, Goulinet S, Matsuura Y, Eder G, Schaff Z, Chapman M, Miyamura T *et al.*: **Hepatitis C virus core protein shows a cytoplasmic localization and associates to cellular lipid storage droplets**. *Proc Natl Acad Sci U S A* 1997, **94**:1200-1205.

9. Sabile A, Perlemuter G, Bonbo F, Kohara K, Demaugre F, Matsuura Y, Brechot C, Barba G: **Hepatitis C virus core protein binds to apolipoprotein A11 and its secretion is modulated by fibrates**. *Hepatology* 1999, **4**:1064-1076.

10. Targett-Adams P, Hope G, Boulant S, McLauchlan J: **Maturation of hepatitis C virus core protein by signal peptide peptidase is required for virus production**. *J Biol Chem* 2008, **283**:16850-16859.

11. •• Miyanari Y, Atsuzawa K, Usuda N, Watashi K, Hishiki T, Zayas M, Bartenschlager R, Wakita T, Hijikata M, Shimotohno K: **The lipid droplet is an important organelle for hepatitis C virus production**. *Nat Cell Biol* 2007, **9**:1089-1097.

Increased lipid droplet by HCV infection was shown to be important for virus production.

12. • Shavinskaya A, Boulant S, Penin F, McLauchlan J, Bartenschlager R: **The lipid droplet binding domain of hepatitis C virus core protein is a major determinant for efficient virus assembly**. *J Biol Chem* 2007, **282**:37158-37169.

Association of HCV core with the lipid droplet requires HCV production.

13. Perlemuter G, Sabile A, Letteron P, Vona G, Topilco A, Chrétien Y, Koike K, Pessayre D, Chapman J, Barba G *et al.*: **Hepatitis C virus core protein inhibits microsomal triglyceride transfer protein activity and very low density lipoprotein secretion: a model of viral-related steatosis**. *FASEB J* 2002, **16**:185-194.

14. Domitrovich AM, Femlee DJ, Siddiqui A: **Hepatitis C virus nonstructural proteins inhibit apolipoprotein B100 secretion**. *J Biol Chem* 2005, **280**:39802-39808.

15. Partin JS, Partin JC, Schubert WK, McAdams AJ: **Liver ultrastructure in abetalipoproteinemia: evolution of micronodular cirrhosis**. *Gastroenterology* 1974, **67**:107-118.

16. Mirandola S, Realdon S, Iqbal J, Gerotto M, Dal Pero F, Bortoletto G, Marcolongo M, Vario A, Datz MM, Hussain MM *et al.*: **Liver microsomal trigriceride transfer protein is involved in hepatitis C liver steatosis**. *Gastroenterology* 2006, **130**:1661-1669.

17. Moriishi K, Mochizuki R, Moriya K, Miyamoto H, Mori Y, Abe T, Murata S, Tanaka K, Miyamura T, Suzuki T *et al.*: **Critical role of PA28gannma in hepatitis C virus-associated steatogenesis and hepatocellular carcinoma**. *Proc Natl Acad Sci U S A* 2007, **104**:1661-1666.

18. Thomssen R, Bonk S, Thiele A: **Density heterogeneities of hepatitis C virus in human sera due to the binding of β-lipoproteins and immunoglobulins**. *Med Microbiol Immunol* 1993, **182**:329-334.

19. André P, Komurian-Pradel F, Deforges S, Perret M, Berland JL, Sodoyer M, Pol S, Bréchot C, Paranhos-Baccalà G, Lotteau V: **Characterization of low- and very-low-density hepatitis C virus RNA-containing particles**. *J Virol* 2002, **76**:6919-6928.

20. Agnello V, Abel G, Elfahal M, Knight GB, Zhang QX: **Hepatitis C virus and other flaviviridae viruses enter cells via low density lipoprotein receptor**. *Proc Natl Acad Sci U S A* 1999, **96**:12766-12771.

21. Molina S, Castet V, Fournier-Wirth C, Pichard-Garcia L, Avner R, Harats D, Roitelman J, Barbaras R, Graber P, Ghersa P *et al.*: **The low-density lipoprotein receptor plays a role in the infection of primary human hepatocytes by hepatitis C virus**. *J Hepatol* 2007, **46**:411-419.

22. Thomssen R, Bonk S, Propfe C, Heermann K-H, Köchel HG, Uy A: **Association of hepatitis C virus in human sera with β-lipoprotein**. *Med Microbiol Immunol* 1992, **181**:293-300.

23. Lindenbach BD, Evans MJ, Syder AJ, Wolk B, Tellinghuisen TL, Liu CC, Maruyama T, Hynes RO, Burton DR, McKeating JA *et al.*: **Complete replication of hepatitis C virus in cell culture**. *Science* 2005, **309**:623-626.

24. Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, Murthy K, Habermann A, Krausslich HG, Mizokami M *et al.*: **Production of infectious hepatitis C virus in tissue culture from a cloned viral genome**. *Nat Med* 2005, **11**:791-796.

25. Zhong J, Gastaminza P, Cheng G, Kapadia S, Kato T, Burton DR, Wieland SF, Uprichard SL, Wakita T, Chisari FV: **Robust hepatitis C virus infection in vitro**. *Proc Natl Acad Sci U S A* 2005, **102**:9294-9299.

26. • Masaki T, Suzuki R, Murakami K, Aizaki H, Ishii K, Murayama A, Date T, Matsuura Y, Miyamura T, Wakita T *et al.*: **Interaction of hepatitis C virus nonstructural protein 5A with core protein is critical for the production of infectious virus particles**. *J Virol* 2008, **82**:7964-7976.

Point mutation in the domain 3 of HCV NS5A protein abrogates production of virus particle.

27. Yi M, Ma Y, Yates J, Lemon SM: **Trans-complementation of an NS2 defect in a late step in hepatitis C virus (HCV) particle assembly and maturation**. *PLoS Pathog* 2009, **5**:e1000403.

28. Jirasko V, Montserret R, Lee JY, Gouttenoire J, Moradpour D, Penin F, Bartenschlager R: **Structural and functional studies of nonstructural protein 2 of the hepatitis C virus reveal its key role as organizer of virion assembly**. *PLoS Pathog* 2010, **6**:e1001233.

29. Ma Y, Anantpadma M, Timpe JM, Shanmugam S, Singh SM, Lemon SM, Yi M: **Hepatitis C virus NS2 protein serves as a scaffold for virus assembly by interacting with both structural and nonstructural proteins**. *J Virol* 2011, **85**:1706-1717.

30. Miyanari Y, Hijikata M, Yamaji M, Hosaka M, Takahashi H, Shimotohno K: **Hepatitis C virus non-structural proteins in the probable membranous compartment function in viral genome replication**. *J Biol Chem* 2003, **278**:50301-50308.

31. • Huang H, Sun F, Owen DM, Li W, Chen Y, Gale M Jr, Ye J: **Hepatitis C virus production by human hepatocytes**

dependent on assembly and secretion of very low-density lipoproteins. *Proc Natl Acad Sci U S A* 2007, **104**:5848-5853.
Functional association of HCV egress and VLDL has been shown.

32. Gastaminza P, Cheng G, Wieland S, Zhong J, Liao W, Chisari FV:
• **Cellular determinants of hepatitis C virus assembly, maturation, degradation, and secretion**. *J Virol* 2008, **82**:2120-2129.
See annotation to [31•].

33. Icard V, Diaz O, Scholtes C, -Cocon P, Ramière L,
• Bartenschlager C, Penin R, Lotteau F, André VP: **Secretion of hepatitis C virus envelope glycoproteins depends on assembly of apolipoprotein B positive lipoproteins**. *PLos ONE* 2009, **4**:e4223.
Association of HCV envelope protein and lipoprotein is disclosed.

34. Jing J, Luo G: **Apolipoprotein E but not B is required for the**
• **formation of infectious hepatitis C virus particles**. *J Virol* 2009, **83**:12680-12691.
Importance of apolipoprotein E rather than apolipoprotein B in infectious HCV production is described.

35. Chang K-S, Jiang J, Cai Z, Luo G: **Human apolipoprotein E is**
•• **required for infectivity and production of hepatitis C virus in cell culture**. *J Virol* 2007, **81**:13783-13793.
Apolipoprotein E plays important role in HCV infectivity.

36. Hishiki T, Shimizu Y, Tobita R, Sugiyama K, Ogawa K, Funami K,
•• Ohsaki Y, Fujimoto T, Takaku H, Wakita T *et al.*: **Infectivity of hepatitis C virus is influenced by association with apolipoprotein E isoforms**. *J Virol* 2010, **84**:12048-12057.
HCV infectivity is influenced by apolipoprotein E isoforms.

37. Mancone C, Steindler C, Santangelo L, Simonte G, Vlassi C, Longo MA, D'Offizi G, Di Giacomo C, Pucillo LP, Amicone L *et al.*: **Hepatitis C virus production requires apolipoprotein A-I and affects its association with nascent low-density lipoproteins**. *Gut* 2011, **60**:378-386.

38. Shimizu Y, Hishiki T, Sugiyama K, Ogawa K, Funami K, Kato A,
• Ohsaki Y, Fujimoto T, Takaku H, Shimotohno K: **Lipoprotein lipase and hepatic triglyceride lipase reduce the infectivity of hepatitis C virus (HCV) through their catalytic activities on HCV-associated lipoproteins**. *Virology* 2010, **407**:152-159.
Association of HCV with lipoprotein is shown by biochemical analysis.

39. Gosert R, Egger D, Lohmann V, Bartenschlager R, Blum HE, Bienz K, Moradpour D: **Identification of the hepatitis C virus RNA replication complex in Huh-7 cells harboring subgenomic replicons**. *J Virol* 2003, **77**:5487-5492.

40. Benga WJ, Krieger SE, Dimitrova M, Zeisel MB, Parnot M, Lupberger J, Hildt E, Luo G, McLauchlan J, Baumert TF *et al.*: **Apolipoprotein E interacts with hepatitis C virus nonstructural protein 5A and determines assembly of infectious particles**. *Hepatology* 2010, **51**:43-53.

41. Pileri P, Uematsu Y, Campagnoli S, Galli G, Falugi F, Petracca R, Weiner AJ, Houghton M, Rosa D, Grandi G *et al.*: **Binding of hepatitis C virus to CD81**. *Science* 1998, **282**:938-941.

42. Bartosch B, Vitelli A, Granier C, Goujon C, Dubuisson J, Pascale S, Scarselli E, Cortese R, Nicosia A, Cosset F-L: **Cell entry of hepatitis C virus requires a set of co-receptors that include the CD81 tetraspanin and the SR-B1 scavenger receptor**. *J Biol Chem* 2003, **278**:41624-41630.

43. Evans MJ, von Hahn T, Tscherne DM, Syder AJ, Panis M, Wolk B,
• Hatziioannou T, McKeating JA, Bieniasz PD, Rice CM: **Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry**. *Nature* 2007, **446**:801-805.
Claudin-1 has been shown to be a receptor for HCV.

44. Owen DM, Huang H, Ye J, Gale M Jr: **Apolipoprotein E on**
•• **hepatitis C virion facilitates infection through interaction with low-density lipoprotein receptor**. *Virology* 2009, **394**:99-108.
LDLR is shown to be a receptor for HCV.

45. Barth H, Schafer C, Adah MI, Zhang F, Linhardt RJ, Toyoda H, K-Toyoda A, Toida T, van Kuppevelt TH, Depla E *et al.*: **Cellular binding of hepatitis C virus envelope glycoprotein E2 requires cell surface heparan sulfate**. *J Biol Chem* 2003, **278**:41003-41012.

46. Zeisel MB, Koutsoudakis G, Schnober EK, Haberstroh A, Blum HE, Cosset FL, Wakita T, Jaeck D, Doffoel M, Royer C *et al.*: **Scavenger receptor class B type I is a key host factor for hepatitis C virus infection required for an entry step closely linked to CD81**. *Hepatology* 2007, **46**:1722-1731.

47. Scarselli E, Ansuini H, Cerino R, Roccasecca RM, Acali S, Filocamo G, Traboni C, Nicosia A, Cortese R, Vitell A: **The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus**. *EMBO J* 2002, **21**:5017-5025.

48. Li X, Kan HY, Lavrentiadou S, Krieger M, Zannis V: **Reconstituted discoidal ApoE-phospholipid particles are ligands for the scavenger receptor BI**. *J Biol Chem* 2002, **277**:21149-21157.

Virus Research 163 (2012) 390–395

Contents lists available at SciVerse ScienceDirect

Virus Research

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

Short communication

The interaction between human initiation factor eIF3 subunit c and heat-shock protein 90: A necessary factor for translation mediated by the hepatitis C virus internal ribosome entry site

Saneyuki Ujino[a], Hironori Nishitsuji[a], Ryuichi Sugiyama[a], Hitoshi Suzuki[a], Takayuki Hishiki[b], Kazuo Sugiyama[c], Kunitada Shimotohno[b], Hiroshi Takaku[a,*]

[a] Department of Life and Environmental Sciences, Chiba Institute of Technology, 2-17-1 Tsudanuma, Narashino-shi, Chiba 275-0016, Japan
[b] Research Institute, Chiba Institute of Technology, 2-17-1 Tsudanuma, Narashino, Chiba 275-0016, Japan
[c] Center for Integrated Medical Research, School of Medicine, Keio University, 35 Shinanomachi, Shinjyuku-ku Tokyo 160-8582, Japan

ARTICLE INFO

Article history:
Received 10 June 2011
Received in revised form 4 October 2011
Accepted 6 October 2011
Available online 14 October 2011

Keywords:
eIF3c
Hsp90
HCV IRES
17-AAG
Translation initiation

ABSTRACT

Heat-shock protein 90 (Hsp90) is a molecular chaperone that plays a key role in the conformational maturation of various transcription factors and protein kinases in signal transduction. The hepatitis C virus (HCV) internal ribosome entry site (IRES) RNA drives translation by directly recruiting the 40S ribosomal subunits that bind to eukaryotic initiation factor 3 (eIF3). Our data indicate that Hsp90 binds indirectly to eIF3 subunit c by interacting with it through the HCV IRES RNA, and the functional consequence of this Hsp90–eIF3c–HCV-IRES RNA interaction is the prevention of ubiquitination and the proteasome-dependent degradation of eIF3c. Hsp90 activity interference by Hsp90 inhibitors appears to be the result of the dissociation of eIF3c from Hsp90 in the presence of HCV IRES RNA and the resultant induction of the degradation of the free forms of eIF3c. Moreover, the interaction between Hsp90 and eIF3c is dependent on HCV IRES RNA binding. Furthermore, we demonstrate, by knockdown of eIF3c, that the silencing of eIF3c results in inhibitory effects on translation of HCV-derived RNA but does not affect cap-dependent translation. These results indicate that the interaction between Hsp90 and eIF3c may play an important role in HCV IRES-mediated translation.

The hepatitis C virus (HCV), a member of the *Flaviviridae* family, has a positive-strand RNA genome (Taylor et al., 1999; Bartenschlager and Lohmann, 2001) encoding a large precursor polyprotein that is cleaved by host and viral proteases to generate at least 10 functional viral proteins: core, envelope 1 (E1), E2, p7, nonstructural protein (NS2), NS3, NS4A, NS4B, NS5A, and NS5B (Grakoui et al., 1993; Hijikata et al., 1993). Ishii et al. identified an HCV replicon system in which the full HCV genomic RNA autonomously replicates in the Huh-7 human hepatoma cell line (Fig. 1A) (Ishii et al., 2006). This HCV replicon system allows researchers to study HCV genome replication in cell culture. HCV protein synthesis is initiated by the HCV RNA genome. This genome contains a conserved structure in its 5′-untranslated region (5′-UTR) that acts as an internal ribosome entry site (IRES) (Lukavsky, 2008). Briefly, the small ribosomal subunit (40S) and the eukaryotic initiation factor eIF3 bind specifically to the HCV IRES RNA, allowing for direct recognition of the start codon present in the 5′-UTR of the viral mRNA (Spahn et al., 2001; Collier et al., 2002; Kieft et al., 2002; Fraser and Doudna, 2007; Julien et al., 2009). Consistent with its diverse functions, eIF3 is the largest and most complex initiation factor. The mammalian version, for example, contains 13 nonidentical subunits designated eIF3a to eIF3m. The eIF3 core subunit (eIF3a–c, g, and i) is essential for translation (Kieft et al., 2002; Hinnebusch, 2006; Masutani et al., 2007; Zhou et al., 2008), and eIF3 specifically associates with the apical half of domain III of the HCV IRES (Kieft et al., 2001, 2002; Siridechadilok et al., 2005; Fraser and Doudna, 2007).

Hsp90 is a heat-shock protein that is abundant in the cytosol of eukaryotes and prokaryotes. In contrast to other chaperones, a number of substrates are known to contain Hsp90 (Schulte et al., 1995). Studies of eukaryotes have revealed that these Hsp90 client proteins include a variety of transcription factors (Coumailleau et al., 1995; Garcia-Cardena et al., 1998; Nagata et al., 1999; Sato et al., 2000; Richter and Buchner, 2001; Xu et al., 2001; Waza et al., 2005). Recently, many studies have reported that Hsp90 is involved with not only HCV RNA replication and viral protein but also HCV IRES-mediated translation (Waxman et al., 2001; Kim et al., 2006; Okamoto et al., 2006; Nakagawa et al., 2007; Ujino et al., 2009). In the present study, we demonstrate that eIF3 forms a complex with Hsp90 that is critical for HCV IRES-mediated translation.

* Corresponding author. Tel.: +81 47 478 0407; fax: +81 47 478 0407.
E-mail address: hiroshi.takaku@it-chiba.ac.jp (H. Takaku).

0168-1702/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.virusres.2011.10.003

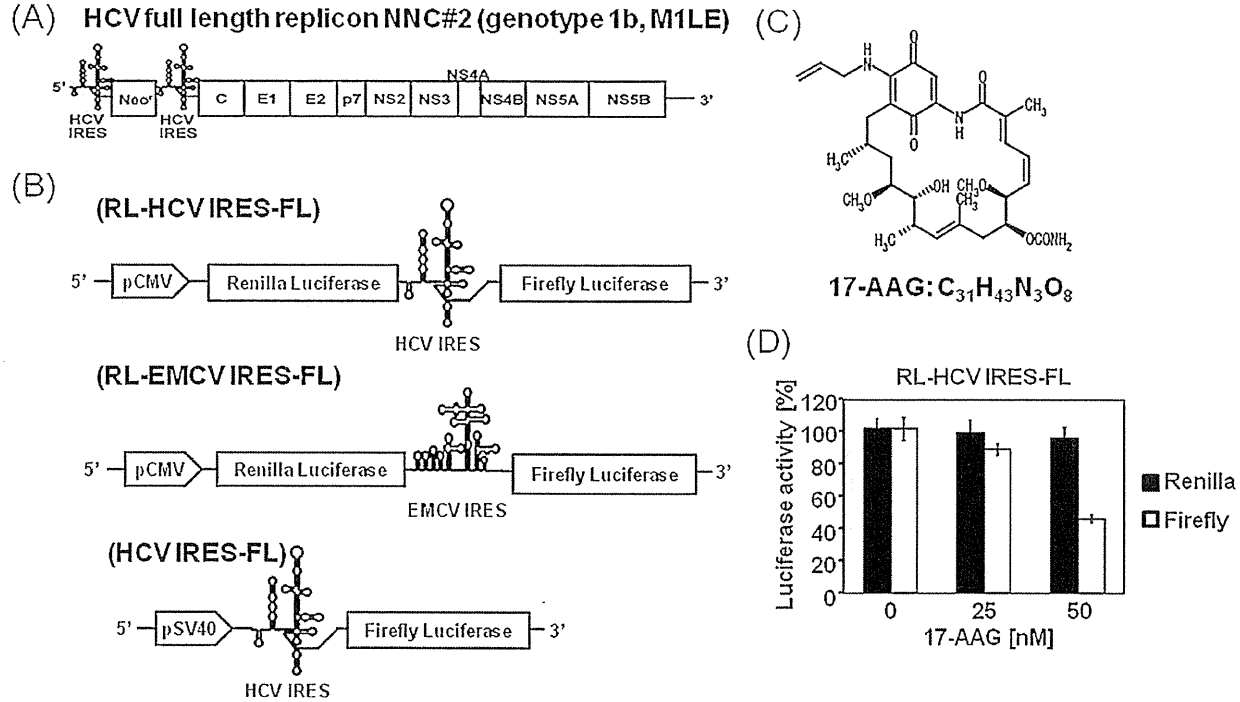


Fig. 1. Inhibition of IRES-mediated translation by an Hsp90 inhibitor. (A) The structure of the HCV replicon RNA molecules comprising the HCV 5′-UTR, including the HCV IRES, the neomycin phosphotransferase gene (Neo^r), and the coding region for the HCV proteins core to NS5B (in the HCV full-length replicon). (B) A schematic representation of the bicistronic HCV IRES or EMCV IRES reporter construct pRenilla-HCV IRES-firefly luciferase (RL-HCV IRES-FL) or pRenilla-EMCV IRES-firefly luciferase (RL-EMCV IRES-FL) driven by the CMV promoter to direct cap-dependent translation of renilla luciferase (RL) and HCV IRES or EMCV IRES-dependent translation of firefly luciferase (FL). The vector construct for HCV IRES-mediated translation of firefly luciferase, pHCV IRES-firefly luciferase (HCV IRES-FL) (Ujino et al., 2010). (C) The structure of the Hsp90 inhibitor 17-AAG (17-allylamino-17-demethoxygeldanamycin, Sigma–Aldrich Chemical Co.). (D) Inhibition of IRES-mediated translation by 17-AAG. Huh-7 cells (1×10^5 cells/well on 12-well plates) treated with 17-AAG (25 and 50 nM) and DMSO as a control for 24 h, and were then transfected with pRenilla-HCV IRES-firefly luciferase (RL-HCV IRES-FL) using Lipofectamine 2000 (Invitrogen). At 24 h post-transfection, Renilla luciferase (cap-dependent translation) and firefly luciferase (HCV IRES-dependent translation) activities were measured with a Dual-Luciferase Reporter Assay System (Promega). The data represent the mean ± standard deviations (SDs) from the experiments performed in triplicate.

To investigate the effects of the *Hsp90* inhibitor 17-AAG on HCV IRES translation, a bicistronic reporter system was used that consisted of an upstream reporter, Renilla luciferase (RL), expressed by cap-dependent translation and a downstream reporter, firefly luciferase (FL), which is under the translational control of the HCV IRES. To construct pcDNA-HCV IRES-firefly Luc, pHCV IRES-firefly Luc (HCV IRES-FL) (Ujino et al., 2010) (Fig. 1B) was digested with BamHI and SalI. The IRES-firefly Luc fragments were inserted into the BamHI-XhoI site of pcDNA3.1 (Invitrogen, Carlsbad, CA). To construct pRenilla-HCV IRES-firefly luciferase (RL-HCV IRES-FL), Renilla luciferase fragments were amplified by PCR from a pFN11A Flexi vector (Promega, Madison, WI), and the PCR products were inserted into the BamHI site of pcDNA-HCV IRES-firefly Luc. The human hepatoma cell line Huh-7 was maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) containing 10% fetal bovine serum (FBS). The benzoquinone ansamycin, the antibiotic geldanamycin (GA) and its less toxic analogue 17-allylamino-17-demethoxygeldanamycin (17-AAG) (Fig. 1C) (Sigma–Aldrich Chemical Co., St Louis, MO) directly bind to the ATP/ADP binding pocket of Hsp90, thus preventing ATP binding and the completion of client protein refolding (Neckers, 2003). The client proteins of Hsp90 appear to shift the role of the primary chaperone from Hsp90 to Hsp70 in cells treated with Hsp90 inhibitors (Doong et al., 2003). It is also well known that 17-AAG causes a modest increase in Hsp70 levels (Morimoto, 1998; Bagatell et al., 2000; Guo et al., 2005). In our previous report, a significant induction of Hsp70 was detected (Ujino et al., 2009).

For the reporter gene assay, Huh-7 cells were treated with different concentrations of the Hsp90 inhibitor, 17-AAG, or DMSO as a control for 24 h. They were then transfected with the bicistronic reporter construct RL-HCV IRES-FL using Lipofectamine 2000 (Invitrogen), which directs cap-dependent translation of the RL gene and HCV IRES-dependent translation of FL genes (Invitrogen). At 24 h post-transfection, the Renilla luciferase (cap-dependent translation) and firefly luciferase (HCV IRES-dependent translation) activities were measured with a Dual-Luciferase Reporter Assay System (Promega, Madison, WI). In cells treated with 50 nM 17-AAG, firefly luciferase activity was reduced by 55% with RL-HCV IRES-FL, whereas Renilla luciferase activity was mostly maintained (Fig. 1D). The inhibition of HCV IRES-mediated translation occurred in a dose-dependent manner. Recently, Kim et al. (2006) demonstrated that Hsp90 regulates ribosomal function by maintaining the stability of 40S ribosomal proteins such as rpS3 and rpS6. The interaction between the 40S ribosomal proteins and Hsp90 has also been associated with ribosomal activities such as protein synthesis. We also found that the Hsp90 inhibitor 17-AAG influences HCV IRES-mediated luciferase activity, suggesting that 17-AAG inhibited HCV RNA replication and HCV IRES-mediated translation.

The HCV IRES is recognized specifically by the small ribosomal subunit and eIF3 before the initiation of viral translation. Although the degradation of rpS3, a component of the small ribosomal subunit, has been shown to occur in the presence of the Hsp90 inhibitor (Kim et al., 2006), the influence of Hsp90 inhibition on eIF3 is not understood. To determine whether 17-AAG affects the expression of the eIF3 subunit, we analyzed eIF3a, eIF3b, eIF3c, eIF3g and eIF3i protein expression by western blot analysis. The HCV replicon cell line NNC#2 (NN/1b/FL), which carries a full genome replicon, was cultured in DMEM with 10% FBS, nonessential amino acids,

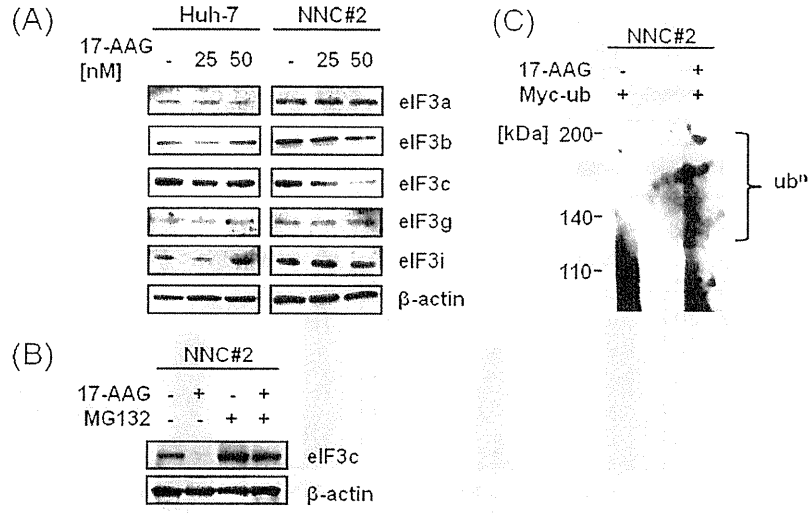


Fig. 2. Effect of 17-AAG treatment on eIF3 expression. (A) Western blot analysis of eIF3 protein expression in Huh-7 or NNC#2 cells treated with 17-AAG (25 nM and 50 nM). The cell lysates were analyzed by western blot 48 h after treatment. The primary antibodies used were monoclonal or polyclonal antibodies against eIF3a, eIF3b, eIF3c, eIF3g, and eIF3i (Santa Cruz Biotechnology). Horseradish peroxidase-conjugated anti-rabbit antibody (Sigma–Aldrich Chemical Co.) was used as the secondary antibody. (B) The reduction of eIF3c expression was prevented by proteasome inhibitor treatment. NNC#2 cells treated with 17-AAG (50 nM) or DMSO as a control. After 8 h treatment, the cells were treated with the proteasome inhibitor MG-132 (5 μM) or DMSO as a control. The cell lysates were analyzed by western blot 40 h after treatment. The primary antibody used was the eIF3c or β-actin (Santa Cruz Biotechnology). Horseradish peroxidase-conjugated anti-rabbit antibody (Sigma–Aldrich Chemical Co.) was used as the secondary antibody. (C) eIF3c degradation is mediated by the ubiquitin-dependent protease pathway. NNC#2 cells were transfected with pCMV-Myc-Ubi using Lipofectamine 2000 (Invitrogen). At 24 h post-transfection, the cells were treated with 17-AAG (50 nM) or DMSO as a control for 8 h and were then treated with the proteasome inhibitor MG-132 (5 μM) for 16 h. The cell lysates were subjected to an immunoprecipitation assay using an anti-αMyc antibody (Cell Signaling) followed by an immunoblot analysis using anti-eIF3c antibody.

L-glutamine, penicillin/streptomycin, and 1 mg/mL G418 (Invitrogen) at 37 °C in 5% CO_2 (Ishii et al., 2006). For western blot analysis, NNC#2 cells and Huh-7 cells were lysed in 1× chloramphenicol acetyltransferase (CAT) enzyme-linked immunosorbent assay buffer (Roche, Basel, Switzerland). The cell lysates were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and blocked with 5% skimmed milk. The primary antibodies used were monoclonal or polyclonal antibodies against FLAG-M2 (Sigma–Aldrich Chemical Co.), Hsp90 (Cell Signaling Tech., Beverly, MA), eIF3a, eIF3b, eIF3c, eIF3g, and eIF3i (Santa Cruz Biotechnology, Santa Cruz, CA). Horseradish peroxidase-conjugated anti-rabbit antibody (Sigma–Aldrich Chemical Co.) was used as the secondary antibody. When the HCV replicon cell line NNC#2 (NN/1b/FL) and Huh-7 cells were treated with increasing doses of 17-AAG, the expression of the eIF3c subunit was markedly reduced in NNC#2 cells, but the expression in Huh-7 cells was unaffected (Fig. 2A). These results suggest that Hsp90 is involved in eIF3c stability through a physical interaction in the presence of HCV IRES RNA.

Protein degradation in cells is mediated by several protease systems; however, the stability of most proteins is regulated by Hsp90, and they appear to be degraded by proteasomes. To investigate whether the reduction of eIF3c was due to proteasomal degradation, we treated NNC#2 cells with a proteasome inhibitor, MG132, to prevent the 17-AAG-induced degradation of eIF3c. Our results indicated that 17-AAG-induced eIF3c degradation can be blocked by proteasome inhibitors (Fig. 2B). Proteasome inhibitors substantially prevented the degradation of eIF3c in cells treated with 17-AAG. This is most likely because the disruption of Hsp90 by the Hsp90 inhibitor treatment destabilized the eIF3c protein. Therefore, it is clear that proteasome-dependent degradation results in the decreased level of eIF3c protein. This indicates that the stability of eIF3c was supported by Hsp90, and unstable eIF3c was removed by proteasomes. Furthermore, we investigated whether the ubiquitination of eIF3c was affected by the Hsp90 inhibitor, 17-AAG. We transfected pCMV-Myc-Ubi (provided by Dr. A. Ryo) using Lipofectamine 2000 (Invitrogen) into NNC#2 cells, which were then treated with 17-AAG (50 μM). After treatment, the cells were then treated with 5 μM MG132 and subjected to immunoprecipitation with an anti-αMyc antibody (Cell Signaling) followed by an immunoblot analysis using an anti-eIF3c antibody. Notably, polyubiquitinated forms of eiF3c was detected in cells treated with 17-AAG (Fig. 2C). These results suggest that the destabilized eIF3c protein is degraded by proteasome-dependent proteolysis mediated by ubiquitin conjugation, and Hsp90 plays an important role in maintaining the stable form of the eIF3c protein in vivo.

To investigate the influence of eIF3c silencing on HCV IRES-mediated translation, Huh-7 cells were transfected with siRNA targeted to eIF3c at a concentration of 50 nM using Lipofectamine 2000 (Invitrogen), and they were then transfected with RL-HCV IRES-FL. Control small interference RNA (siRNA) and eIF3 p110 (eukaryotic translation initiation factor 3, subunit 8, 110 kDa) siRNA were purchased from Santa Cruz Biotechnology. The protein levels of eIF3c were examined by western blot analysis, and HCV IRES-mediated translation was analyzed with a Dual-Luciferase Assay. As demonstrated in Fig. 3A, when compared to Huh-7 cells treated with control siRNA, the eIF3c protein level was markedly reduced in Huh-7 cells transfected with eIF3 p110 siRNA targeting eIF3c. Furthermore, firefly luciferase activity in RL-HCV IRES-FL was also reduced by approximately 63% in the cells treated with siRNA targeted to eIF3c, whereas Renilla luciferase activity was mostly maintained (Fig. 3B). The inhibition of HCV IRES-mediated translation by siRNA against eIF3c indicates that the suppression of HCV IRES-mediated translation by Hsp90 inhibition leads to a reduction in eIF3c. To further characterize the HCV IRES inhibitory effect of siRNA targeting eIF3c, we used additional bicistronic reporter plasmids for transient transfection assays with Huh-7 cells. Since we were mainly interested in viral IRESs, we chose to investigate the effect of the luciferase activities on translation derived from the IRES of EMCV in place of the HCV IRES (Fig. 1B). To generate pcDNA-EMCV IRES-firefly Luc, EMCV IRES fragments were created by PCR using the following primers: 5′-GAC TGG ATC CCC CCC CCT AAC-3′ and 5′-CAG TGG GCC CTA TTA TCG TGT TTT TCA AAG GAA AAC C-3′. The PCR products were inserted into the BamHI and

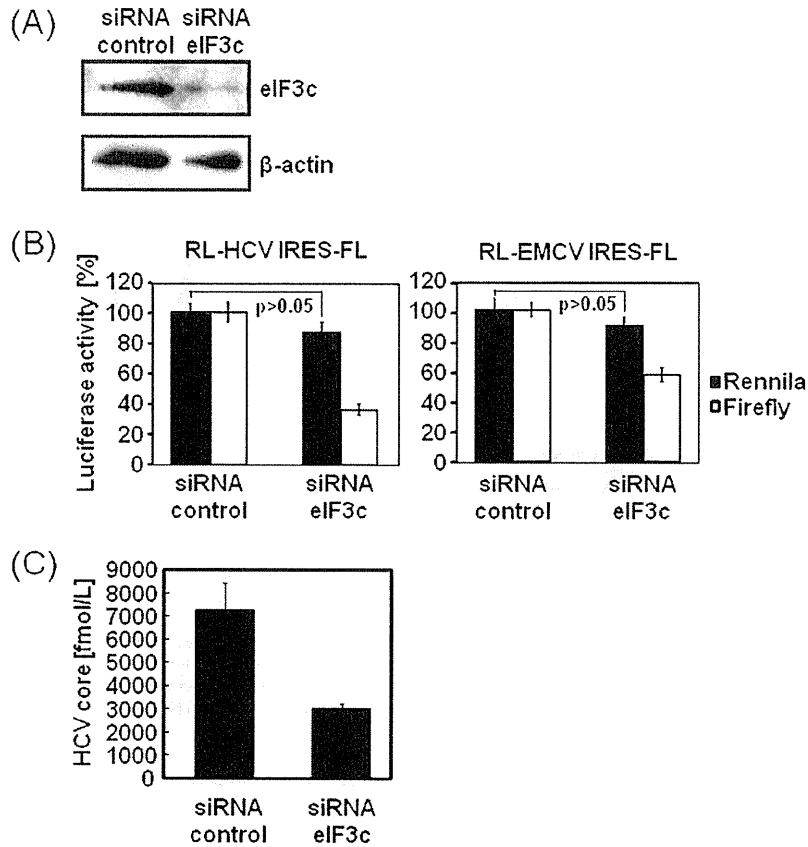


Fig. 3. Knockdown of eIF3c expression inhibits HCV IRES-mediated translation. (A) eIF3c protein expression in Huh-7 cells transfected with control siRNA or eIF3c siRNA at a concentration of 50 nM using Lipofectamine 2000 (Invitrogen). Cell lysates were analyzed by western blot 24 h post-treatment. eIF3c or β-actin antibodies were used as the primary antibodies (Santa Cruz Biotechnology). Horseradish peroxidase-conjugated anti-rabbit antibody (Sigma–Aldrich Chemical Co.) was used as the secondary antibody. (B) Huh-7 cells were transfected with siRNA targeted to eIF3c (50 nM) or nontarget control siRNA (50 nM) using Lipofectamine 2000 (Invitrogen). At 24 h post-transfection, the cells were then transfected with RL-HCV IRES-FL or RL-EMCV IRES-FL using Lipofectamine 2000 (Invitrogen). At 24 h post-transfection, the Renilla luciferase (cap-dependent translation) and firefly luciferase (HCV IRES or EMCV IRES-dependent translation) activities were measured with a Dual-Luciferase Reporter Assay System (Promega). Results are representative of three independent experiments, and error bars indicate the ± standard deviations (SDs) of the means. $P > 0.05$ (Student's t-test). (C) HCV IRES-mediated translational inhibition with eIF3 p110 siRNA by HCV full-genome RNA (NN/1b/FL). Huh-7 cells were transfected with eIF3 p110 siRNA or control siRNA at a concentration of 50 nM using Lipofectamine 2000 (Invitrogen). At 24 h post-transfection, the cells were transfected with 2 μg of HCV full-genome RNA (NN/1b/FL) using Lipofectamine 2000 (Invitrogen). After 24 h, intracellular HCV core-protein levels were measured using a fully automated HCV core-protein antigen chemiluminescent enzyme immunoassay (CLEIA) according to the manufacturer's instructions (Aoyagi et al., 1999). The relative chemiluminescence units were measured and used to determine the concentration of the HCV core antigen according to a standard curve generated using recombinant HCV core antigen. The concentration was expressed in units of femtomole/L (fmol/L). The data represent the mean ± standard deviations (SDs) from the experiments performed in triplicate.

ApaI sites of pcDNA3.1, and firefly Luc fragments were cloned into the ApaI site of the resulting plasmid. To construct pcDNA-Renilla-EMCV IRES-firefly Luc (RL-EMCV IRES-FL), pcDNA-Renilla-EMCV IRES-firefly Luc was digested with BamHI, and the renilla Luc fragments were inserted into BamHI site of pcDNA-EMCV IRES-firefly Luc (Fig. 1B). Huh-7 cells were transfected with control small interference RNA (siRNA) or eIF3 p110 siRNA at a concentration of 50 nM using Lipofectamine 2000 (Invitrogen) and then transfected with RL-EMCV IRES-FL. Following transient transfection in Huh-7 cells, firefly luciferase activity in RL-EMCV IRES-FL was also reduced by approximately 43% in cells treated with siRNA targeting eIF3c, but Renilla luciferase activity was mostly maintained (Fig. 3B). The knockdown of eIF3c expression also resulted in inhibitory effects on translation derived from HCV and EMCV (Fig. 3B) but did not affect cap-dependent translation. These results indicate that eIF3c may play a more important initiation factor in IRES-mediated translation than cap-dependent translation. However, it remains the subject of future investigation to determine whether only eIF3c proteins are subject to the IRES-mediated translation.

We also examined the HCV IRES-mediated translational inhibition with eIF3 p110 siRNA by HCV full-genome RNA ((NN/1b/FL) (Ishii et al., 2006). Huh-7 cells were transfected with eIF3 p110 siRNA or control siRNA at a concentration of 50 nM using Lipofectamine 2000 (Invitrogen). At 24 h post-transfection, the cells were transfected with HCV full-genome RNA (NN/1b/FL). After 24 h, the intracellular HCV core-protein levels were measured using a fully automated HCV core-protein antigen chemiluminescent enzyme immunoassay (CLEIA) according to the manufacturer's instructions (Aoyagi et al., 1999). The core-protein expression in cells treated with eIF3 p110 siRNA was reduced by approximately 61% when compared to cells treated with control siRNA (Fig. 3C). These findings further confirmed that HCV IRES-mediated translational inhibition occurs through a reduction of eIF3c expression caused by the Hsp90 inhibitor-mediated disruption of the interaction between eIF3c and Hsp90 with HCV IRES RNA.

To investigate the role of Hsp90 in HCV IRES-mediated translation further, we confirmed the interaction of eIF3c and Hsp90 by immunoprecipitation. The pFLAG-eIF3c vector was constructed by subcloning a DNA fragment encoding full-length human eiF3c into the EcoRI and XbaI sites of the pFLAG CMV™-2 expression vector (Sigma–Aldrich Chemical Co.) so that the amino-terminal FLAG epitope was fused in-frame with eIF3c. The pFLAG-eIF3c expression vector or the control vector pFLAG-CMV2 was transfected into NNC#2 cells or Huh-7 cells. After 48 h, the immunoprecipitates

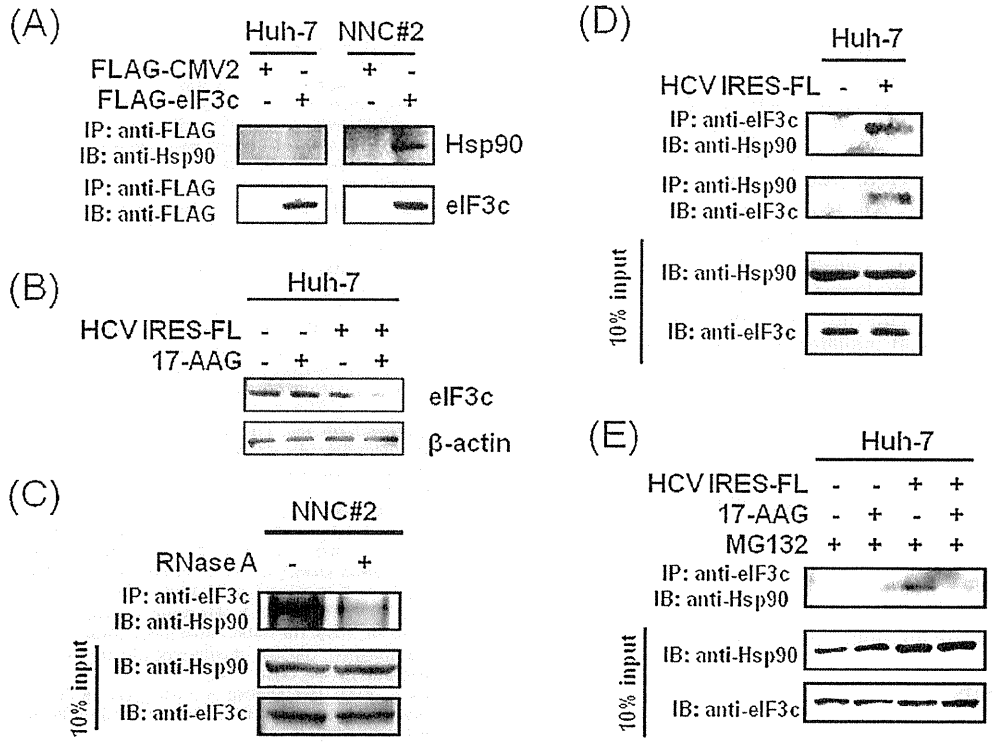


Fig. 4. An interaction between eIF3c and Hsp90 was induced by HCV IRES. (A) The pFLAG-eIF3c vector was constructed by subcloning a DNA fragment encoding full-length human eiF3c into the EcoRI and XbaI sites of the pFLAG CMV™-2 expression vector (Sigma–Aldrich Chemical Co.) such that the amino-terminal FLAG epitope was fused in-frame with eIF3c. Huh-7 and NNC#2 cells were transfected with pFLAG-eIF3c or pFLAG-CMV2 control plasmids using Lipofectamine 2000 (Invitrogen). Cell lysates were immunoprecipitated by the anti-FLAG M2 antibody 48 h after transfection. The precipitates were analyzed by western blot using the anti-Hsp90 antibody. (B) The Huh-7 cells were transfected with or without pHCV IRES-firefly luciferase (HCV IRES-FL) (Fig. 1B) and then treated with or without 17-AAG (50 nM). Cell lysates were analyzed by western blot 48 h post-treatment with an eIF3c primary antibody (Santa Cruz Biotechnology). Horseradish peroxidase-conjugated anti-rabbit antibody (Sigma-Aldrich Chemical Co.) was used as the secondary antibody. β-Actin was used as an internal control. (C) The interruption of Hsp90–eIF3c interaction by RNase A treatment. NNC#2 cell lysates were treated with RNase A (5 U/μL) (Sigma–Aldrich Chemical Co.). After 4 h, the cell lysates were subjected to immunoprecipitation using an anti-eIF3c antibody, followed by immunoblot analysis using an anti-Hsp90 antibody. (D) eIF3c or Hsp90 co-immunoprecipitates with Hsp90 or eIF3c from cells transfected or untransfected with pHCV IRES-FL. Huh-7 cells were transfected with pHCV IRES-FL using Lipofectamine 2000 (Invitrogen) and subject to immunoprecipitation using the indicated antibodies at 24 h post-transfection. The precipitates were analyzed by western blot using the indicated antibodies. (E) The inhibitor 17-AAG dissociates Hsp90 and eIF3c from the HCV IRES complex. Huh-7 cells were transfected with pHCV IRES-FL using Lipofectamine 2000 (Invitrogen). At 24 h post-transfection, cells were treated with 17-AAG (50 nM) or DMSO as a control for 8 h and were then treated with MG132 (5 μM) for 16 h. Cell lysates were subjected to immunoprecipitation using an anti-eIF3c antibody, and the precipitates were analyzed by western blot using the anti-Hsp90 antibody.

(anti-FLAG antibody) were determined by western blot analysis (Fig. 4A). The western blot analysis clearly indicated that eIF3c and Hsp90 coprecipitated in NNC#2 cells, whereas they did not coprecipitate in Huh-7 cells, suggesting that eIF3c was bound to the chaperone complex that formed with Hsp90 in NNC#2 cells (Fig. 4A). This interaction between Hsp90 and eIF3c in NNC#2 cells suggests that HCV translation is due to the interaction between eIF3c and Hsp90. Given the observed binding of eIF3 with the HCV IRES RNA, this Hsp90-eIF3c interaction occurring in HCV replicon cells was likely mediated by HCV IRES RNA. To address this question, Huh-7 cells were transfected with pHCV IRES-firefly luciferase (HCV IRES-FL) (Fig. 1B) using Lipofectamine 2000 (Invitrogen) and then treated with 17-AAG (50 nM) or DMSO as a control. After treatment, the cell lysates were analyzed by western blot (Fig. 4B). The level of eIF3c was reduced by the 17-AAG treatment in cells transfected with pHCV IRES-FL compared to control DMSO, but eIF3c was not reduced in cells transfected with pHCV IRES-FL. The disruption of Hsp90 activity by the Hsp90 inhibitor, 17-AAG, appears to dissociate eIF3c from the Hsp90-eIF3c-HCV IRES complex and induce the degradation of the free forms of eIF3c. To verify this result, NNC#2 cell lysates were further treated with RNase A (5 U/μL) (Sigma–Aldrich Chemical Co.). After treatment, anti-eIF3c antibody immunoprecipitates were determined by western blot analysis (Fig. 4C). The analysis clearly indicated that eIF3c and Hsp90 coprecipitated in NNC#2 cell lysates, whereas they did not coprecipitate in NNC#2 cells lysates treated with RNase A. The interaction between Hsp90 and eIF3c was interrupted by RNase A treatment. These results suggest that the interaction of eIF3c and Hsp90 is dependent on HCV IRES binding. Next, to further demonstrate whether HCV IRES is required for the interaction between eIF3c and Hsp90, we performed a co-immunoprecipitation assay using the extracts of cells transfected with pHCV IRES-FL. eIF3c co-immunoprecipitated with anti-Hsp90 (Fig. 4D). The interaction of eIF3c and Hsp90 was further confirmed by reverse co-immunoprecipitation of Hsp90 and eIF3c (Fig. 4D). These results also indicated that the interaction of eIF3c and Hsp90 was supported by the HCV IRES. Furthermore, we performed an immunoprecipitation assay to confirm that eIF3c and Hsp90 interaction was influenced by the treatment with 17-AAG in cells transfected with pHCV IRES-FL. Huh-7 cells were transfected with pHCV IRES-FL using Lipofectamine 2000 (Invitrogen). At 24 h post-transfection, the cells were treated with 17-AAG (50 nM) or DMSO as a control for 8 h and then MG132 (5 μM) for 16 h. An immunoprecipitation assay with the cell lysates was performed using the anti-eIF3c antibody, and the precipitates were analyzed by western blot using the anti-Hsp90 antibody. Although treatment with both 17-AAG and MG132 in cells transfected with pHCV IRES-FL resulted in a reduction in the interaction of Hsp90 and eIF3c, eIF3c expression recovered upon treatment with the proteasome inhibitor MG132 (Fig. 4E), but the interaction between

Hsp90 and eIF3c was not restored (Fig. 4E). Furthermore, eIF3c was not detected in cells not treated with MG132 (Fig. 2B), which indicates that eIF3c is a client protein for active Hsp90 (Fig. 2C). In contrast, an interaction between Hsp90 and eIF3c was observed in cells not treated with 17-AAG (Fig. 4E). These results suggest that the interaction between Hsp90 and eIF3c is specific to HCV IRES-expressing cells. However, the interaction of Hsp90 and eIF3c in the presence of 17-AAG, which blocks the association of Hsp90 with eIF3c, may dissociate Hsp90 and eIF3c from the overall HCV IRES complex (Figs. 2B and C, and 4B and E).

In conclusion, our results demonstrate that HCV IRES-mediated translational inhibition occurs through a reduction of eIF3c expression caused by the Hsp90 inhibitor-mediated disruption of the interaction between eIF3c and Hsp90 with HCV IRES RNA. Furthermore, the interaction between Hsp90 and eIF3c requires HCV IRES RNA. Taken together, our results suggest that the interaction between Hsp90 and eIF3c plays an important role in HCV IRES-mediated translation. More experiments are needed to verify the relationship between eukaryotic initiation factor 3 (eIF3) and Hsp90.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

We thank Dr. A. Ryo for providing pCMV-Myc-Ubi. We are grateful to S. Yamaguchi, M. Sato, and Y. Katumura for their excellent technical assistance. This work was supported by a Grant-in-Aid for HCV Research from the Ministry of Health, Labor, and Welfare of Japan, by a Grant-in-Aid for High Technology Research (HTR) from the Ministry of Education, Science, Sports, and Culture of Japan, and a Grant from the Strategic Research Foundation Grant-aided Project for Private Universities from the Ministry of Education, Culture, Sport, Science, and Technology, Japan (MEXT).

References

Aoyagi, K., Ohue, C., Iida, K., Kimura, T., Tanaka, E., Kiyosawa, K., Yagi, S., 1999. Development of a simple and highly sensitive enzyme immunoassay for hepatitis C virus core antigen. J. Clin. Microbiol. 37 (6), 1802–1808.

Bagatell, R., Paine-Murrieta, G.D., Taylor, C.W., Pulcini, E.J., Akinaga, S., Benjamin, J., Whitesell, L., 2000. Induction of a heat shock factor 1-dependent stress response alters the cytotoxic activity of hsp90-binding agents. Clin. Cancer Res. 6 (8), 3312–3318.

Bartenschlager, R., Lohmann, V., 2001. Novel cell culture systems for the hepatitis C virus. Antivir. Res. 52 (1), 1–17.

Collier, A.J., Gallego, J., Klinck, R., Cole, P.T., Harris, S.J., Harrison, G.P., Aboul-Ela, F., Varani, G., Walker, S., 2002. A conserved RNA structure within the HCV IRES eiF3-binding site. Nat. Struct. Biol. 9 (5), 375–380.

Coumailleau, P., Poellinger, L., Gustafsson, J.A., Whitelaw, M.L., 1995. Definition of a minimal domain of the dioxin receptor that is associated with Hsp90 and maintains wild type ligand binding affinity and specificity. J. Biol. Chem. 270 (42), 25291–25300.

Doong, H., Rizzo, K., Fang, S., Kulpa, V., Weissman, A.M., Kohn, E.C., 2003. CAIR-1/BAG-3 abrogates heat shock protein-70 chaperone complex-mediated protein degradation: accumulation of poly-ubiquitinated Hsp90 client proteins. J. Biol. Chem. 278 (31), 28490–28500.

Fraser, C.S., Doudna, J.A., 2007. Structural and mechanistic insights into hepatitis C viral translation initiation. Nat. Rev. Microbiol. 5 (1), 29–38.

Garcia-Cardena, G., Fan, R., Shah, V., Sorrentino, R., Cirino, G., Papapetropoulos, A., Sessa, W.C., 1998. Dynamic activation of endothelial nitric oxide synthase by Hsp90. Nature 392, 821–824.

Grakoui, A., Wychowski, C., Lin, C., Feinstone, S.M., Rice, C.M., 1993. Expression and identification of hepatitis C virus polyprotein cleavage products. J. Virol. 67 (3), 1385–1395.

Guo, F., Rocha, K., Bali, P., Pranpat, M., Fiskus, W., Boyapalle, S., Kumaraswamy, S., Balasis, M., Greedy, B., Armitage, E.S., Lawrence, N., Bhalla, K., 2005. Abrogation of heat shock protein 70 induction as a strategy to increase antileukemia activity of heat shock protein 90 inhibitor 17-allylamino-demethoxy geldanamycin. Cancer Res. 65 (22), 10536–10544.

Hijikata, M., Mizushima, H., Shimotohno, K., 1993. Two distinct proteinase activities required for the processing of a putative nonstructural precursor protein of hepatitis C virus. J. Virol. 67 (8), 4665–4675.

Hinnebusch, A.G., 2006. eIF3: a versatile scaffold for translation initiation complexes. Trends Biochem. Sci. 10, 553–562.

Ishii, N., Watashi, K., Hishiki, T., Goto, K., Inoue, D., Hijikata, M., Wakita, T., Kato, N., Shimotohno, K., 2006. Diverse effects of cyclosporine on hepatitis C virus strain replication. J. Virol. 80 (9), 4510–4520.

Julien, P., Rodolfo, R., Jan, M., Isabel, A., Jerome, B., Emmanuel, D., Florence, B., 2009. Human initiation factor eIF3 subunit b interacts with HCV IRES RNA through its N-terminal RNA recognition motif. FEBS Lett. 583 (1), 70–74.

Kieft, J.S., Zhou, K., Jubin, R., Doudna, J.A., 2001. Mechanism of ribosome recruitment by hepatitis C IRES RNA. RNA 7 (2), 194–206.

Kieft, J.S., Zhou, K., Grech, A., Jubin, R., Doudna, J.A., 2002. Crystal structure of an RNA tertiary domain essential to HCV IRES-mediated translation initiation. Nat. Struct. Biol. 9 (5), 370–374.

Kim, T.S., Jang, K., Kim, C.-Y., Lee, H.D., Ahn, J.Y., Kim, B.-Y., 2006. J. Interaction of Hsp90 with ribosomal proteins protects from ubiquitination and proteasome-dependent degradation. Mol. Biol. Cell 17 (2), 824–833.

Lukavsky, P.J., 2008. Structure and function of HCV IRES domains. Virus Res. 139 (2), 166–171.

Masutani, M., Sonenberg, N., Yokoyama, S., Imataka, H., 2007. Reconstitution reveals the functional core of mammalian eIF3. EMBO J. 26 (14), 3373–3383.

Morimoto, R.I., 1998. Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. Genes Dev. 12 (24), 3788–3796.

Nagata, Y., Anan, T., Yoshida, T., Mizukami, T., Taya, Y., Fujiwara, T., Kato, H., Saya, H., Nakao, M., 1999. The stabilization mechanism of mutant-type p53 by impaired ubiquitination: the loss of wild-type p53 function and the hsp90 association. Oncogene 18 (44), 6037–6049.

Neckers, L., 2003. Development of small molecule Hsp90 inhibitors: utilizing both forward and reverse chemical genomics for drug identification. Curr. Med. Chem. 10 (9), 733–739.

Nakagawa, S., Umehara, T., Matsuda, C., Kuge, S., Sudoh, M., Kohara, M., 2007. Hsp90 inhibitors suppress HCV replication in replicon cells and humanized liver mice. Biochem. Biophys. Res. Commun. 353 (4), 882–888.

Okamoto, T., Nishimura, Y., Ichimura, T., Suzuki, K., Miyamura, T., Suzuki, T., Moriishi, K., Matsuura, Y., 2006. hepatitis C virus RNA replication is regulated by FKBP8 and Hsp90. EMBO J. 25 (20), 5015–5025.

Richter, K., Buchner, J., 2001. Hsp90, chaperoning signal transduction. J. Cell. Physiol. 188 (3), 281–290.

Sato, S., Fujita, N., Tsuruo, T., 2000. Modulation of Akt kinase activity by binding to Hsp90. Proc. Natl. Acad. Sci. U.S.A. 97 (20), 10832–10837.

Schulte, T.W., Blagosklonny, M.V., Ingui, C., Neckers, L., 1995. Disruption of the Raf-1-Hsp90 molecular complex results in destabilization of Raf-1 and loss of Raf-1-Ras association. J. Biol. Chem. 270 (41), 24585–24588.

Siridechadilok, B., Fraser, C.S., Hall, R.J., Doudna, J.A., Nogales, E., 2005. Structural roles for human translation factor eIF3 in initiation of protein synthesis. Science 310, 1513–1515.

Spahn, C.M., Kieft, J.S., Grassucci, R.A., Penczek, P.A., Zhou, K., Doudna, J.A., Frank, J., 2001. Hepatitis C virus IRES RNA-induced changes in the conformation of the 40s ribosomal subunit. Science 291, 1959–1962.

Taylor, D.R., Shi, S.T., Tomano, P.R., Barber, G.N., Lai, M.M., 1999. Inhibition of the interferon-inducible protein kinase PKR by HCV E2 protein. Science 285, 107–110.

Ujino, S., Yamaguchi, S., Shimotohno, K., Takaku, H., 2009. Heat-shock protein 90 is essential for stabilization of the hepatitis C virus nonstructural protein NS3. J. Biol. Chem. 284 (11), 6841–6846.

Ujino, S., Yamaguchi, S., Shimotohno, K., Takaku, H., 2010. Combination therapy for hepatitis C virus with heat-shock protein 90 inhibitor 17-AAG and proteasome inhibitor MG132. Antivir. Chem. Chemother. 20 (4), 161–167.

Waxman, L., Whitney, M., Pollok, B.A., Kuo, L.C., Darke, P.L., 2001. Host cell factor requirement for hepatitis C virus enzyme maturation. Proc. Natl. Acad. Sci. U.S.A. 98 (24), 13931–13935.

Waza, M., Adachi, H., Katsuno, M., Minamiyama, M., Sang, C., Tanaka, F., Inukai, A., Doyu, M., Sobue, G., 2005. 17-AAG, an Hsp90 inhibitor, ameliorates polyglutamine-mediated motor neuron degeneration. Nat. Med. 11 (10), 1088–1095.

Xu, W., Mimnaugh, E., Rosser, M.F., Nicchitta, C., Marcu, M., Yarden, Y., Neckers, L., 2001. Sensitivity of mature Erbb2 to geldanamycin is conferred by its kinase domain and is mediated by the chaperone protein Hsp90. J. Biol. Chem. 276 (5), 3702–3708.

Zhou, M., Sandercock, A.M., Fraser, C.S., Ridlova, G., Stephens, E., Schenauer, M.R., Yokoi-Fong, T., Barsky, D., Leary, J.A., Hershey, J.W., Doudna, J.A., Robinson, C.V., 2008. Mass spectrometry reveals modularity and a complete subunit interaction map of the eukaryotic translation factor eIF3. Proc. Natl. Acad. Sci. U.S.A. 105 (47), 18139–18144.

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 286, NO. 12, pp. 10051–10057, March 25, 2011

Heat Shock Protein 70 Inhibits HIV-1 Vif-mediated Ubiquitination and Degradation of APOBEC3G*

Received for publication, July 19, 2010, and in revised form, January 10, 2011 Published, JBC Papers in Press, January 12, 2011, DOI 10.1074/jbc.M110.166108

Ryuichi Sugiyama[‡], Hironori Nishitsuji[‡], Ayako Furukawa[§], Masato Katahira[§1], Yuichiro Habu[¶], Hiroaki Takeuchi[‖], Akihide Ryo[**], and Hiroshi Takaku[‡2]

From the [‡]Department of Life and Environmental Sciences, Chiba Institute of Technology, 2-17-1 Tsudanuma, Narashino, Chiba 275-0016, Japan, the [§]Institute of Advanced Energy, Kyoto University, Gokasho, Uji, Kyoto 611-0011, Japan, the [¶]Department of Microbiology, Immunology, and Pathology, Colorado State University, Fort Collins, Colorado 80523-1619, the [‖]International Research Center for Infectious Diseases, The Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan, and the [**]Department of Microbiology, Yokohama City University School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 236-0004, Japan

The cytidine deaminase APOBEC3G, which is incorporated into nascent virus particles, possesses potent antiviral activity and restricts Vif-deficient HIV-1 replication at the reverse transcription step through deamination-dependent and -independent effects. HIV-1 Vif counteracts the antiviral activity of APOBEC3G by inducing APOBEC3G polyubiquitination and its subsequent proteasomal degradation. In this study, we show that overexpression of heat shock protein 70 (HSP70) blocked the degradation of APOBEC3G in the ubiquitin-proteasome pathway by HIV-1 Vif, rendering the viral particles non-infectious. In addition, siRNA targeted knock-down of HSP70 expression enhanced the Vif-mediated degradation of APOBEC3G. A co-immunoprecipitation study revealed that overexpression of HSP70 inhibited APOBEC3G binding to HIV-1 Vif. Thus, we provide evidence for a host protein-mediated suppression of HIV-1 replication in an APOBEC3G-dependent manner.

Human immunodeficiency virus type-1 (HIV-1),[3] the retrovirus that causes AIDS, efficiently replicates within human $CD4^+$ T cells. However, Vif-deficient virions produced by non-permissive cells, including $CD4^+$ T cells and immortalized lines, such as Hut78 or CEM, are non-infections, whereas virions produced in permissive cells, such as SupT1 or CEM-SS, are infectious (1, 2). Previous studies have demonstrated that HIV-1 Vif counteracts the innate antiviral activity of APOBEC3G, a member of the APOBEC family of cytidine deaminase-editing enzymes (3). In the absence of Vif, APOBEC3G induces the deamination of cytidine (C) and its conversion to uridine (U) (4, 5), which can be packaged into budding retroviruses through a direct interaction with the Gag polyprotein (6–11). The C to U conversion in the HIV-1 minus strand leads to a G to A hypermutation, preferentially at CCCA sequences. This motif corresponds to TGGG in the plus-strand sequence, thereby mutating the TGG tryptophan codon to a TAG stop codon and affecting subsequent stages of the viral life cycle (12). Vif predominant mechanism for overcoming the antiviral activity of APOBEC3G is to form an E3 ubiquitin ligase with cullin 5 (Cul5), elongin B (EloB), and elongin C (EloC) and target these proteins for degradation by the ubiquitin-proteasome pathway (13–16). Vif may also inhibit APOBEC3G activity through mechanisms independent of proteasomal degradation (17–19).

Heat shock proteins play critical roles in the life cycle of a variety of RNA and DNA viruses (20–23). For example, heat shock protein 70 (HSP70) is specifically incorporated into HIV-1 virions (24). However, the formation of the P-TEFb/Tat/TAR complex is required to stabilize the CDK9/cyclinT1 heterodimer by HSP70 and HSP90 (25).

To better develop potential novel therapeutic strategies to exploit the APOBEC3G antiviral function, we investigated the role of HSP70 in APOBEC3G function. We found that siRNA against HSP70 significantly reduced the level of APOBEC3G in the presence of HIV-1 Vif, but not in the absence of Vif. In addition, overexpression of HSP70 in 293T cells reduced the Vif-mediated degradation of APOBEC3G by inhibiting APOBEC3G polyubiquitination. This effect is attributed to the impairment of APOBEC3G-Vif binding. Furthermore, overexpression of HSP70 in the presence, but not in the absence, of APOBEC3G clearly suppressed the infectivity of virions in a dose-dependent manner. These results suggest that HSP70 acts as a potential antiviral host factor through interaction with APOBEC3G and may form the basis for new anti-HIV-1 therapies.

EXPERIMENTAL PROCEDURES

Immunoprecipitation—293T cells (5×10^5) were transfected with 1.0 μg of each Vif expression plasmid using Lipofectamine2000 (Invitrogen). At 48 h post-transfection, cells were suspended in a lysis buffer (50 mM Tris-HCl, pH 7.0, 150 mM NaCl, 1% Nonidet P-40, and 10% glycerol) and incubated with 5 μl of anti-HSP70 antibody (Santa Cruz Biotechnology) and 30 μl of Dynabeads-protein G (Invitrogen). The beads were

* This work was supported in part by a Grant-in-aid for High Technology Research (No. 09309011) from the Ministry of Education, Science, Sports, and Culture of Japan and by a grant-in-aid for AIDS research from the Ministry of Health, Labor, and Welfare, Japan.
[1] Supported by Grants from MEXT (21370047 & 22121517), JST-SENTAN, Naito Foundation, and YCU-AMRC.
[2] To whom correspondence should be addressed: 2-17-1 Tsudanuma, Narashino, Chiba 275-0016, Japan. Tel.: 81-47-478-0407; Fax: 81-47-471-8764; E-mail: hiroshi.takaku@it-chiba.ac.jp.
[3] The abbreviations used are: HIV-1, human immunodeficiency virus type-1; RNP, ribonucleoprotein; HSP, heat shock protein; Ub, ubiquitin.

washed with PBS containing 0.02% Tween 20. The immunocomplex was eluted by boiling with 20 μl of 5× sample buffer and analyzed by SDS-PAGE and Western blot.

Protein Stability Assay—293T cells (5×10^5) were co-transfected with 1.0 μg of pc-Hu-APOBEC3G-HA and 0.5 μg of a GFP expression plasmid (CS-CDF-CG-PRE), or 1.0 μg of pc-Hu-APOBEC3G-HA, 0.5 μg of CS-CDF-CG-PRE and 0.5 μg of pcDNA-Vif along with either 2.0 μg of pFLAG-HSP70 or an empty plasmid. At 24 h post-transfection, cells were treated with 100 μg/ml of cycloheximide. Cells were harvested, and cell lysates were analyzed by Western blotting with horseradish peroxidase-conjugated anti-HA antibody (Roche Diagnostics) and anti-GFP antibody (Medical & Biological Laboratories). The blots were semi-quantified using ImageJ 1.43u software.

Polyubiquitination Assay—293T cells (3×10^6) were co-transfected with 2.0 μg of pc-Hu-APOBEC3G-HA, 2.0 μg of pVif-V5, 4.0 μg of pFLAG-HSP70, and 2.0 μg of pCMV-Myc-Ubi (26). At 24 h post-transfection, cells were treated with 5 μM MG-132 for 24 h. Cells were suspended in a lysis buffer. Cell lysates were immunoprecipitated using anti-Myc antibody (Cell Signaling) followed by Western blotting with horseradish peroxidase-conjugated anti-HA antibody.

MAGI Assay—MAGI cells were plated in 96-well plates at 1×10^4 cells per well in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. The next day, cells were infected with dilutions of the virus in a total volume of 50 μl in the presence of 20 μg/ml DEAE-dextran for 2 h. At 2 days post-infection, cells were fixed with 100 μl of fix solution (1% formaldehyde/0.2% glutaraldehyde in PBS) at room temperature for 5 min and then washed twice with PBS. Cells were incubated with 100 μl of staining solution (4 mM potassium ferrocyanide, 4 mM potassium ferricyanide, 2 mM $MgCl_2$, and 0.4 mg/ml X-Gal) for 50 min at 37 °C. The reaction was stopped by removing the staining solution, and blue cells were counted under a microscope.

Construction of Plasmids—To generate pcDNA-Vif, HIV-1 Vif fragments were amplified from pNL4-3 by PCR with the following primers: forward 5′-GAT ATC ATG GAA AAC AGA TGG CAG GTG ATG-3′ and reverse 5′-CTC GAG CTA GTG TCC ATT CAT TGT ATG CT-3′. The PCR products were inserted into pcDNA3.1 (Invitrogen).

To construct pFLAG-HSP70, whole RNA was isolated from 293T cells with TRIzol (Invitrogen) and amplified by RT-PCR with the following primers: forward 5′-GTT GAA TTC CGC CAA AGC CGC GGC GAT-3′ and reverse 5′-CGC GGA TCC CTA ATC TAC CTC CTC AAT-3′. The products were inserted into pFLAG-CMV2 (Sigma).

To generate pVif-V5, HIV-1 Vif fragments were amplified from pNL4-3. The PCR products were inserted into pENTR using TOPO Cloning kits (Invitrogen) and transferred into pLenti6/V5-DEST (Invitrogen) by LR recombination. This construct contains the β-globin intron sequence of pMDL-g/p-RRE downstream of the CMV promoter. A plasmid construct encoding human APOBEC3G tagged with the influenza hemagglutinin (HA) sequence was a kind gift from Darlene Chen (The Salk Institute for Biological Studies). Vif-defective variants of NL4-3 have been described previously (27).

To generate pCS-U6, the U6 promoter was amplified by PCR. The resulting products were inserted into pCS-CDF-CG-PRE. pCS-U6-shControl or pCS-U6-shHSP70 was constructed by ligating the annealed product of sense oligonucleotide 5′-GAT CCT TCT CCG AAC GTG TCA CGT TTC AAG AGA ACG TGA CAC GTT CGG AGA ATT T-3′ and antisense oligonucleotide 5′-CTA GAA ATT CTC CGA ACG TGT CAC GTT CTC TTG AAA CGT GAC ACG TTC GGA GAA G-3′ or sense oligonucleotide 5′-GAT CCC ACG GCA AGG TGG AGA TCA TTC AAG AGA TGA TCT CCA CCT TGC CGT GTT T-3′ and antisense oligonucleotide 5′-CTA GAA ACA CGG CAA GGT GGA GAT CAT CTC TTG AAT GAT CTC CAC CTT GCC GTG G-3′, respectively, with the BamHI-XbaI fragment from pCS-U6. These plasmids contain a transcriptional termination signal sequence downstream of the shControl and shHSP70 sequences.

Transfection of siRNA—293T cells (3×10^6) were transfected with siRNAs (100 nM) using Lipofectamine2000. Control siRNA (5′-UUC UCC GAA CGU GUC ACG UdTdT-3′) and HSP70-siRNA (5′-CAC GGC AAG GUG GAG AUC AdTdT-3′) were purchased from B-Bridge International.

Preparation of Lentiviral Vectors—293T cells (5×10^5) were cotransfected with the lentiviral vector (1.6 μg), vesicular stomatitis virus G expression vector pMD.G (0.4 μg), *rev* expression vector pRSV-Rev (0.8 μg), and *gag-pol* expression vector pMDLg/pRRE (1.2 μg) using Lipofectamine2000. At 48 h after transfection, culture supernatants were harvested and filtered through 0.45-μm filters. In all experiments, H9 cells (3×10^5) were transduced with equal amounts of the lentivirus vector.

Statistical Analysis—The results are shown as means ± S.D., and statistical analysis was performed using the paired Student's *t* test. A p value of <0.05 was considered significant. At least three replicates were performed for each experiment.

RESULTS

HSP70 Leads to Stabilization of APOBEC3G—HSPs are induced by a variety of stress-related stimuli, including heat, UV radiation, and microbial/viral infections (28). HSPs are involved in the folding and translocation of cellular proteins under normal conditions, whereas under stressful conditions, HSPs bind to proteins and inhibit their misfolding or irreversible aggregation (29). Recent studies revealed that the binding of HSPs to HIV-1 proteins enhances antiviral immunity (30). HSP70 is selectively expressed soon after HIV-1 infection, suggesting that these proteins might be involved in the innate cellular antiviral immune response (31). However, the specific targets of HSPs and their role in the response to HIV infection remain unclear.

HIV-1 Vif targets APOBEC3G for ubiquitination by forming an Skp1-cullin-F-box (SCF)-like complex, which subsequently leads to the degradation of APOBEC3G. To evaluate how HSP70 affects Vif-dependent ubiquitination and degradation, we examined the steady-state level of APOBEC3G in 293T cells co-transfected with FLAG-tagged HSP70 (FLAG-HSP70) and pNL4-3. The expression of HSP70 in 293T cells significantly increased the amount of APOBEC3G in a dose-dependent manner but not the amount of the HIV-1 Gag p24 antigen (Fig. 1*A*, *lanes 1–4* and Fig. 1*B*). Importantly, HSP70 had no effect on

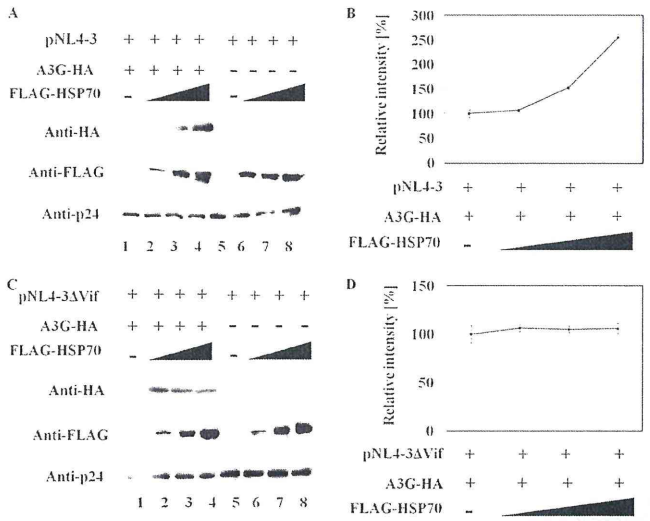


FIGURE 1. **Expression of FLAG-tagged HSP70 blocks APOBEC3G degradation in cells transfected with pNL4-3, but not those transfected with pNL4-3-delta-Vif.** 293T cells (5×10^5) were co-transfected with 1.0 μg of pc-Hu-APOBEC3G-HA and increasing amounts of pFLAG-HSP70 (0, 0.5, 1.0, or 2.0 μg), adjusted with an empty vector to 2.0 μg of total, along with either 0.1 μg of pNL4-3 (*A*) or 0.1 μg of pNL4-3-delta-Vif (*C*). At 48 h post-transfection, cell lysates were analyzed by Western blotting. The relative intensity of APOBEC3G-HA bands was determined by densitometry (*B* and *D*). Results are representative of three independent experiments, and error bars show the standard deviations of the means.

the expression level of APOBEC3G in 293T cells transfected with Vif-deleted HIV-1 proviral plasmid (Fig. 1, *C*, *lanes 1–4* and *D*). Our results suggest that HSP70 may inhibit the degradation of APOBEC3G by HIV-1 Vif.

HSP70 Blocks HIV-1 Vif-mediated Degradation of APOBEC3G—Next, we investigated whether expression of HSP70 directly blocks APOBEC3G degradation by HIV-1 Vif. 293T cells were co-transfected with pc-Hu-APOBEC3G-HA and pFLAG-HSP70 in the absence or presence of pcDNA-Vif. We found that the steady-state levels of APOBEC3G in the presence of pcDNA-Vif were increased by the expression of HSP70 in a dose-dependent manner (Fig. 2, *A*, *lanes 6–10* and *B*, *right panel*). By contrast, HSP70 did not significantly affect the amount of APOBEC3G expression in the absence of pcDNA-Vif (Fig. 2, *A*, *lanes 1–5* and *B*, *left panel*). These data indicate that the effects of HSP70 on APOBEC3G expression depend on HIV-1 Vif.

Previous studies have reported that microbial HSP70 up-regulates APOBEC3G mRNA (32, 33). To rule out this possibility, pulse-chase experiments were performed using 293T cells that were co-transfected with pc-Hu-APOBEC3G-HA, pVif-V5, pFLAG-HSP70, and a GFP expression plasmid (CS-CDF-CG-PRE). Cycloheximide was used to block protein synthesis. When 293T cells were transfected with pc-Hu-APOBEC3G-HA alone, there was no change in the level of APOBEC3G (Fig. 2, *C*, *lanes 1–4* and *D*, *left panel*). Consistent with previous reports, degradation of APOBEC3G-HA was induced in the presence of HIV-1 Vif (Fig. 2, *C*, *lanes 5–8* and *D*, *middle panel*). In contrast, HSP70 expression significantly suppressed the degradation of APOBEC3G by HIV-1 Vif (Fig. 2, *C*, *lanes 9–12* and *D*, *right panel*). To further evaluate whether HSP70 expression inhibits the ubiquitination of APOBEC3G by HIV-1 Vif, we performed ubiquitination assays. Lysates of cells co-expressing pVif-V5, Myc-tagged ubiquitin (Myc-Ub), pc-Hu-APOBEC3G-HA and either empty plasmid or pFLAG-HSP70 were analyzed for the polyubiquitination of APOBEC3G. We detected the ubiquitination of APOBEC3G as a ladder band (Fig. 2*E*, *lane 2*). The expression of HSP70 resulted in a significant reduction in polyubiquitinated APOBEC3G (Fig. 2*E*, *lane 1*). Thus, the expression of HSP70 causes an increase in the steady-state levels of APOBEC3G by blocking the Vif-mediated ubiquitination and degradation of APOBEC3G.

HSP70 Interacts with Both APOBEC3G and HIV-1 Vif—We performed an immunoprecipitation assay to evaluate the binding between HSP70 and APOBEC3G or HIV-1 Vif (Fig. 3). 293T cells were transfected with pc-Hu-APOBEC3G-HA, pcDNA-Vif, pNL4-3, or pNL4-3-delta-Vif. Cell lysates were precipitated with anti-HSP70 antibody, followed by immunoblotting with anti-ApoC17 or anti-Vif antibody. HSP70 interacted with both APOBEC3G (Fig. 3*A*) and HIV-1 Vif (Fig. 3*B*). These interactions and the intracellular localization of HSP70 and HA-tagged APOBEC3G were confirmed by immunostaining assays (data not shown). To further investigate the role of HSP70 in APOBEC3G-Vif interactions, 293T cells were co-transfected with pc-Hu-APOBEC3G-HA and pVif-V5 along with either an empty plasmid or pFLAG-HSP70 in the presence of a proteasome inhibitor (MG-132). Consistent with previous studies, HIV-1 Vif was bound to APOBEC3G (Fig. 3*C*, *lane 2*). Strikingly, the expression of HSP70 in 293T cells led to the inhibition of APOBEC3G-Vif binding. (Fig. 3*C*, *lane 1*). Because a previous study reported that APOBEC3G binds the N-terminal region of HIV-1 Vif (34), we tested the hypothesis that HSP70 competes with APOBEC3G for binding to the N-terminal region of HIV-1 Vif. We found that FLAG-HSP70 efficiently co-immunoprecipitated with the N-terminal region of Vif (amino acids 1–107) (Fig. 3*D*, *lane 1*). However, the C-terminal region of Vif (amino acids 108–192) exhibited no detectable interaction with FLAG-HSP70 (Fig. 3*D*, *lane 2*). These results suggest that APOBEC3G-Vif binding is reduced by HSP70 through an interaction with the N-terminal region of Vif, resulting in the inhibition of the Vif-mediated ubiquitination and the degradation of APOBEC3G.

Knock-down of HSP70 in 293T Cells Enhances APOBEC3G Degradation by HIV-1 Vif—To further investigate the effect of endogenous HSP70 on the stability of APOBEC3G, we silenced endogenous HSP70 expression by RNA interference. 293T cells were transfected with control siRNA (siCtrl) or HSP70-specific siRNA (siHSP70) for 4 h prior to transfection along with pc-Hu-APOBEC3G-HA and either pNL4-3 or pNL4-3-delta-Vif. At 48 h post-transfection, cells were harvested and subjected to Western blotting. As expected, the level of APOBEC3G in pNL4-3-transfected cells was less stable than that in the pNL4-3-delta-Vif transfected cells (Fig. 4*A*, compare *lane 1* to *lane 3*). Quantification of the relative intensities revealed that transfection with pNL4-3 induced APOBEC3G degradation with a potency ~1.8 times higher than that of pNL4-3-delta-Vif (Fig. 4*B*). Moreover, in the case of transfection with pNL4-3, the level of APOBEC3G, but not the level of HIV-1 Gag, in the siHSP70-transduced cells were lower than in

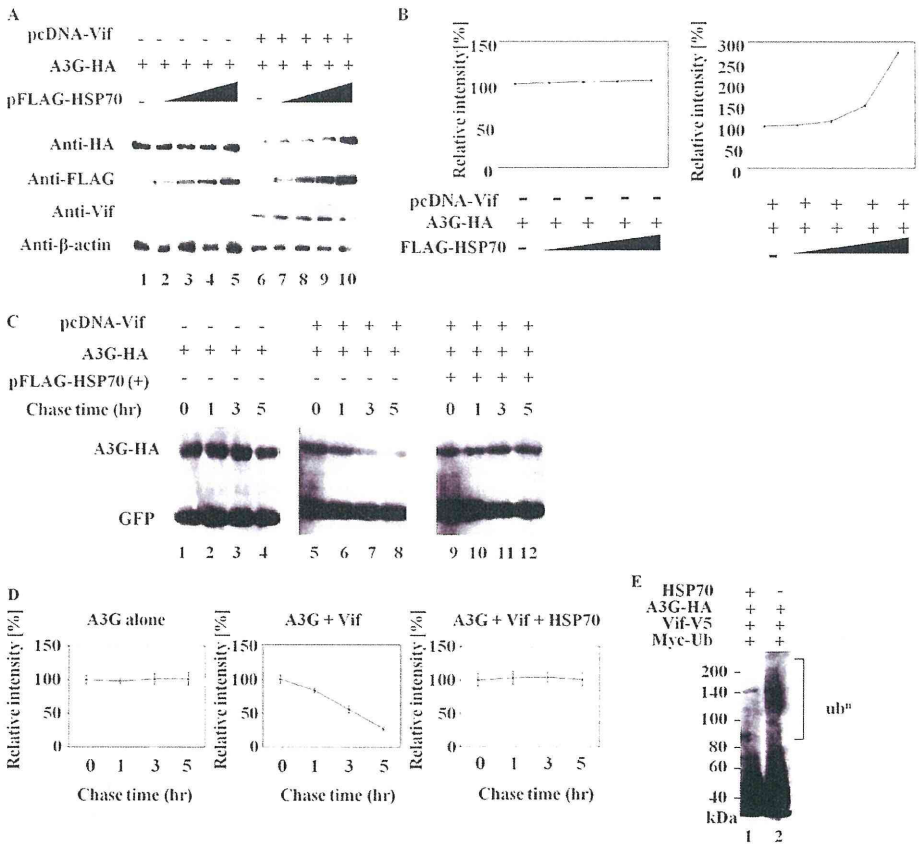


FIGURE 2. **HSP70 expression inhibits Vif-mediated APOBEC3G ubiquitination and degradation.** *A*, 293T cells (5×10^5) were co-transfected with 1.0 μg of pc-Hu-APOBEC3G-HA and increasing amounts of pFLAG-HSP70 (0, 0.5, 1.0, or 2.0 μg), adjusted to 2.0 μg of total DNA with 0.5 μg of an empty plasmid (pcDNA3.1) or pcDNA-Vif. At 48 h post-transfection, cell lysates were subjected to Western blotting and were then analyzed with the indicated antibody. β-Actin was used as a control for protein levels. *B*, relative intensity of APOBEC3G-HA bands in *A* was determined by densitometry. *C*, 293T cells (5×10^5) were transfected with 1.0 μg of pc-Hu-APOBEC3G-HA alone (*lanes 1–4*); 0.5 μg of pcDNA-Vif and 1.0 μg of pc-Hu-APOBEC3G-HA (*lanes 5–8*); and 0.5 μg of pcDNA-Vif, 1.0 μg of pc-Hu-APOBEC3G-HA and 2.0 μg of pFLAG-HSP70 (*lanes 9–12*). The transfected cells were treated with cycloheximide to block *de novo* protein synthesis. The level of APOBEC3G was detected by immunoblotting after cycloheximide treatment lasting 1, 3, or 5 h. CS-CDF-CG-PRE (0.5 μg), which expresses the green fluorescent protein (GFP), was co-transfected with each plasmid into 293T cells as a control plasmid. *D*, relative intensity of APOBEC3G-HA bands in *C* was determined by densitometry. *E*, 293T cells (3×10^6) were co-transfected with 2.0 μg of pCMV-Myc-Ubi, 2.0 μg of pVif-V5, and 2.0 μg of pc-Hu-APOBEC3G-HA along with 4.0 μg of an empty plasmid or pFLAG-HSP70. At 24 h post-transfection, cells were treated with 5 μM MG132. After 24 h, cell lysates were immunoprecipitated with anti-Myc antibody, followed by immunoblotting analysis with horseradish peroxidase-conjugated anti-HA antibody. Results are representative of three independent experiments, and error bars show the standard deviations of the means.

the siCtrl-transduced cells (Fig. 4*A*, compare *lane 1* to *lane 2*). The amount of APOBEC3G in HSP70 knock-down cells decreased to half the amount in the control cells (Fig. 4*B*). However, in terms of transfection with pNL4-3-delta-Vif, treatment with siHSP70 had no effect on the stability of APOBEC3G (Fig. 4*A*, compare *lane 3* to *lane 4*). These data indicate that depletion of HSP70 facilitates Vif-mediated degradation of APOBEC3G.

HSP70 Suppresses HIV-1 Vif-mediated Degradation of Endogenous APOBEC3G in Non-permissive Cells—Most experiments in this study used permissive cells. To investigate whether our findings have physiologic relevance in non-permissive cells, we used a lentiviral vector encoding FLAG-HSP70 or HIV-1 Vif-V5. In the absence of Vif-V5, there was no significant effect of FLAG-HSP70 on the level of endogenous APOBEC3G in H9 cells (Fig. 5*A*, compare *lane 1* to *lane 2*). When Vif-V5 was expressed in H9 cells, expression of FLAG-HSP70 increased the amount of endogenous APOBEC3G (Fig. 5*A*, compare *lane 3* to *lane 4*). Next, we suppressed the expression of HSP70 using a lentiviral vector to express shHSP70 under the control of the human U6 promoter in H9 cells. APOBEC3G expression in shHSP70-transduced H9 cells was similar to that in shControl-transduced H9 cells (Fig. 5*B*, compare *lane 1* to *lane 2*). The level of endogenous APOBEC3G was lower in H9 cells transduced with shHSP70 than in H9 cells transduced with shControl by expression of Vif-V5 (Fig. 5*B*, compare *lane 3* to *lane 4*). Therefore, HSP70 suppresses Vif-mediated degradation of endogenous APOBEC3G in non-permissive cells.

Expression of HSP70 in the Presence of APOBEC3G Augments APOBEC3G Restriction of HIV-1—To examine whether HSP70 expression influences the function of APOBEC3G, pNL4-3, or pNL4-3-delta-Vif was transfected into 293T cells along with either pFLAG-HSP70 alone or pFLAG-HSP70 and pc-Hu-APOBEC3G-HA. The viral infectivity was measured by MAGI assay. As shown in Fig. 6*A*, expression of FLAG-HSP70 clearly suppressed the infectivity of wild-type HIV-1 in the presence of APOBEC3G in a dose-dependent manner. In the absence of APOBEC3G, FLAG-HSP70 did not affect the infectivity of the wild-type HIV-1. Unexpectedly, HSP70 expression in

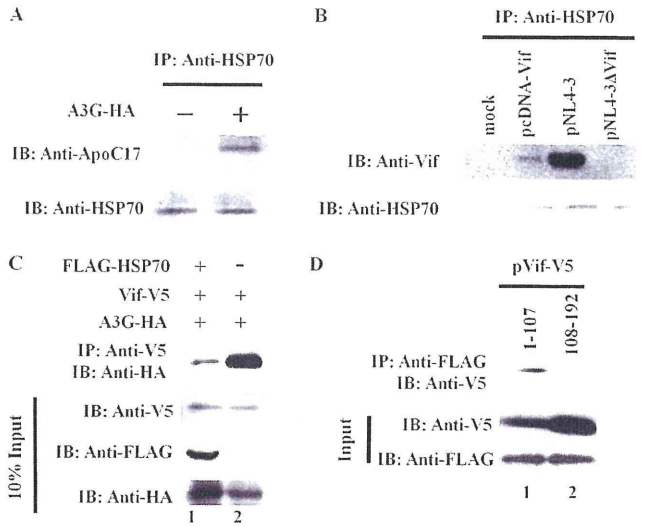


FIGURE 3. **HSP70 interacts with APOBEC3G and HIV-1 Vif.** *A*, 293T cells (5×10^5) were transfected with 1.0 μg of pc-Hu-APOBEC3G-HA. After 48 h, cell lysates were immunoprecipitated with anti-HSP70 antibody, followed by immunoblotting analysis with anti-ApoC17 antibody. *B*, 293T cells (5×10^5) were transfected with 1.0 μg of the indicated plasmids. At 48 h post-transfection, cell lysates were subjected to immunoprecipitation using anti-HSP70 antibody, followed by immunoblotting analysis with anti-Vif antibody. *C*, 293T cells (5×10^5) were co-transfected with 1.0 μg of pc-Hu-APOBEC3G-HA and 1.0 μg of pVif-V5 together with 2.0 μg of either an empty plasmid or pFLAG-HSP70. At 24 h post-transfection, cells were treated with 5 μM MG132. At 24 h post-treatment, cell lysates were immunoprecipitated with anti-V5 antibody, followed by immunoblotting analysis with horseradish peroxidase-conjugated anti-HA antibody. *D*, 293T cells (5×10^5) were co-transfected with 1.0 μg of pFLAG-HSP70 and either 1.0 μg of pVif-1–107-V5 or pVif-108–192-V5. At 48 h post-transfection, cell lysates were immunoprecipitated with anti-FLAG antibody, followed by immunoblotting analysis with anti-V5 antibody. Results are representative of three independent experiments.

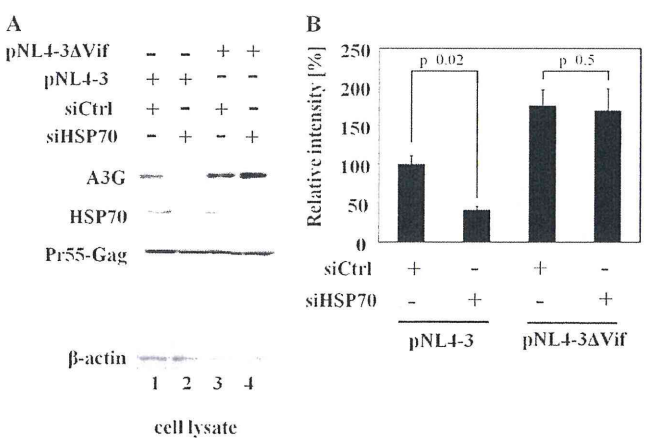


FIGURE 4. **Depletion of HSP70 in 293T cells impairs the stability of APOBEC3G.** *A*, 293T cells (3×10^6) were treated with 100 nM HSP70-siRNA (siHSP70) or 100 nM control-siRNA (siCtrl) for 4 h, prior to co-transfection with 1.0 μg of pc-Hu-APOBEC3G-HA with either 1.0 μg of pNL4-3 or pNL4-3-delta-Vif. At 48 h post-transfection, cell lysates were analyzed by Western blotting using the indicated antibodies. *B*, relative intensity of APOBEC3G bands in *A* was determined by densitometry. Results are representative of three independent experiments, and error bars show the standard deviations of the means.

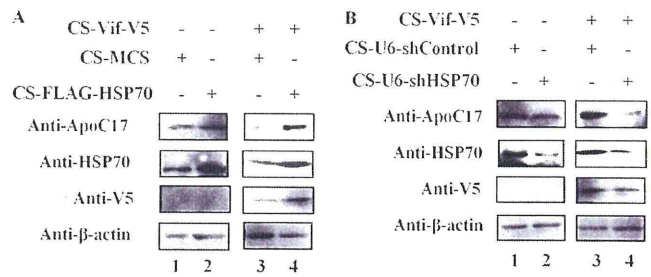


FIGURE 5. **HSP70 affects the level of endogenous APOBEC3G expression in non-permissive T cells expressing HIV-1 Vif.** *A*, H9 cells (3×10^5) were infected with a lentiviral vector encoding an artificial multiple cloning site (MCS) or FLAG-HSP70 in the presence of 8 μg/ml of polybrene. At 48 h after infection, cells were suspended with lysis buffer (*left panel*) or transduced with HIV-1 Vif using a lentivirus vector system (*right panel*). At 48 h post-transduction, cell lysates were analyzed by Western blotting using the indicated antibodies. *B*, H9 cells (3×10^5) were infected with lentivirus-based vectors to express shControl or shHSP70 under the control of the human U6 promoter in the presence of 8 μg/ml of polybrene. At 48 h post-infection, cells were treated as in *A*. Data are representative of three independent experiments.

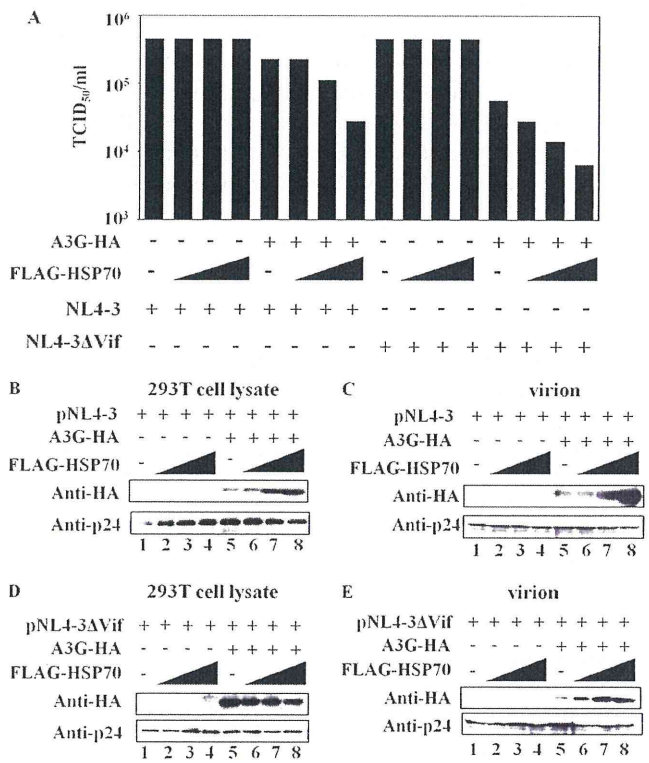


FIGURE 6. **HSP70 regulates HIV-1 infectivity in an APOBEC3G-dependent manner.** *A*, 293T cells (5×10^5) were co-transfected with 0.1 μg of pNL4-3 or pNL4-3-delta-Vif and 1.0 μg of pc-Hu-APOBEC3G-HA alone, pFLAG-HSP70 (0.5, 1.0, or 2.0 μg) alone or 1.0 μg of pc-Hu-APOBEC3G-HA and pFLAG-HSP70 (0.5, 1.0 or 2.0 μg). At 48 h post-transfection, supernatants were harvested, and the amount of each virus was normalized to the equivalent level of p24. MAGI cells (1×10^4) were infected with serially diluting supernatants of each stock of virus, and infected cells were stained with X-Gal 2 days later. 50% tissue culture infective doses (TCID50) is determined by the last virus dilution that is still capable of infecting the cells. *B*, each stock of cell lysate or virus in *A* was subjected to Western blotting and was then analyzed with the indicated antibody. All data are representative of three independent experiments.

APOBEC3G-HA-transfected 293T cells led to a dose-dependent inhibition of the infectivity of Vif-deficient HIV-1 particles (Fig. 6*A*). Moreover, no effect of HSP70 expression on the infectivity of the Vif-deficient HIV-1 particles produced by mock-transfected 293T cells was observed. To further demonstrate whether expression of HSP70 affects virion packaging of APOBEC3G, viral particles produced by 293T cells expressing

HSP70 were analyzed for APOBEC3G expression by Western blotting. We found that expression of HSP70 significantly increased the amount of intracellular and wild type virion-associated APOBEC3G (Fig. 6, *B* and *C*). Interestingly, HSP70 expression enhanced the level of APOBEC3G packaging in Vif-deficient virions, but had no effect on intracellular APOBEC3G and viral release (Fig. 6, *D* and *E*). These results indicate that HSP70 blocks Vif-mediated APOBEC3G degradation and enhances the incorporation of APOBEC3G into both wild type and Vif-deficient virions, which result from inhibition of HIV-1 replication through HSP70 interaction with APOBEC3G.

DISCUSSION

APOBEC3G, which is incorporated into progeny virus particles, restricts the replication of Vif-deficient HIV-1 through cytidine deamination-dependent and independent mechanisms (3–5, 17, 35–41). This restriction can be overcome by HIV-1 Vif, which induces the polyubiquitination of APOBEC3G through recruitment of a ubiquitin E3 ligase complex composed of cullin 5, elongin B, elongin C, and Ring box-1 and facilitates the proteasomal degradation of APOBEC3G (13, 14, 16, 42–45). Thus, mechanistic insights into the quality control of APOBEC3G protein are important for understanding the molecular basis of APOBEC3G-mediated HIV-1 restriction. In this study, we showed that HSP70 suppressed Vif-mediated APOBEC3G degradation. In contrast to our results for HSP70, Pin1 suppresses the HIV restriction activity of APOBEC3G (46). Overexpression of Pin1 reduces the levels of intracellular APOBEC3G. One possibility is that HSP70 regulates Pin1 function, which results in the stimulation of APOBEC3G function, although further analysis is needed to properly address this question.

Pido-Lopez *et al.* (32) have reported that microbial HSP70 up-regulates APOBEC3G mRNA and protein expression in human $CD4^+$ T cells. Our data indicate that in 293T cells, overexpression of human HSP70 in the absence of HIV-1 Vif did not affect the amount of APOBEC3G protein. The stabilization of APOBEC3G is attributed to a reduction in the Vif-dependent polyubiquitination of APOBEC3G (Fig. 2*E*). Whereas we have focused on human HSP70 activity on APOBEC3G stability, it would be interesting to investigate whether human HSP70 can affect the level of endogenous APOBEC3G mRNA.

APOBEC3G associates with ribonucleoprotein (RNP) complexes and is not only dispersed throughout the cytoplasm but is also markedly concentrated in cytoplasmic foci that are identified as mRNA-processing bodies (P bodies) (47). Localization of APOBEC3G in P bodies is not important for its LINE-1 suppression activity (48). However, Y3 and 7SL RNAs, which compose RNP complexes, are required for efficient APOBEC3G packaging (49). Stimulation of cells at 44 °C induces the rapid accumulation of APOBEC3G and many cellular RNA-binding proteins (50). We examined whether HSP70 plays a role in packaging APOBEC3G into virus particles and found that overexpression of HSP70 enhanced APOBEC3G packaging in the absence of Vif (Fig. 6*E*). It is possible that HSP70 interacts with cytoplasmic APOBEC3G, but it remains unclear whether HSP70 induces the accumulation of APOBEC3G in P bodies and increases the association of APOBEC3G with RNP complexes. Further studies will be required to clarify the details of how, where and when HSP70 and APOBEC3G co-localize within cells.

Recently, Nathans *et al.* (51) have identified a small molecule, termed RN-18, that degrades HIV-1 Vif only in the presence of APOBEC3G, resulting in enhanced APOBEC3G abundance and virion incorporation, similar to the function of HSP70. The possibility has been raised that HSP70 may be the target of RN-18. However, HSP70 has no significant effect on HIV-1 Vif expression and leads to the increase of APOBEC3G packaging into virions in a Vif-independent manner. Moreover, RN-18 exhibits a strong dependence on APOBEC3G, whereas HSP70 can interact directly with both HIV-1 Vif and APOBEC3G. Thus, RN-18 probably does not target HSP70. Taken together, the results of the present study suggest that stimulation of innate immunity, such as that mediated by APOBEC3G, may aid in the development of antiviral therapies.

Acknowledgments—We thank Dr. Darlene Chen for providing pc-Hu-APOBEC3G-HA, Dr. Miyoshi Hiroyuki for providing pCS-CDF-CG-PRE, and Haruki Naganuma and Hiroshi Koseki for technical assistance.

REFERENCES

1. Gabuzda, D. H., Lawrence, K., Langhoff, E., Terwilliger, E., Dorfman, T., Haseltine, W. A., and Sodroski, J. (1992) *J. Virol.* **66,** 6489–6495
2. von Schwedler, U., Song, J., Aiken, C., and Trono, D. (1993) *J. Virol.* **67,** 4945–4955
3. Sheehy, A. M., Gaddis, N. C., Choi, J. D., and Malim, M. H. (2002) *Nature* **418,** 646–650
4. Zhang, H., Yang, B., Pomerantz, R. J., Zhang, C., Arunachalam, S. C., and Gao, L. (2003) *Nature* **424,** 94–98
5. Harris, R. S., Bishop, K. N., Sheehy, A. M., Craig, H. M., Petersen-Mahrt, S. K., Watt, I. N., Neuberger, M. S., and Malim, M. H. (2003) *Cell* **113,** 803–809
6. Cen, S., Guo, F., Niu, M., Saadatmand, J., Deflassieux, J., and Kleiman, L. (2004) *J. Biol. Chem.* **279,** 33177–33184
7. Svarovskaia, E. S., Xu, H., Mbisa, J. L., Barr, R., Gorelick, R. J., Ono, A., Freed, E. O., Hu, W. S., and Pathak, V. K. (2004) *J. Biol. Chem.* **279,** 35822–35828
8. Alce, T. M., and Popik, W. (2004) *J. Biol. Chem.* **279,** 34083–34086
9. Douaisi, M., Dussart, S., Courcoul, M., Bessou, G., Vigne, R., and Decroly, E. (2004) *Biochem. Biophys. Res. Commun.* **321,** 566–573
10. Schäfer, A., Bogerd, H. P., and Cullen, B. R. (2004) *Virology* **328,** 163–168
11. Kremer, M., Bittner, A., and Schnierle, B. S. (2005) *Virology* **337,** 175–182
12. Yu, Q., König, R., Pillai, S., Chiles, K., Kearney, M., Palmer, S., Richman, D., Coffin, J. M., and Landau, N. R. (2004) *Nat. Struct. Mol. Biol.* **11,** 435–442
13. Yu, X., Yu, Y., Liu, B., Luo, K., Kong, W., Mao, P., and Yu, X. F. (2003) *Science* **302,** 1056–1060
14. Mehle, A., Goncalves, J., Santa-Marta, M., McPike, M., and Gabuzda, D. (2004) *Genes Dev.* **18,** 2861–2866
15. Yu, Y., Xiao, Z., Ehrlich, E. S., Yu, X., and Yu, X. F. (2004) *Genes Dev.* **18,** 2867–2872
16. Kobayashi, M., Takaori-Kondo, A., Miyauchi, Y., Iwai, K., and Uchiyama, T. (2005) *J. Biol. Chem.* **280,** 18573–18578
17. Iwatani, Y., Chan, D. S., Wang, F., Maynard, K. S., Sugiura, W., Gronenborn, A. M., Rouzina, I., Williams, M. C., Musier-Forsyth, K., and Levin, J. G. (2007) *Nucleic. Acids Res.* **35,** 7096–7108
18. Stopak, K., de Noronha, C., Yonemoto, W., and Greene, W. C. (2003) *Mol. Cell* **12,** 591–601
19. Mercenne, G., Bernacchi, S., Richer, D., Bec, G., Henriet, S., Paillart, J. C., and Marquet, R. (2010) *Nucleic. Acids Res.* **38,** 633–646
20. Parent, R., Qu, X., Petit, M. A., and Beretta, L. (2009) *Hepatology* **49,**

1798–1809
21. Padwad, Y. S., Mishra, K. P., Jain, M., Chanda, S., Karan, D., and Ganju, L. (2009) *Immunobiology* **214,** 422–429
22. Ujino, S., Yamaguchi, S., Shimotohno, K., and Takaku, H. (2009) *J. Biol. Chem.* **284,** 6841–6846
23. Chase, G., Deng, T., Fodor, E., Leung, B. W., Mayer, D., Schwemmle, M., and Brownlee, G. (2008) *Virology* **377,** 431–439
24. Gurer, C., Cimarelli, A., and Luban, J. (2002) *J. Virol.* **76,** 4666–4670
25. O'Keeffe, B., Fong, Y., Chen, D., Zhou, S., and Zhou, Q. (2000) *J. Biol. Chem.* **275,** 279–287
26. Ryo, A., Suizu, F., Yoshida, Y., Perrem, K., Liou, Y. C., Wulf, G., Rottapel, R., Yamaoka, S., and Lu, K. P. (2003) *Mol. Cell* **12,** 1413–1426
27. Karczewski, M. K., and Strebel, K. (1996) *J. Virol.* **70,** 494–507
28. Neidhardt, F. C., VanBogelen, R. A., and Vaughn, V. (1984) *Annu. Rev. Genet.* **18,** 295–329
29. Bukau, B., Weissman, J., and Horwich, A. (2006) *Cell* **125,** 443–451
30. SenGupta, D., Norris, P. J., Suscovich, T. J., Hassan-Zahraee, M., Moffett, H. F., Trocha, A., Draenert, R., Goulder, P. J., Binder, R. J., Levey, D. L., Walker, B. D., Srivastava, P. K., and Brander, C. (2004) *J. Immunol.* **173,** 1987–1993
31. Wainberg, Z., Oliveira, M., Lerner, S., Tao, Y., and Brenner, B. G. (1997) *Virology* **233,** 364–373
32. Pido-Lopez, J., Whittall, T., Wang, Y., Bergmeier, L. A., Babaahmady, K., Singh, M., and Lehner, T. (2007) *J. Immunol.* **178,** 1671–1679
33. Babaahmady, K., Oehlmann, W., Singh, M., and Lehner, T. (2007) *J. Virol.* **81,** 3354–3360
34. Mehle, A., Wilson, H., Zhang, C., Brazier, A. J., McPike, M., Pery, E., and Gabuzda, D. (2007) *J. Virol.* **81,** 13235–13241
35. Bishop, K. N., Holmes, R. K., and Malim, M. H. (2006) *J. Virol.* **80,** 8450–8458
36. Bishop, K. N., Verma, M., Kim, E. Y., Wolinsky, S. M., and Malim, M. H. (2008) *PLoS Pathog.* **4,** e1000231
37. Guo, F., Cen, S., Niu, M., Yang, Y., Gorelick, R. J., and Kleiman, L. (2007) *J. Virol.* **81,** 11322–11331
38. Lecossier, D., Bouchonnet, F., Clavel, F., and Hance, A. J. (2003) *Science* **300,** 1112
39. Li, X. Y., Guo, F., Zhang, L., Kleiman, L., and Cen, S. (2007) *J. Biol. Chem.* **282,** 32065–32074
40. Mangeat, B., Turelli, P., Caron, G., Friedli, M., Perrin, L., and Trono, D. (2003) *Nature* **424,** 99–103
41. Suspène, R., Sommer, P., Henry, M., Ferris, S., Guétard, D., Pochet, S., Chester, A., Navaratnam, N., Wain-Hobson, S., and Vartanian, J. P. (2004) *Nucleic. Acids Res.* **32,** 2421–2429
42. Sheehy, A. M., Gaddis, N. C., and Malim, M. H. (2003) *Nat. Med.* **9,** 1404–1407
43. Marin, M., Rose, K. M., Kozak, S. L., and Kabat, D. (2003) *Nat. Med.* **9,** 1398–1403
44. Conticello, S. G., Harris, R. S., and Neuberger, M. S. (2003) *Curr. Biol.* **13,** 2009–2013
45. Mehle, A., Strack, B., Ancuta, P., Zhang, C., McPike, M., and Gabuzda, D. (2004) *J. Biol. Chem.* **279,** 7792–7798
46. Watashi, K., Khan, M., Yedavalli, V. R., Yeung, M. L., Strebel, K., and Jeang, K. T. (2008) *J. Virol.* **82,** 9928–9936
47. Gallois-Montbrun, S., Kramer, B., Swanson, C. M., Byers, H., Lynham, S., Ward, M., and Malim, M. H. (2007) *J. Virol.* **81,** 2165–2178
48. Niewiadomska, A. M., Tian, C., Tan, L., Wang, T., Sarkis, P. T., and Yu, X. F. (2007) *J. Virol.* **81,** 9577–9583
49. Wang, T., Tian, C., Zhang, W., Luo, K., Sarkis, P. T., Yu, L., Liu, B., Yu, Y., and Yu, X. F. (2007) *J. Virol.* **81,** 13112–13124
50. Gallois-Montbrun, S., Holmes, R. K., Swanson, C. M., Fernández-Ocaña, M., Byers, H. L., Ward, M. A., and Malim, M. H. (2008) *J. Virol.* **82,** 5636–5642
51. Nathans, R., Cao, H., Sharova, N., Ali, A., Sharkey, M., Stranska, R., Stevenson, M., and Rana, T. M. (2008) *Nat. Biotechnol.* **26,** 1187–1192