Alternative endocytosis pathway for productive entry of hepatitis C virus	Matsuda M, Suzuki R, Kataoka C, Watashi K, Aizaki H, Kato N, Matsuura Y, Suzuki T, Wakita T	J Gen Virol 95(Pt 12):2658-67	2014	国際
Cyclophilin inhibitors reduce phosphorylation of RNA-dependent protein kinase to restore expression of IFN-stimulated genes in HCV-infected cells	Daito T, Watashi K*, Sluder A, Ohashi H, Nakajima S, Borroto-Esoda K, Fujita T, Wakita T (* corresponding author)	Gastroenterology 147(2):463-72	2014	国際
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III. 研究成果の刊行物・別刷

Gastroenterology 2014;147:463–472

Cyclophilin Inhibitors Reduce Phosphorylation of RNA-Dependent Protein Kinase to Restore Expression of IFN-Stimulated Genes in HCV-Infected Cells

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BACKGROUND & AIMS: Cyclophilin inhibitors are being developed for treatment of hepatitis C virus (HCV) infection. They are believed to inhibit the HCV replication complex. We investigated whether cyclophilin inhibitors interact with interferon (IFN) signaling in cultured cells infected with HCV. **METHODS:** We used immunoblot assays to compare expression of IFN-stimulated genes (ISGs) and of components of IFN signaling in HCV-infected and uninfected cells. **RESULTS:** Incubation with IFN alfa induced expression of ISGs in noninfected cells and, to a lesser extent, in HCV-infected cells; addition of the cyclophilin inhibitor SCY-635 restored expression of ISG products in HCV-infected cells. SCY-635 reduced phosphorylation of double-strand RNA-dependent protein kinase (PKR) and its downstream factor eIF2α; the phosphorylated forms of these proteins are negative regulators of ISG translation. Cyclophilin A interacted physically with PKR; this interaction was disrupted by SCY-635. SCY-635 also suppressed PKR-mediated formation of stress granules. Cyclophilin inhibitors were found to inhibit PKR phosphorylation and stress granule formation in HCV-infected and uninfected cells. **CONCLUSIONS:** In cultured cells, cyclophilin inhibitors reverse the attenuation of the IFN response by HCV, in addition to their effects on HCV replication complex. Cyclophilin A regulation of PKR has been proposed as a mechanism for observed effects of cyclophilin inhibitors on IFN signaling. We found that cyclophilin inhibitors reduce phosphorylation of PKR and eIF2α during HCV infection to allow for translation of ISG products. Proteins in this pathway might be developed as targets for treatment of HCV infection.

Keywords: Signal Transduction; Cyclosporin; Innate Immunity; Replication.

Hepatitis C virus (HCV) infection, which affects approximately 170 million people worldwide, is a leading cause of liver cirrhosis and hepatocellular carcinoma.[1–6] The current standard anti-HCV treatment employs pegylated interferon (IFN) and ribavirin, in combination with newly approved protease inhibitors.[7–10] In addition to these clinically available drugs, a variety of anti-HCV compounds are under clinical development. Direct-acting antiviral agents that target viral proteins to suppress HCV replication include protease inhibitors, polymerase inhibitors, and NS5A inhibitors.[4,11] Host-targeting antiviral agents are alternative classes of anti-HCV candidates that act by inhibiting host factors essential for HCV replication.[3,4,11,12]

Cyclophilin (CyP) inhibitors, including alisporivir (Debio 025), NIM811, and SCY-635, are a class of host-targeting antiviral agents showing a significant anti-HCV effect in HCV-infected patients.[13] These agents target cellular CyPs, which are peptidyl prolyl cis-trans isomerases catalyzing conformational changes in proteins. The CyP family consists of >15 subtypes, including CyPA, CyPB, and CyPD.[14] We initially reported that cyclosporin A (CsA), the prototype CyP inhibitor, suppressed HCV RNA replication in the HCV subgenomic replicon system.[15–18] Subsequent studies suggested that CyPA is likely to be the main CyP acting in HCV RNA replication.[19–22] Although the precise mechanism by which CyPs regulate HCV RNA replication is still under investigation, this protein family is likely to directly regulate the function or formation of the RNA replication machinery.[13]

Interestingly, recent clinical studies of the CyP inhibitors, alisporivir and SCY-635, without IFN showed that the decline of HCV viral load after CyP inhibitor administration was likely to be influenced by interleukin (IL)28B genotype, viral load reduction was drastic in CC genotype patients, and was relatively moderate in CT and TT patients, similar to the case with IFN-based treatment.[23,24] In addition, studies with SCY-635 reported that administration of SCY-635 monotherapy up-regulated the serum levels of IFN-stimulated gene (ISG) protein products in HCV-infected patients. These data suggest that CyP inhibitors may cross talk with the IFN signaling pathway(s) in HCV-infected cells and patients.

In general, the antiviral activity of IFN alfa is mediated by downstream genes of the IFN signaling pathway, which are classified as ISGs.[25–27] IFN-alfa stimulation triggers

Abbreviations used in this paper: **CsA, cyclosporin A; CyP, cyclophilin; HCV, hepatitis C virus; IFN, interferon; IL, interleukin; ISG, IFN-stimulated gene; mRNA, messenger RNA; PKR, double-strand RNA-dependent protein kinase; SG, stress granule; STAT, signal transducers and activators of transcription.**

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the Janus-activated kinase/signal transducers and activators of transcription (STAT) signaling pathway, in which STAT1 and STAT2 are phosphorylated by Janus-activated kinase family proteins and then form a complex with ISGF3γ to translocate into the nucleus. The ISGF3 complex drives IFN-stimulated response element–mediated transcription to induce messenger RNA (mRNA) for ISGs, which is then translated into ISG proteins. Recently, it was reported that protein translation from mRNA for ISGs triggered by IFN, as well as for other host proteins, was negatively regulated in HCV-infected cells by the phosphorylation of double-stranded RNA-dependent protein kinase (PKR) and its downstream target eIF2α.[28–30] It was also reported that the formation of stress granules (SG) triggered by phosphorylated PKR induced translational termination of proteins, including ISGs.[29–31] However, the therapeutic relevance of this phenomenon regulated by phosphorylated PKR has not yet been demonstrated.

An understanding of the mechanisms for antiviral agents is important for predicting the antiviral efficacy, as well as providing appropriate treatment to patients. In this study, we analyzed the interaction of CyP inhibitors and the IFN signaling pathway in HCV-infected cells. CyP inhibitors restored ISG protein production in HCV-infected cells through impairment of PKR phosphorylation. CyPA was involved in the regulation of PKR phosphorylation. CyPA interacted with PKR in both HCV-infected and uninfected cells. SG formation triggered by PKR was inhibited by CyP inhibitors. These findings suggest that CyPA is a functional regulator of the IFN signaling pathway at the translational level, and that CyP inhibitors can unexpectedly exhibit a dual mechanism for their anti-HCV activity.

Materials and Methods

Materials and Methods are shown in the Supplemental Material.

Results

SCY-635 Restored Interferon-Alfa–Induced Interferon-Stimulated Gene Protein Production in Hepatitis C Virus–Infected Cells

In HCV-infected patients, ISG protein production, monitored by 2′5′ oligoadenylate synthetase–1 protein level in serum, was augmented along with SCY-635 concentration (Supplementary Figure 1) as reported previously.[23] Productions of IFN alfa and IFN gamma 1, which are also induced as ISGs,[32] were consistently increased after serum SCY-635 level (Supplementary Figure 1). These ISG protein inductions by SCY-635 treatment were not observed in noninfected healthy volunteers. These data raised the unexpected possibility that SCY-635 facilitated the production of ISG proteins upon HCV infection.

We then investigated the ISG induction triggered by IFN-alfa stimulation of HCV-infected cells. Huh-7 cells were infected with HCV JFH1 at a multiplicity of infection of 0.2 or left uninfected. After 4 days, when >70% of the cell population was HCV infected, the cells were stimulated with IFNα for 16 hours to induce ISG proteins; parallel control cultures were not treated with IFN alfa. Protein production of representative ISGs, ISG15 and MxA, but not of actin as an internal control, was increased after IFN-alfa treatment in both HCV-infected and uninfected cells (Figure 1*A*, compare *lanes 1* and *2*; *lanes 3* and *4*). However, ISG induction in HCV-infected cells was impaired compared with that in uninfected cells (Figure 1*A*, compare *lanes 2* and *4*), consistent

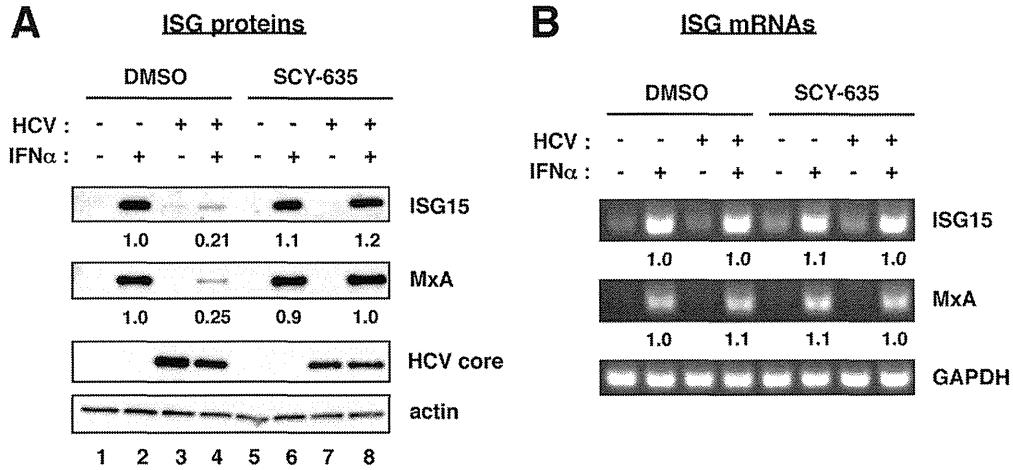


Figure 1. Protein production of ISGs in HCV-infected cells was restored by treatment with a CyP inhibitor, SCY-635. (*A*) HCV-infected (*lanes 3, 4, 7,* and *8*) or uninfected Huh-7 cells (*lanes 1, 2, 5,* and *6*) were pretreated with dimethyl sulfoxide (DMSO), 0.05% (*lanes 1–4*) or SCY-635, 2 μM (*lanes 5–8*) for 24 hours, and then treated with (*lanes 2, 4, 6, 8*) or without (*lanes 1, 3, 5, 7*) IFN alfa, 100 IU/mL for 16 hours (*A*) or 8 hours (*B*). Protein levels of ISG15, MxA, HCV core, and actin were detected by Western blot (*A*). mRNA expression levels of ISG15, MxA, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were detected by reverse transcription polymerase chain reaction (*B*). Relative band intensities (see Materials and Methods) for these proteins and mRNAs by setting those in treatment with IFN alfa plus DMSO as 1.0 are shown below the panels. We performed 3 independent experiments for each figure in this study and obtained similar results in each replicate.

with previous observations.[29] Intriguingly, pretreatment with a CyP inhibitor, SCY-635,[23] restored the IFN-alfa–induced up-regulation of ISG protein production in HCV-infected cells (Figure 1*A*, *lane 8*). In contrast, the mRNA levels for these ISGs were not significantly changed by SCY-635 (Figure 1*B*). These results suggest that SCY-635 restores IFN-alfa–induced ISG induction in HCV-infected cells at a post-transcriptional level.

SCY-635 Inhibited the Phosphorylation of Double-Strand RNA-Dependent Protein Kinase

ISG protein production can be regulated at 2 levels, transcriptionally and post transcriptionally (see Figure 7). Stimulation of cells with type I IFNs induces phosphorylation of STAT1 and STAT2, which then form a complex with ISGF3γ that transactivates gene transcription via the IFN-stimulated response element to induce ISG mRNAs.[33] In addition, the subsequent translational level is regulated by PKR and its downstream target eIF2α.[29,30] To explore the mechanism of ISG-production restoration by SCY-635, we treated HCV-infected cells with another anti-HCV drug, telaprevir,[34] an HCV protease inhibitor, as well as SCY-635, and examined the IFN response of the treated cells. Protein production of ISG15 and ISG56 on IFN-alfa stimulation was augmented in the cells pretreated with SCY-635, however, the effect of telaprevir was less pronounced (Figure 2*Ai* and *ii*, Supplementary Figure 2 for statistics). Given that the anti-HCV effect of telaprevir was greater than that of SCY-635, as monitored by HCV core protein production (Figure 2*Avii*) and HCV RNA level (Supplementary Figure 3*A*) (50% effective concentrations of SCY-635 and telaprevir were 0.51 and 0.36 μM, respectively), these data suggest that the SCY-635 effect on ISG up-regulation was not primarily mediated by the elimination of HCV from the cells, but rather by a more direct interaction of SCY-635 with the IFN pathway. Intriguingly, SCY-635 drastically decreased the level of PKR that was phosphorylated at amino acid threonine 446, without reducing the total amount of PKR (Figure 2*Aiii, iv*). In addition, downstream phosphorylation of eIF2α was consistently inhibited by treatment with SCY-635 (Figure 2*Av*). The modest effect of telaprevir on the

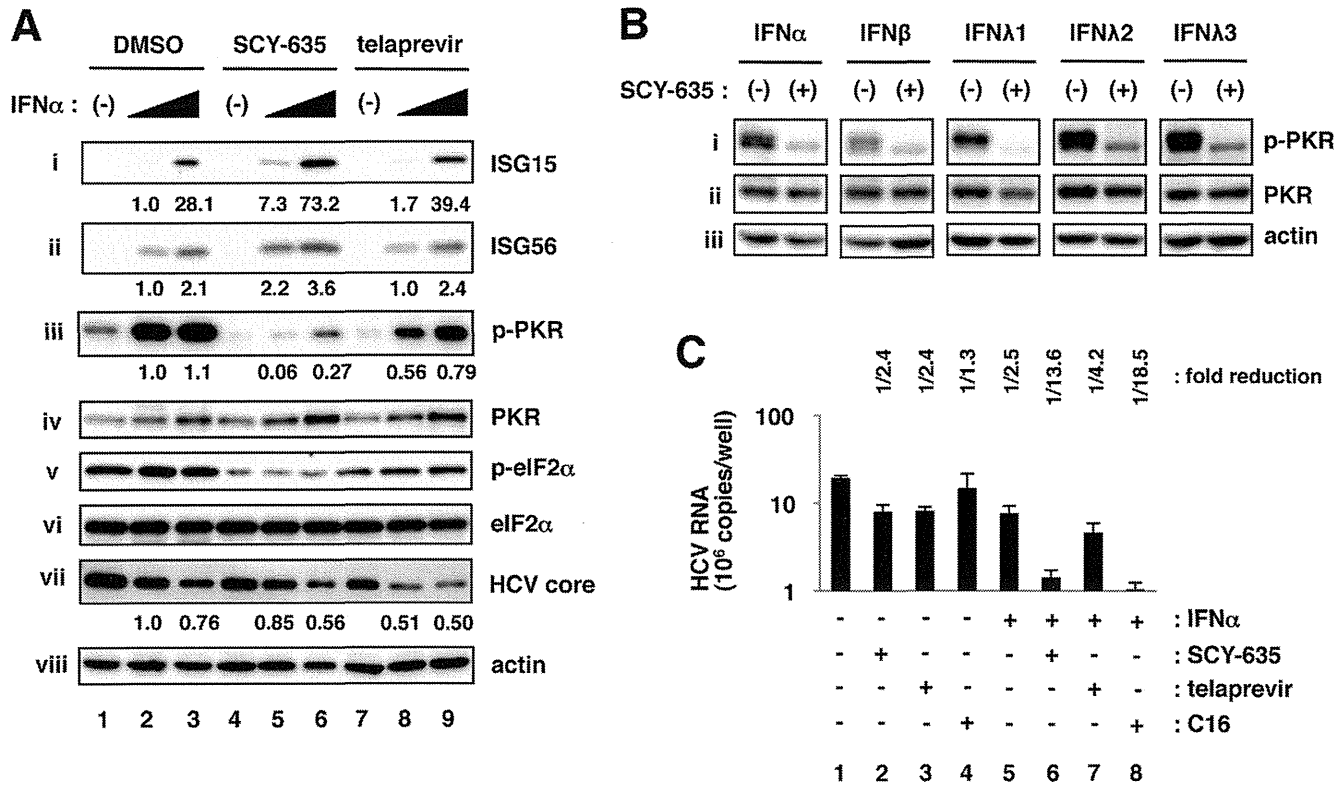
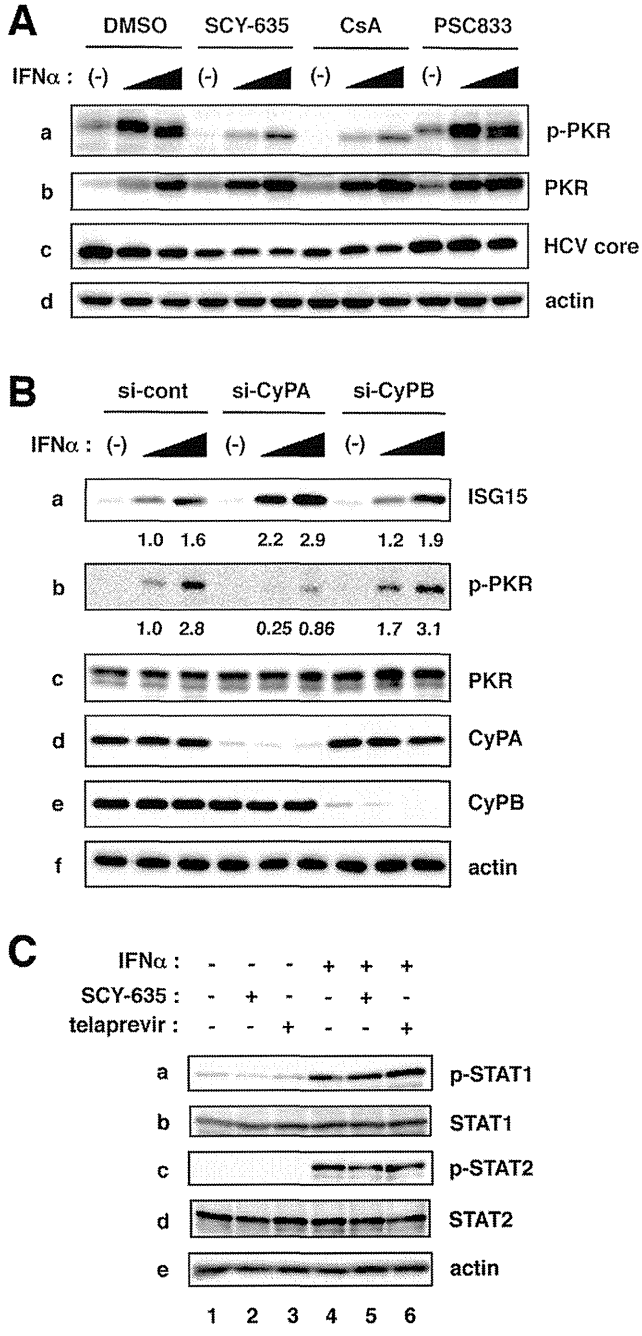


Figure 2. Phosphorylation of PKR was inhibited by SCY-635. (*A*) HCV-infected Huh-7 cells were treated with dimethyl sulfoxide (DMSO), 0.05%; SCY-635, 2 μM; or a protease inhibitor telaprevir, 2 μM for 24 hours, followed by treatment with or without IFNα (10 or 100 IU/mL) for 16 hours. Protein production of ISG15 (*i*), ISG56 (*ii*), phosphorylated PKR (T446) (*iii*), PKR (*iv*), phosphorylated eIF2α (S51) (*v*), eIF2α (*vi*), HCV core (*vii*), and actin (*viii*) are shown. Relative band intensities for these proteins by setting those in treatment with IFN alfa, 10 IU/mL plus DMSO as 1.0 are shown below the panels. (*B*) HCV-infected Huh-7 cells were pretreated with DMSO, 0.05% or SCY-635, 2 μM for 24 hours, and then treated with IFN alfa, 10 IU/mL; IFNβ, 10 IU/mL; IFNλ1 100 ng/mL; IFNλ2, 100ng/mL; or IFNλ3, 100 ng/mL for 16 hours. Proteins for phosphorylated PKR (T446) (*i*), PKR (*ii*), and actin (*iii*) were detected by Western blot. (*C*) SCY-635 as well as PKR inhibitor C16 enhanced the anti-HCV activity of IFN alfa. HCV-infected Huh-7 cells were treated with or without DMSO, 0.05% (*lanes 1* and *5*); SCY-635, 0.51 μM (*lanes 2* and *6*); telaprevir, 0.36 μM (*lanes 3* and *7*); or C16, 2 μM (*lanes 4* and *8*) together with (*lanes 5–8*) or without (*lanes 1–4*) IFN alfa, 10 IU/mL for 24 hours. HCV RNA in the cells was quantified by real-time reverse transcription polymerase chain reaction. Fold reduction values are also indicated above the graph.

levels of ISG proteins and phosphorylated PKR and eIF2α, in contrast, can be mediated by the elimination of HCV from the cells. SCY-635 also inhibited PKR phosphorylation triggered by another type-I IFN, IFN beta, and by the type-III IFNs, IFN lambda 1, IFN lambda 2, and IFN lambda 3 (Figure 2*Bi*). Previous reports have shown that highly phosphorylated PKR in HCV-infected cells reduced expression of ISG proteins on IFN-alfa treatment in an eIF2α-dependent manner.[39] These results suggest that SCY-635 restores ISG protein induction by preventing phosphorylation of PKR in HCV-infected cells.



To examine whether the inhibition of PKR phosphorylation is really related to the anti-HCV activity, we treated HCV-infected cells with SCY-635 or telaprevir at the 50% effective concentrations, as well as C16, a PKR inhibitor, in combination with IFN alfa for 24 hours, and determined the HCV RNA level in these cells. Treatment with C16, while having only a slight anti-HCV activity on its own (Figure 2*C, lane 4*), drastically potentiated the anti-HCV activity of IFN alfa (Figure 2*C, lane 8*), supporting a suppressive role for phosphorylated PKR in IFN signaling as reported.[29] Importantly, treatment with SCY-635 together with IFN alfa dramatically reduced HCV RNA levels (Figure 2*C, lane 6*), although treatment with SCY-635 alone at this condition had only a limited anti-HCV effect (Figure 2*C, lane 2*). Cotreatment with telaprevir did not as notably augment the anti-HCV activity of IFN alfa (Figure 2*C, lane 7*), suggesting that the impairment of PKR phosphorylation contributed to the synergism for the anti-HCV effect of IFN-alfa treatment. Cotreatment with the identical concentrations of SCY-635 (2 μM) and IFN alfa (10 IU/mL) to those used in Figure 2*A* also showed a synergistic anti-HCV effect (Supplementary Figure 3*B*). Thus, PKR inhibition by SCY-635 can contribute to the elimination of HCV from infected cells in the presence of IFN alfa.

Cyclophilin A Played a Significant Role in Double-Strand RNA-Dependent Protein Kinase Phosphorylation and Interferon-Stimulated Gene Expression

To determine the factor responsible for the SCY-635-mediated impairment of PKR phosphorylation, we investigated the effect of 2 related compounds, CsA and PSC833. CsA is the prototype compound of SCY-635 and can inhibit CyP, while PSC833 is a CsA derivative deficient for CyP inhibition.[18] As shown in Figure 3*A*, pretreatment with SCY-635 or CsA, but not with PSC833, reduced the

Figure 3. CyPA was important for modulating PKR phosphorylation and ISG expression. (*A*) HCV-infected Huh-7 cells were treated with or without CsA or its derivatives, SCY-635 or PSC833. At 24 hours after treatment, cells were stimulated with IFN alfa at 10 or 100 IU/mL or left untreated for 16 hours. Proteins for phosphorylated PKR (T446) (*a*), PKR (*b*), HCV core (*c*), and actin (*d*) were detected by Western blot. (*B*) HCV-infected cells were transfected with small interfering (si) RNAs for CyP subtypes, CyPA (si-CyPA) or CyPB (si-CyPB), or with a nontargeting scrambled siRNA (si-cont). After 48 hours, cells were treated with or without IFN alfa at 10 or 100 IU/mL for 16 hours. Protein production of ISG15 (*a*), phosphorylated PKR (T446) (*b*), PKR (*c*), CyPA (*d*), CyPB (*e*), and actin (*f*) are shown. Relative band intensities are shown below the panels as in Figure 1*A*. (*C*) HCV-infected Huh-7 cells pretreated with dimethyl sulfoxide, 0.05% (*lanes 1* and *4*); SCY635, 2 μM (*lanes 2* and *5*); or telaprevir, 2 μM (*lanes 3* and *6*) for 48 hours were stimulated with (*lanes 4–6*) or without (*lanes 1–3*) 100 IU/mL of IFNα. Cell lysates were recovered at 60 minutes after treatment with IFN alfa. Proteins for phosphorylated STAT1 (Y701) (*a*), STAT1 (*b*), phosphorylated STAT2 (Y690) (*c*), STAT2 (*d*), and actin (*e*) were detected.

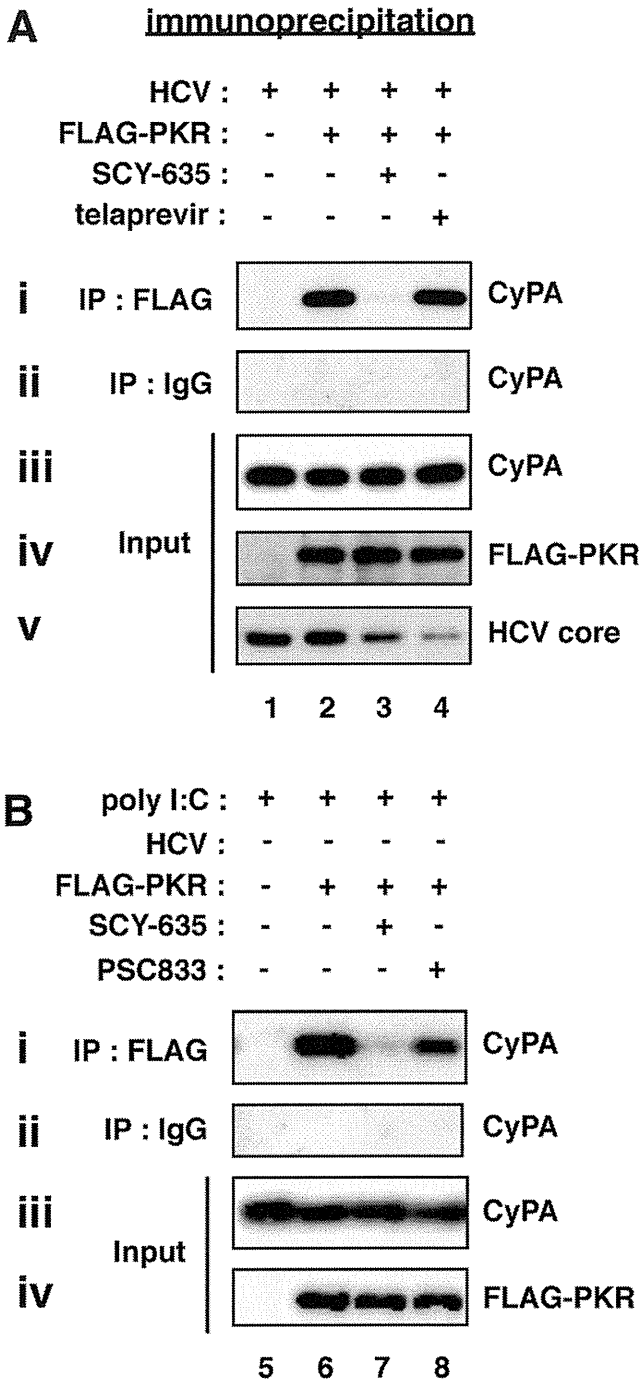


Figure 4. Interaction of PKR and CyPA in HCV-infected and poly I:C–stimulated cells. HCV-infected (*lanes 1–4*) and uninfected Huh7 cells (*lanes 5–8*) transfected with an expression plasmid for FLAG-tagged PKR (*lanes 2–4* and *6–8*) or the empty vector (*lanes 1* and *5*) for 6 hours were pretreated with dimethyl sulfoxide, 0.05%; SCY-635, 2 μM; telaprevir, 2 μM (*A*), or PSC833 2 μM (*B*) together with (*lanes 5–8*) or without (*lanes 1–4*) poly I:C for 24 hours. The cells were then stimulated with 100 IU/mL IFN alfa for 16 hours. Cell lysates were immunoprecipitated (IP) as described in Methods. Input, IP: FLAG, and IP: IgG indicate 10% input (*iii–v* in *A* and *iii–iv* in *B*), immunoprecipitation with an anti-FLAG antibody (*i*), and with a control anti-mouse normal IgG (*ii*), respectively. CyPA (*i–iii*), FLAG-PKR (*iv*), and HCV core (*v* in *A*) were detected by Western blot.

phosphorylation of PKR (Figure 3*Aa*), suggesting a critical role of CyP inhibition in the PKR dysregulation. Small interfering RNA-mediated knockdown of different CyP subtypes, CyPA and CyPB, indicated that a depletion of CyPB resulted in a slight increase in ISG15 production, as well as slightly increased phosphorylation of PKR (Figure 3*Ba*, and *b*), suggesting regulation of ISG15 production by a different mechanism independent of PKR. However, a knockdown of CyPA clearly augmented ISG protein production (Figure 3*Ba*), accompanied by a drastic reduction of PKR phosphorylation (Figure 3*Bb*). In general, CyPA is the most abundant protein among CyP subtypes and serves as the primary target of CyP inhibitors.[14] These data suggest that at least CyPA played a significant role in the regulation of PKR phosphorylation and the resultant ISG protein production.

SCY-635 Did Not Affect the Phosphorylation Status of Signal Transducers and Activators of Transcription 1 and 2

We investigated whether SCY-635 affected the phosphorylation status of other signaling components of the IFN pathway. Treatment with IFN alfa induced phosphorylation of STAT1 and STAT2 as shown in Figure 3*C* (*panels a* and *c, lane 4*). In this setting, pretreatment with SCY-635 or telaprevir did not have a significant effect on IFN alfa–induced phosphorylation of either STAT1 or STAT2 (Figure 3*C*, *panels a* and *c*, *lanes 5* and *6*). This is consistent with the result that SCY-635 did not change the transcription of ISG mRNAs (Figure 1*B*). Therefore, regulation of protein phosphorylation by CyPA was likely to be specific for PKR.

Cyclophilin A Interacted With Double-Strand RNA-Dependent Protein Kinase and Was Dissociated on SCY-635 Treatment

In general, CyPs regulate the function of their substrate proteins, such as IL2 tyrosine kinase, steroid hormone receptors, and adenine-nucleotide translocator, through direct molecular interaction.[35–37] We therefore investigated whether CyPA physically interacted with PKR in HCV-infected and uninfected cells. A co-immunoprecipitation assay from HCV-infected cells showed that endogenous CyPA co-precipitated with FLAG-tagged PKR (Figure 4*Ai*, *lane 2*). This interaction was dissociated by treatment with SCY-635, but not telaprevir (Figure 4*Ai*, *lanes 3 and 4*). To address whether the interaction between PKR and CyPA depends on the products derived from HCV, we conducted a co-immunoprecipitation assay in Huh-7 cells treated with poly I:C, which is generally used as a double-strand RNA mimic that can activate the IFN pathway, instead of with HCV. As shown in Figure 4*B*, endogenous CyPA co-precipitated with FLAG-tagged PKR in poly I:C–transfected cells, and this was abrogated by SCY-635 but not PSC833, a CsA derivative inactive for CyP inhibition (Figure 4*Bi*, *lanes 6–8*). In contrast, the interaction between CyPA and PKR was much less in the absence of HCV or poly I:C

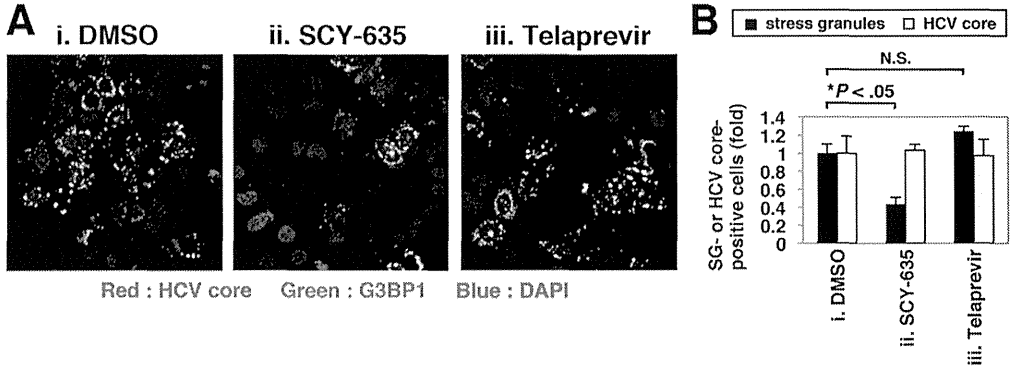


Figure 5. SCY-635 inhibited the formation of SGs. (*A*) HCV-infected Huh-7 cells were treated with dimethyl sulfoxide, 0.05% (*i*); SCY-635, 2 μM (*ii*); or telaprevir, 2 μM (*iii*) together with IFNα 100 IU/mL for 12 hours, and then were detected for G3BP1, an SG marker (*green*), HCV core protein (*red*), and the nucleus with 4′,6-diamidino-2-phenylindole (*blue*) by immunofluorescence analysis. The pictures show the merged pattern of 3 signals. (*B*) Numbers of SG-containing cells (*black bars*), as well as HCV core–positive cells (*gray bars*) were counted as Methods and are shown as relative values.

(Supplementary Figure 4). Therefore, the interaction of CyPA with PKR was likely to be more general to cells carrying double-strand RNA rather than specific for HCV-infected cells (also see Figure 6).

SCY-635 Inhibited Stress Granule Formation in Hepatitis C Virus–Infected Cells

Recent reports suggest that phosphorylated PKR plays a key role in the formation of SGs in HCV-infected cells.[31] The assembled SGs contribute to the suppression of IFN-alfa–triggered ISG translation.[30] We therefore examined whether SCY-635–mediated suppression of PKR phosphorylation and restoration of ISG translation were accompanied by an alteration of SG formation. Figure 5*A* shows the results of cells stained with G3BP1, a marker for SGs (*green*), as well as HCV core protein (*red*) and the nucleus (*blue*) in Huh-7 cells infected with HCV on IFN alfa treatment (Figure 5*A*). As shown in Figure 5*A* and *B*, treatment with SCY-635 decreased the number of cells forming SGs to approximately 40% of the control treated with IFN alfa and dimethyl sulfoxide (Figure 5*A* and *Bii*). In this condition in which the treatment time of anti-HCV agents was short, the number of HCV core-positive cells was not affected (Figure 5*A* and *Bii, iii*). In contrast, telaprevir did not decrease SG formation (Figure 5*A* and *Biii*), suggesting that the SCY-635 effect on SG formation was not the result of elimination of HCV from the cells, but rather through a direct effect on SG formation mediated by PKR.

Modulation of Double-Strand RNA-Dependent Protein Kinase by SCY-635 in the Absence of Hepatitis C Virus

To address whether the regulation of PKR by CyPA depends on products derived from HCV or not, we examined

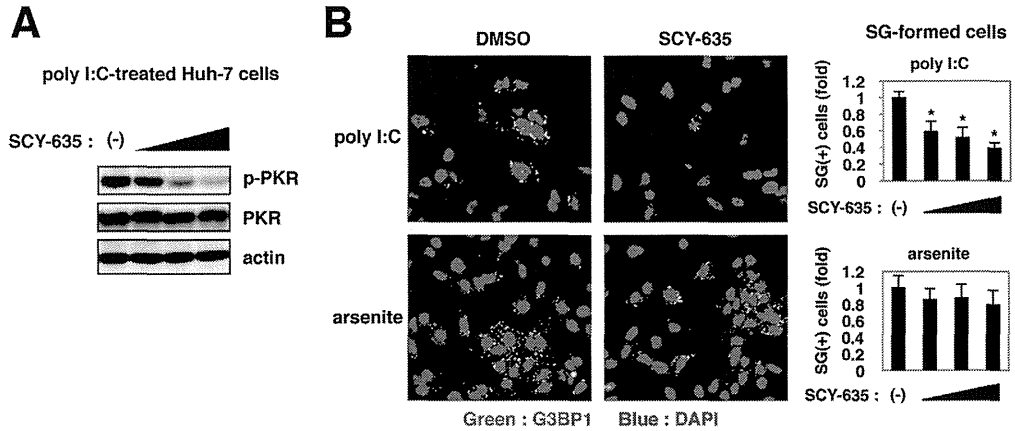


Figure 6. Modulation of PKR and SG formation by SCY-635 in the absence of HCV. (*A*) Huh-7.5.1 cells stimulated with poly I:C 0.5 μg/mL for 6 hours were pretreated with varying concentrations of SCY-635 (0, 2, 4, and 8 μM) for 18 hours. The cells were then treated with IFNα 100 IU/mL. At 24 hours later, phosphorylated PKR (T446), PKR, and actin were detected by Western blot (*A*). At 12 hours post treatment with IFN alfa, G3BP1 (*green*) and the nucleus (*blue*) were detected by immunofluorescence (*B, upper pictures*). For detecting PKR-independent SG, Huh-7.5.1 cells were pretreated with SCY-635 or dimethyl sulfoxide for 24 hours, followed by stimulation with arsenite 100 μM for 30 minutes to detect G3BP1 (*green*) and the nucleus (*blue*) (*B, lower pictures*).

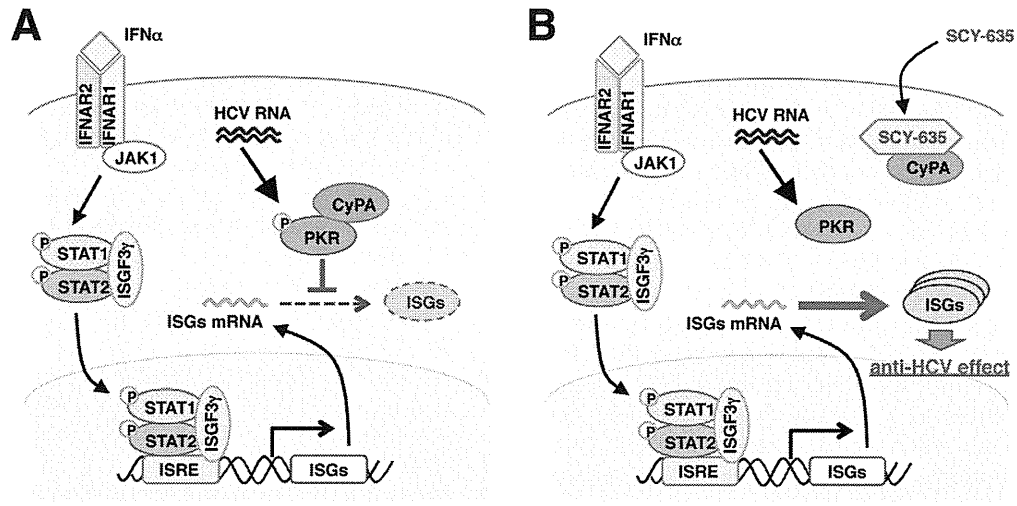


Figure 7. Schematic representation of the IFN signal transduction and the role of CyPA in regulating this pathway. (*A*) In the absence of CyP inhibitors, IFN stimulation triggers the activation of Janus-activated kinase 1, which phosphorylates STAT1 and STAT2, and translocates into the nucleus in association with ISGF3γ. Transcription of ISGs is regulated by the STAT1/STAT2/ISGF3γ complex. mRNA translation into ISG proteins is then negatively regulated by phosphorylated (activated) PKR. PKR is highly phosphorylated in HCV-infected cells. CyPA is suggested to positively regulate the phosphorylation of PKR. (*B*) In the presence of CyP inhibitors such as SCY-635, CyPA dissociates from PKR, which results in the impairment of the phosphorylation of PKR and releases the negative regulation of ISG protein translation.

the effect of SCY-635 on PKR phosphorylation in Huh-7 cells stimulated with poly I:C instead of infected with HCV. As shown in Figure 6*A*, treatment with SCY-635 reduced poly I:C–induced phosphorylation of PKR in a dose-dependent manner (in this assay, we used higher concentrations of SCY-635 [2, 4, and 8 μM] than those used in the assay with HCV-infected cells) (Figure 6*A*). The SG formation triggered by poly I:C via phosphorylated PKR was consistently inhibited by SCY-635 (Figure 6*B*). These data suggest that the modulation of PKR by SCY-635 was not limited to cells infected with HCV, consistent with the molecular interaction of CyPA with PKR in uninfected cells (Figure 4, *lane 6*). Arsenite is known to induce SG formation but through a mechanism independent of PKR.[38] As shown in Figure 6*B*, SCY-635 did not affect the formation of SGs induced by arsenite (Figure 6*B*), further suggesting that the functional regulation by CyPA is specific to PKR. Therefore, CyPA was suggested to be a positive regulator of PKR.

Discussion

In this study, we showed that CyP inhibitors restored IFN-induced ISG protein production through impairment of PKR phosphorylation. CyPA was specifically required for the regulation of PKR phosphorylation and the formation of stress granules. These results suggest that CyP inhibitors potentiate the anti-HCV effect of IFN in HCV-infected cells. It was reported that the clinical anti-HCV activity of CyP inhibitors (when given as monotherapy) was possibly influenced by IL28B genotypes, as is the case with IFN-based treatment,[23,24] suggesting a cross talk between CyP inhibitors and IFN pathway. In a separate clinical study in difficult to treat patients with IL28B genotype CT or TT, therapy with a CyP inhibitor in combination with IFN resulted in improved rates of sustained virologic response as compared with patients treated with IFN alone.[39] Our study clearly presents a molecular basis for this clinical observation: at least some portion of the anti-HCV activity of CyP inhibitors is mediated by the potentiation of IFN action.

The anti-HCV activity of CyP inhibitors reported to date is attributed mainly to direct inhibition of the function or formation of the RNA replication complex.[13,17,40–42] Huh-7, Huh-7.5, and Huh-7.5.1 cells that are typically used for HCV cell culture studies are partly or fully deficient for IFN induction, and produce little IFN alfa, although the IFN response to ISG induction is active.[43,44] Therefore, results obtained in these cell lines would primarily evaluate direct effects on HCV replication with little IFN-alfa production. In contrast, under conditions of functional IFN-induction pathways, such as in HCV-infected patients, the modulatory effect of CyP inhibitors on IFN signaling pathway might play a more relevant role in achieving anti-HCV activity. In support of this, it has been reported that combination treatment of CyP inhibitors with ectopic IFN alfa exhibited a synergistic anti-HCV activity both in cell culture and in the clinical setting.[16,45–47] We also showed that a CyP inhibitor augmented the anti-HCV activity of IFN alfa (Figure 2*C*). HCV-infected patients treated with SCY-635 alone showed up-regulation of IFN alfa and oligoadenylate synthetase proteins, both of which are representative ISGs, which corresponded with SCY-635 concentrations in serum.[23] Clinically, ectopically administered IFN induces substantial side effects, and IFN-free therapy has been greatly demanded.[4] Interestingly, the induction of endogenous IFN observed with SCY-635 monotherapy did not produce any of the serious side effects typically observed with IFN-based

therapy.[23] Our study raises the possibility that CyP inhibitors can be used as a replacement for exogenous IFN in the treatment of HCV. This is of particular importance because it has been shown that treatment with direct-acting antivirals alone might not be sufficient to cure HCV infection across all HCV genotypes, and that addition of IFN can increase the rates of sustained virologic response.[1,48]

Although it has been reported that HCV E2 and NS5A inhibited PKR activity,[49,50] PKR was highly phosphorylated in HCV-infected cells (Figure 2), as reported previously,[29,43] possibly through stimulation by the 5′-untranslated region of HCV RNA.[51] Activated PKR suppressed host protein translation, including ISGs, without affecting HCV internal ribosome entry site–dependent translation.[29,43] Garaigorta et al further reported that a knockdown of endogenous PKR restored ISG protein induction by IFN alfa in HCV-infected cells to augment the anti-HCV effect of IFN alfa. They speculate that inhibitors blocking PKR activation can be therapeutic agents to eliminate HCV from infected cells.[29] Consistent with this idea, our study revealed that CyP inhibitors suppressed PKR phosphorylation and restored ISG protein induction at the translational level. A clinical study with SCY-635 monotherapy demonstrated an increase in ISG protein production in HCV-infected patients treated with SCY-635.[23] These results are likely to support the proposed mechanism of the CyP inhibitors on the translational regulation of ISG proteins (Figure 7). CyP inhibitors can reverse the IFN-resistant mechanism in HCV-infected cells mediated by a reduced response of ISG protein induction.

In general, through the recognition of double-stranded RNA, PKR is dimerized and then autophosphorylated at T446.[28] The phosphorylated PKR interacts with and phosphorylates the downstream target eIF2α to negatively regulate the translation of proteins. We hypothesize that CyPA is acting as a molecular chaperone and possibly regulates one or more steps in this activation process of PKR, including ligand recognition, dimerization, and phosphorylation. Additional analyses are required to address which step in PKR activation is regulated by CyPA. However, this study indicates that the double-stranded RNA activation mechanism of PKR is a target for CyP inhibitors, which show significant clinical effects in HCV-infected patients. Our results further suggest that PKR can serve as a target for the development of anti-HCV agents.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2014.04.035.

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Conflicts of interest
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Alternative endocytosis pathway for productive entry of hepatitis C virus

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Previous studies have shown that hepatitis C virus (HCV) enters human hepatic cells through interaction with a series of cellular receptors, followed by clathrin-mediated, pH-dependent endocytosis. Here, we investigated the mechanisms of HCV entry into multiple HCV-permissive human hepatocyte-derived cells using trans-complemented HCV particles (HCVtcp). Knockdown of CD81 and claudin-1, or treatment with bafilomycin A1, reduced infection in Huh-7 and Huh7.5.1 cells, suggesting that HCV entered both cell types via receptor-mediated, pH-dependent endocytosis. Interestingly, knockdown of the clathrin heavy chain or dynamin-2 (Dyn2), as well as expression of the dominant-negative form of Dyn2, reduced infection of Huh-7 cells with HCVtcp, whereas infectious entry of HCVtcp into Huh7.5.1 cells was not impaired. Infection of Huh7.5.1 cells with culture-derived HCV (HCVcc) via a clathrin-independent pathway was also observed. Knockdown of caveolin-1, ADP-ribosylation factor 6 (Arf6), flotillin, p21-activated kinase 1 (PAK1) and the PAK1 effector C-terminal binding protein 1 of E1A had no inhibitory effects on HCVtcp infection into Huh7.5.1 cells, thus suggesting that the infectious entry pathway of HCV into Huh7.5.1 cells was not caveolae-mediated, or Arf6- and flotillin-mediated endocytosis and macropinocytosis, but rather may have occurred via an undefined endocytic pathway. Further analysis revealed that HCV entry was clathrin- and dynamin-dependent in ORL8c and HepCD81/miR122 cells, but productive entry of HCV was clathrin- and dynamin-independent in Hep3B/miR122 cells. Collectively, these data indicated that HCV entered different target cells through different entry routes.

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INTRODUCTION

Over 170 million people worldwide are chronically infected with hepatitis C virus (HCV), and are at risk of developing chronic hepatitis, cirrhosis and hepatocellular carcinoma (Hoofnagle, 2002). HCV is an enveloped virus belonging to the family *Flaviviridae*. Its genome is an uncapped 9.6 kb positive-stranded RNA consisting of the 5′-UTR, an ORF encoding viral proteins and the 3′-UTR (Suzuki *et al.*, 2007). A precursor polyprotein is further processed into structural proteins (core, E1, and E2), followed by p7 and non-structural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B), by cellular and viral proteases.

Two supplementary figures are available with the online version of this paper.

Host–virus interactions are required during the initial steps of viral infection. Viruses enter the cells by various pathways, such as receptor-mediated endocytosis followed by pH-dependent or -independent fusion from endocytic compartments, or pH-independent fusion at the plasma membrane coupled with receptor-mediated signalling and coordinated disassembly of the actin cortex (Grove & Marsh, 2011). It was reported previously that CD81 (Bartosch *et al.*, 2003; McKeating *et al.*, 2004; Pileri *et al.*, 1998), scavenger receptor class B type I (SR-BI) (Bartosch *et al.*, 2003; Scarselli *et al.*, 2002), claudin-1 (Evans *et al.*, 2007; Liu *et al.*, 2009) and occludin (Benedicto *et al.*, 2009; Liu *et al.*, 2009; Ploss *et al.*, 2009) are critical molecules for HCV entry into cells. Recently, epidermal growth factor receptor and ephrin receptor type A2 were also identified as host cofactors for HCV entry, possibly by modulating interactions between CD81 and claudin-1 (Lupberger *et al.*,

2011). In addition, Niemann–Pick C1-like 1 (NPC1L1) cholesterol absorption receptor has been shown to play a role in HCV entry, probably at the fusion step (Sainz *et al.*, 2012).

Following receptor binding, HCV has been reported to enter cultured cells via clathrin-mediated endocytosis, the most common and best-characterized mode of endocytosis, following membrane fusion in early endosomes (Blanchard *et al.*, 2006; Codran *et al.*, 2006; Coller *et al.*, 2009; Meertens *et al.*, 2006; Trotard *et al.*, 2009) using retrovirus-based HCV pseudoparticles (HCVpp) and cell culture-produced HCV (HCVcc). Early steps in HCV infection, including the role of HCV glycoprotein heterodimers, receptor binding, internalization and pH-dependent endosomal fusion, have been at least in part mimicked by HCVpp. However, as HCVpp are generated in non-hepatic cells such as human embryo kidney 293T cells, it is likely that the cell-derived component(s) of HCVpp differ from those of HCVcc.

In the present study, we readdressed the HCV endocytosis pathway using trans-complemented HCV particles (HCVtcp) (Suzuki *et al.*, 2012), of which the packaged genome is a subgenomic replicon. HCVtcp, generated in Huh-7 or its derivative cell lines with two plasmids, are infectious, but support only single-round infection, thereby allowing us to examine infectious viral entry without the influence of reinfection. In addition, HCVtcp is useful for quantifying productive infection by measuring luciferase activity. Furthermore, it has been shown that the HCVtcp system is more relevant as a model of HCV infection than HCVpp (Suzuki *et al.*, 2012). Our results demonstrated conclusively that, in addition to the clathrin-mediated endocytosis pathway, HCV was capable of utilizing the clathrin- and dynamin-independent pathways for infectious entry of HCV into human liver-derived cells.

RESULTS

HCV entry depends on receptor-mediated, pH-dependent endocytosis

HCV has been shown to enter permissive cells through clathrin-mediated endocytosis and low pH-dependent fusion with endosomes mostly using HCVpp (Codran *et al.*, 2006; Meertens *et al.*, 2006; Trotard *et al.*, 2009), although some researchers have used HCVcc with limited cell lines (Blanchard *et al.*, 2006; Coller *et al.*, 2009). However, several distinct characteristics between HCVpp and HCVcc have recently been revealed with regard to morphogenesis and entry steps (Helle *et al.*, 2010; Sainz *et al.*, 2012; Suzuki *et al.*, 2012; Vieyres *et al.*, 2010). Therefore, in this study, we used HCVtcp, which exhibit similar characteristics to HCVcc when compared with HCVpp and support single-round infection (Suzuki *et al.*, 2012).

Initially, to determine whether receptor candidates such as CD81, claudin-1, occludin and SR-BI are essential for HCV entry into Huh-7 and Huh7.5.1 cells, we examined the knockdown effect of these molecules on HCVtcp infection. Knockdown of these receptors was confirmed by immunoblotting (Fig. 1a) and FACS analysis (Fig. 1b). It should be noted that the luciferase activity in Huh7.5.1 was approximately four times higher than that in Huh-7 cells when the same amount of inoculum was used for infection (Fig. S1, available in the online Supplementary Material), and knockdown did not affect cell viability (data not shown). Knockdown of CD81 and claudin-1 significantly reduced the infection of Huh-7 and Huh7.5.1 cells with HCVtcp derived from genotype 2a (Fig. 1c). Knockdown of occludin led to a moderate reduction in infection; however, only a marginal effect was observed in SR-BI knockdown in both Huh-7 and Huh7.5.1 cells (Fig. 1c), possibly due to the reduced requirement for SR-BI during virus entry by adaptive mutation in E2 (Grove *et al.*, 2008).

Next, to examine whether HCV entry was pH-dependent, Huh-7 and Huh7.5.1 cells were pretreated with bafilomycin A1, an inhibitor of vacuolar H^+-ATPases that impairs vesicle acidification, and then infected with HCVtcp. At 72 h post-infection, luciferase activity and cell viability were determined. Bafilomycin A1 inhibited HCVtcp infection in a dose-dependent manner without affecting cell viability in both Huh-7 and Huh7.5.1 cells (Fig. 2a, b). We also confirmed that treatment with bafilomycin A1 after HCVtcp infection had a minor effect on luciferase activity (Fig. 2c). These results indicated that the infectious route of HCVtcp into Huh-7 and Huh7.5.1 cells is receptor-mediated and involves pH-dependent endocytosis.

Knockdown of clathrin heavy chain (CHC) or dynamin-2 (Dyn2) reduces HCVtcp infection in Huh-7 cells, but not in Huh7.5.1 cells

Among the known pathways of pH-dependent viral endocytosis, clathrin-mediated dynamin-dependent endocytosis is a major endocytosis pathway. Chlorpromazine, an inhibitor of clathrin-dependent endocytosis, has been commonly used to study clathrin-mediated endocytosis; however, it exerts multiple side-effects on cell function as it targets numerous receptors and intracellular enzymes, and alters plasma membrane characteristics (Sieczkarski & Whittaker, 2002a). Therefore, we examined the HCV endocytosis pathway by knockdown of specific molecules required for the endocytosis pathway. CHC, a major structural protein in clathrin-coated vesicles, and Dyn2, a GTPase essential for clathrin-coated-pit scission from the plasma membrane, play important roles in the clathrin-mediated pathway. Another well-studied model of viral entry is caveolin-mediated endocytosis. The role of dynamin in both clathrin-mediated endocytosis and caveolae-dependent endocytosis has been established (Marsh & Helenius, 2006; Miaczynska & Stenmark, 2008). To examine the endocytosis pathways of HCV, small interfering RNAs (siRNAs) for CHC, Dyn2 and caveolin-1 (Cav1), or scrambled control siRNA, were transfected into Huh-7 or

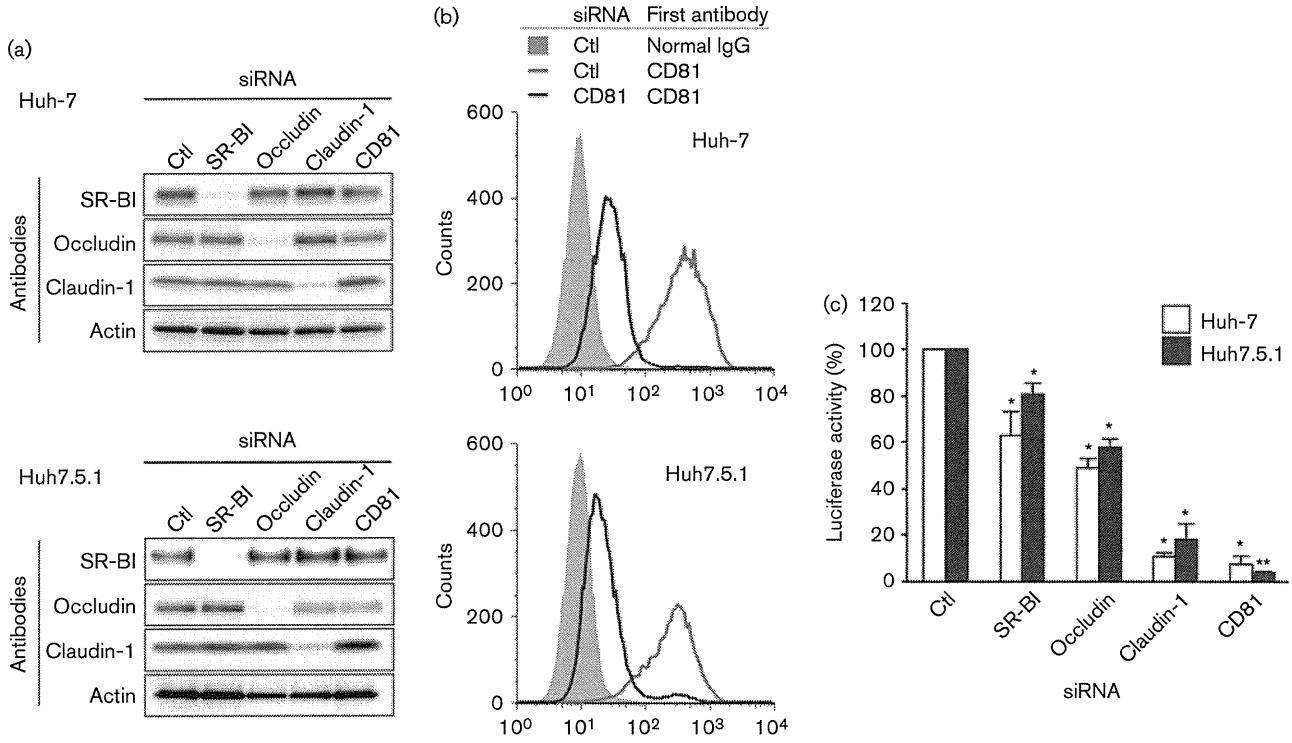

Fig. 1. Knockdown effect of receptor candidate molecules on HCV infection. (a) Huh-7 or Huh7.5.1 cells were transfected with the indicated small interfering RNAs (siRNA), harvested at 48 h post-transfection and the specific knockdown of each protein was verified by immunoblotting. (b) Huh-7 or Huh7.5.1 cells were transfected with CD81 or control siRNAs, harvested at 48 h post-transfection and the cell surface expression of CD81 was verified by FACS analysis. (c) Cells transfected with siRNA were infected with the same amount of HCVtcp at 48 h post-transfection. Firefly luciferase activity in the cells was determined at 72 h post-infection and is expressed relative to the activity with control siRNA transfection. The value for control (Ctl) siRNA was set at 100 %. Data represent the mean ± SD. Statistical differences between controls and each siRNA were evaluated using Student's *t*-test. **P*<0.05, ***P*<0.001 versus control.

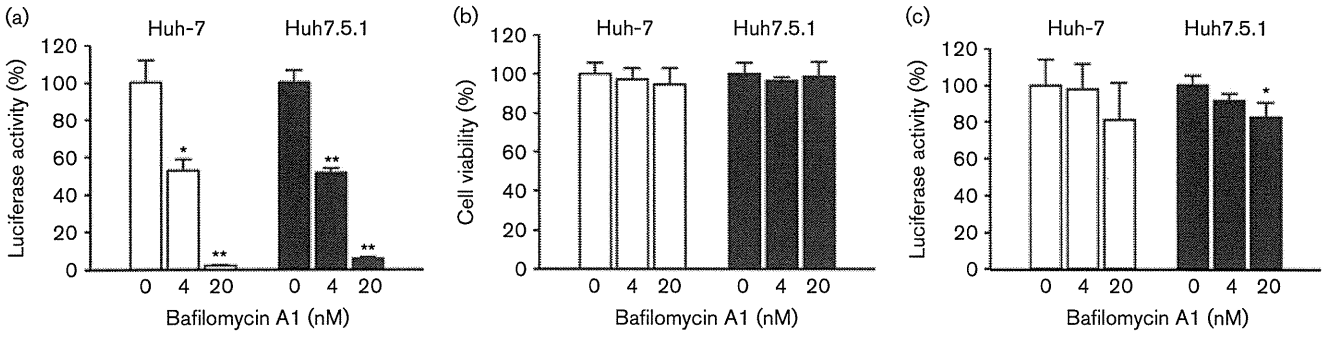

Fig. 2. Role of endosomal low pH in HCV infection. Cells were treated with bafilomycin A1 for 1 h at the indicated concentrations and infected with HCVtcp. (a, b) Luciferase activity (a) and cell viability (b) were determined at 72 h post-infection, and expressed relative to amounts observed in controls. (c) Cells were treated with bafilomycin A1 for 1 h at the indicated concentrations 48 h after HCVtcp infection. Luciferase activity was determined at 10 h post-treatment and expressed relative to amounts observed in controls. Data represent the mean ± SD. Statistical differences between controls and indicated concentrations were evaluated using Student's *t*-test. **P*<0.05, ***P*<0.001 versus control.

Huh7.5.1 cells, followed by infection with HCVtcp. Expression of CHC, Dyn2 and Cav1 was downregulated by transfection of specific siRNAs (Fig. 3a, b), whereas expression of SR-BI, occludin, claudin-1 and CD81 was not reduced (Figs 3a and S2). As indicated in Fig. 3(c), luciferase activity from HCVtcp was significantly reduced by knockdown of CHC and Dyn2 in Huh-7 cells, but not in Huh7.5.1 cells. Knockdown of Cav1 showed no inhibitory effects on HCVtcp entry into either cell line. Dynamin-independent entry in Huh7.5.1 cells was also observed using HCVtcp derived from genotype 1b (data not shown). Knockdown of CHC or Dyn2 also reduced entry of HCVcc in Huh-7 cells, but had no inhibitory effects in Huh7.5.1 (Fig. 3d). To rule out the possibility of effects on CHC and Dyn2 knockdown on viral RNA replication, HCVtcp were also inoculated before siRNA transfection. Luciferase activity was not affected by knockdown of CHC or Dyn2 in either cell line, whereas marked inhibition was observed for phosphatidylinositol 4-kinase (PI4K) (Fig. 3e). These data suggested that HCV entry was clathrin-mediated and dynamin-dependent in Huh-7 cells, but productive entry of HCV was clathrin- and dynamin-independent in Huh7.5.1 cells.

Expression of the dominant-negative form of Dyn2 reduces HCV infection in Huh-7 cells, but not in Huh7.5.1 cells

We also examined the role of dynamin in infectious entry of HCV into Huh-7 and Huh7.5.1 cells by overexpression of the dominant-negative form of Dyn2 (Dyn-K44A), which

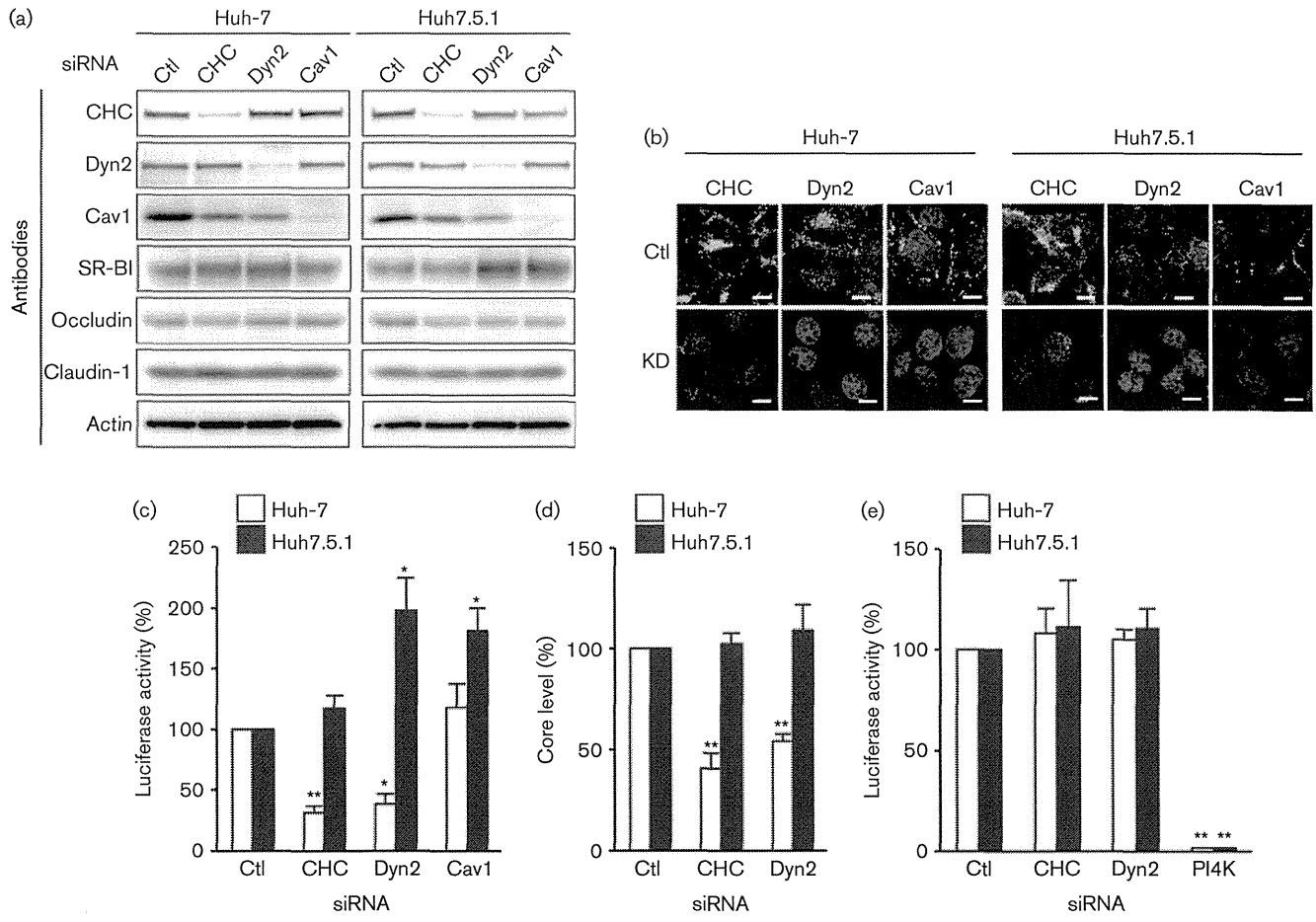

Fig. 3. Effects of CHC, Dyn2 and Cav1 knockdown on HCV infection. (a, b) Huh-7 cells or Huh7.5.1 cells were transfected with the indicated siRNAs and the specific knockdown (KD) of each protein was verified by immunoblotting (a) or immunostaining (b) at 48 h post-transfection. Bar, 50 μm. (c) Cells were transfected with the indicated siRNAs, followed by infection with HCVtcp at 48 h post-transfection. Firefly luciferase activity in the cells was subsequently determined at 3 days post-infection. The value for control (Ctl) siRNA was set at 100 %. Data represent the mean ± SD. (d) Cells were transfected with siRNA, followed by infection with HCVcc at 48 h post-transfection. Intracellular core levels were quantified at 24 h post-infection. The value for control siRNA was set at 100 %. Data represent the mean ± SD. (e) Cells were infected with HCVtcp, followed by transfection with the indicated siRNAs. Luciferase activity in the cells was subsequently determined at 2 days post-transfection. The value for control siRNA was set at 100 %. Data represent the mean ± SD. Statistical differences between controls and each siRNA were evaluated using Student's *t*-test. **P*<0.05, ***P*<0.001 versus control.

has been shown to effectively block clathrin-dependent and caveolar endocytosis (Damke *et al.*, 1995). Expression of haemagglutinin (HA)-tagged Dyn-K44A reduced the number of HCV-infected Huh-7 cells, but not Huh7.5.1 cells, as compared with WT HA-tagged Dyn2 (Dyn-WT), as shown in Fig. 4(a, b). Interestingly, internalization of transferrin, which is known to be mediated by clathrin-dependent endocytosis, was reduced in both Huh-7 and Huh7.5.1 cells expressing Dyn-K44A, whereas cells expressing Dyn-WT showed efficient endocytosis of transferrin (Fig. 4c, d). Collectively, these results suggested that dynamin participated in the internalization of HCV in Huh-7 cells, but was not absolutely required in Huh7.5.1 cells, although transferrin was taken up via dynamin-dependent endocytosis in both Huh-7 and Huh7.5.1 cells.

Flotillin-1 or the GTPase regulator associated with focal adhesion kinase 1 (GRAF1) play no major role during HCV infection of Huh7.5.1 cells

In order to dissect the major endocytosis pathways of HCVtcp in Huh7.5.1 cells, we investigated the role of alternative routes of HCV entry by siRNA knockdown. We silenced essential factors for the clathrin- or dynamin-independent pathways

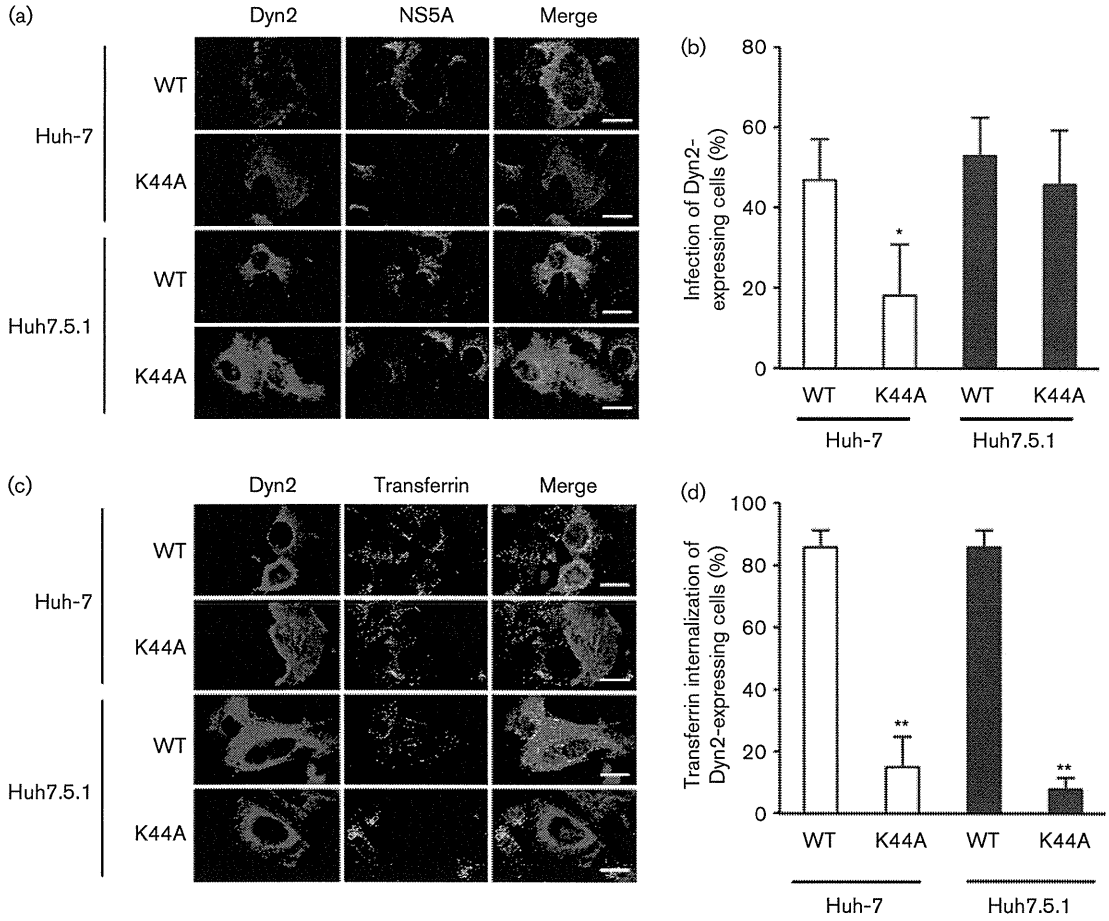

Fig. 4. Dynamin participates in the internalization of HCV in Huh-7 cells, but not in Huh7.5.1 cells. (a) Cells were transfected with HA-tagged WT Dyn2 (Dyn-WT) or dominant-negative Dyn2 (Dyn-K44A) expression plasmids. At 2 days post-transfection, cells were infected with HCVtcp, which possessed a subgenomic replicon without the luciferase gene. After 3 days, cells were fixed and HA-Dyn2 or HCV NS5A stained with anti-HA or anti-NS5A antibodies, respectively. Cell nuclei were counterstained with DAPI. Bar, 100 μm. (b) Data were quantified as the population of HCVtcp-infected cells among HA-positive cells. At least 20 HA-positive cells were evaluated in triplicate experiments. Data represent the mean ± SD. (c) Cells were transfected with HA-tagged Dyn-WT or Dyn-K44A expression plasmids. At 2 days post-transfection, cells were incubated with Alexa Fluor-488 labelled transferrin at 37 °C in a 5 % CO_2 incubator. After 30 min of incubation, cells were washed, fixed and stained with anti-HA antibodies. Cell nuclei were counterstained with DAPI. Bar, 100 μm. (d) Data were quantified as the population of transferrin-internalized cells among HA-positive cells. At least 20 HA-positive cells were evaluated in triplicate experiments. Data represent the mean ± SD. Statistical differences between Dyn-WT and Dyn-K44A were evaluated using Student's *t*-test. **P*<0.05, ***P*<0.001 versus Dyn-WT.

including flotillin-dependent endocytosis, ADP-ribosylation factor 6 (Arf6)-dependent endocytosis, clathrin-independent carrier/glycosylphosphatidylinositol-enriched early endosomal compartment (CLIC/GEEC) endocytic pathway and macropinocytosis in Huh7.5.1 cells. Flotillin-1 and Arf6 are indispensable components of the flotillin and Arf6 pathways, respectively. Knockdown of flotillin-1 or Arf6 had no inhibitory effects on HCVtcp infection in Huh7.5.1 cells (Fig. 5a). The CLIC/GEEC endocytic pathway has recently become better defined and is regulated by the GTPase regulator associated with focal adhesion kinase-1 (GRAF1). However, GRAF1 was not detected in Huh-7 or Huh7.5.1 cells (Fig. 5b); thus, it is unlikely that the CLIC/GEEC pathway was involved in HCV entry in Huh7.5.1 cells. In addition, knockdown of p21-activated kinase 1 (PAK1) and the PAK1 effector C-terminal binding protein 1 of E1A (CtBP1), which play important regulatory roles in the process of macropinocytosis, did not inhibit HCVtcp infection in Huh7.5.1 cells (Fig. 5c). Taken together, these results suggested that the entry of HCVtcp into Huh7.5.1 cells was not mediated mainly by flotillin-dependent endocytosis, Arf6-dependent endocytosis, the CLIC/GEEC endocytic pathway and macropinocytosis.

Clathrin-dependent and -independent pathways for HCV entry in other hepatic cells

We further examined the endocytosis pathways for HCV in non-Huh-7-related human liver-derived cell lines. Three HCVcc permissive hepatocellular carcinoma cell lines, Li23-derived ORL8c (Kato *et al.*, 2009), HepCD81/miR122 cells (HepG2/CD81 cells overexpressing miR122) and Hep3B/miR122 (Kambara *et al.*, 2012), were transfected with siRNA for CHC, Dyn2 or claudin-1, followed by infection with HCVtcp. Immunoblotting was performed in order to confirm knockdown of target proteins (Fig. 6a). Although knockdown of CHC or Dyn2 expression inhibited HCVtcp infection of ORL8c and HepCD81/miR122 cells, HCVtcp infection of Hep3B/miR122 cells was not affected (Fig. 6b), thus suggesting that productive entry of HCV is clathrin- and dynamin-independent in Hep3B/miR122 cells.

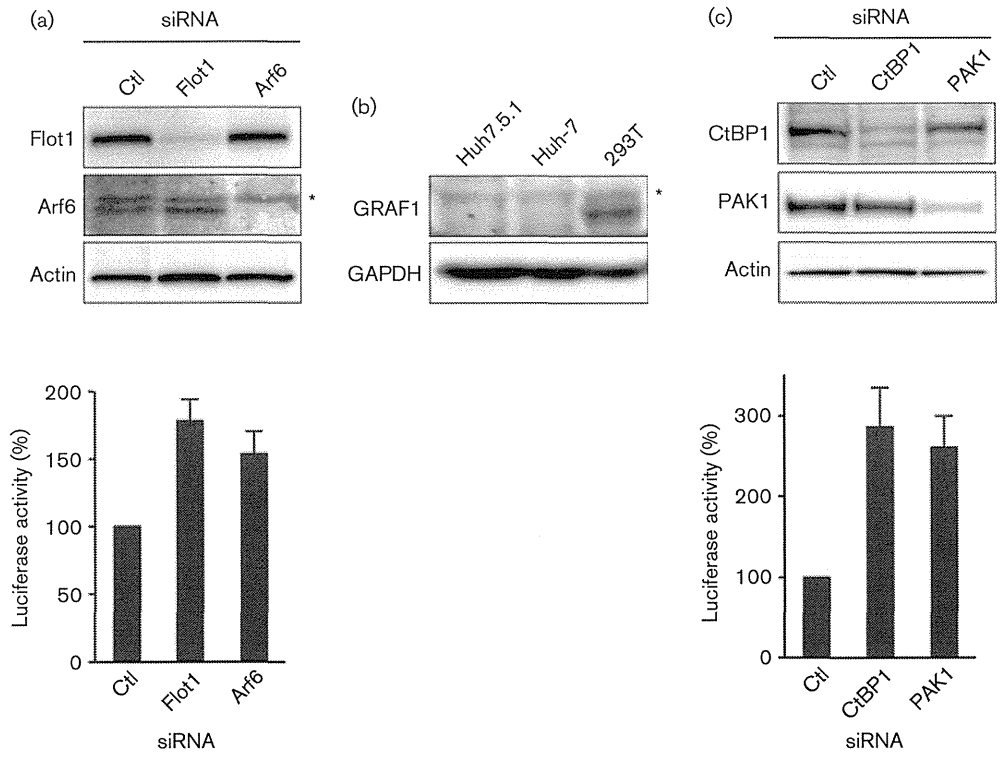

Fig. 5. Role of an alternative endocytosis pathway of HCV in Huh7.5.1 cells. (a) Huh7.5.1 cells were transfected with flotillin-1 (Flot1) or Arf6 siRNAs and specific knockdown of each protein was verified by immunoblotting (upper). Non-specific bands are marked with an asterisk. Cells transfected with siRNA were infected with HCVtcp. Luciferase activity (lower) was determined at 72 h post-infection and expressed relative to the amount observed in control (Ctl) siRNA transfection, Data represent the mean ± SD. (b) Expression of GRAF1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in Huh7.5.1, Huh-7 and 293T cells was analysed by immunoblotting. Non-specific bands are marked with an asterisk. (c) Huh7.5.1 cells were transfected with CtBP1 or PAK1 siRNA and specific knockdown of each protein was verified by immunoblotting (upper). Cells transfected with siRNA were infected with the HCVtcp. Luciferase activity (lower) was determined at 72 h post-infection and expressed relative to the amount observed in control (Ctl) siRNA transfection. Data represent the mean ± SD.

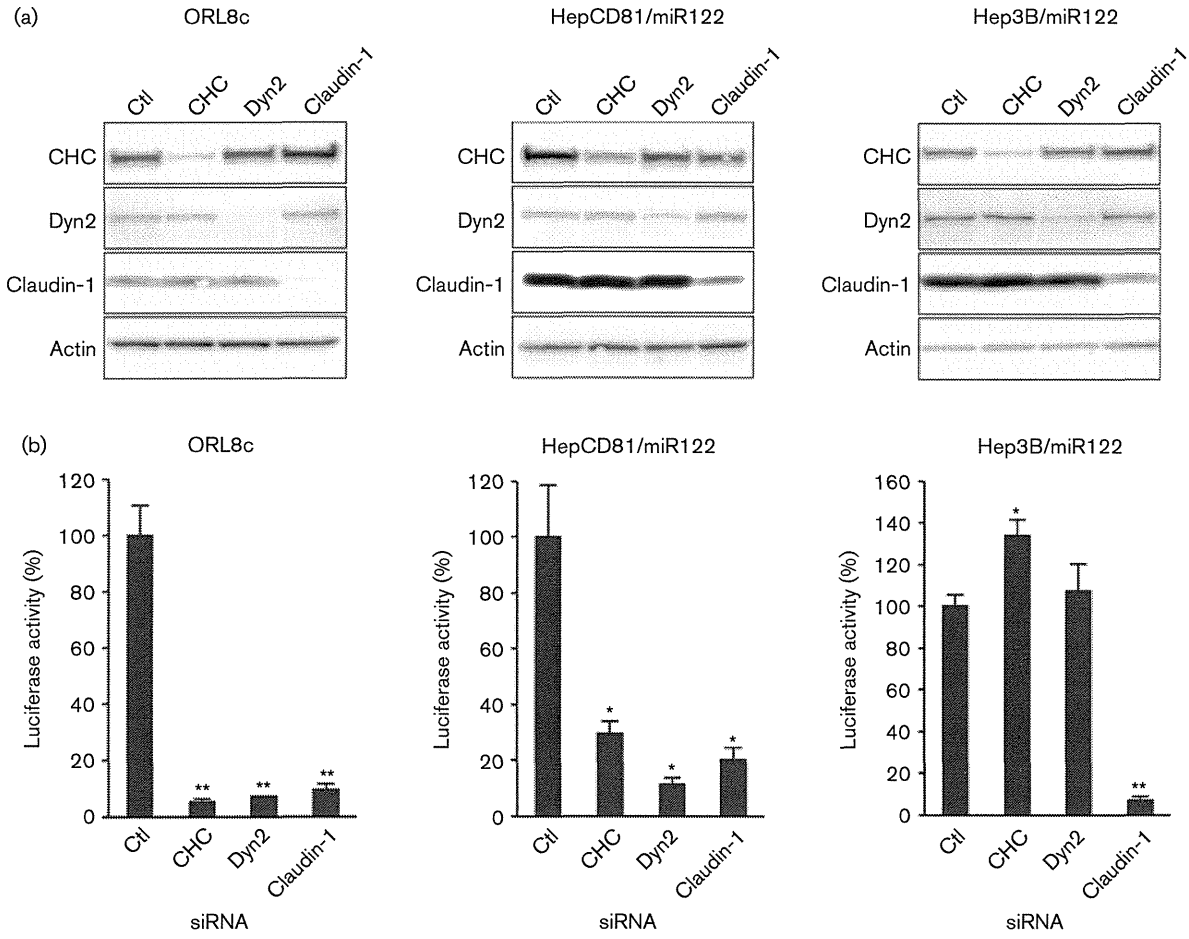

Fig. 6. Clathrin-dependent and -independent pathway of HCV entry in other HCV-permissive cells. The indicated cells were transfected with the indicated siRNAs and then infected with HCVtcp at 48 h post-transfection. (a) Specific knockdown of each protein was verified by immunoblotting. (b) Luciferase activity was determined at 72 h post-infection and expressed relative to the amount observed in the control (Ctl) siRNA transfection. Data represent the mean ± SD. Statistical differences between controls and each siRNA were evaluated using Student's *t*-test. **P*<0.05, ***P*<0.001 versus control.

In summary, we identified an alternative clathrin- and dynamin-independent entry pathway for HCV in at least two independent cell lines, Huh7.5.1 and Hep3B/miR122 cells, in addition to the previously reported clathrin- and dynamin-dependent pathway. These findings provided clues for understanding the molecular mechanisms of the endocytosis pathway for HCV infection.

DISCUSSION

Many viruses have been shown to utilize a number of different endocytic pathways to productively infect their hosts. Clathrin-dependent endocytosis would appear to be the most commonly used, but it is increasingly clear that a number of clathrin-independent endocytosis pathways are also used by several different viruses (Mercer *et al.*, 2010). In the case of HCV, it has been reported that viral entry is mediated by clathrin-dependent endocytosis (Blanchard *et al.*, 2006; Codran *et al.*, 2006; Coller *et al.*, 2009; Meertens *et al.*, 2006; Trotard *et al.*, 2009). In these papers, HCVpp was used at least in part for analysis of HCV entry pathway. However, recent reports have revealed several different characteristics between HCVpp and HCVcc.

Viral entry has been addressed primarily by pharmacologic inhibitor studies, immunofluorescence and electron microscopy, by transfection with dominant-negative constructs, and more recently by siRNA knockdown. Analysis of endocytosis pathways using pharmacological inhibitors has raised concerns about specificity. For example, chlorpromazine, an inhibitor of clathrin-mediated endocytosis, has been shown to exert multiple side-effects on cell function as it targets numerous receptors and intracellular enzymes, and alters plasma membrane characteristics (Sieczkarski & Whittaker, 2002a). Methods for elucidating the viral endocytosis pathway by co-localization of virus particles with host factor also have limitations. Electron and

fluorescence microscopy, which require a high particle number, do not allow the differentiation of infectious and non-infectious particles. Infectious particles of HCV in the supernatant of infected cells appeared to represent only a small portion of secreted virus particles (Akazawa *et al.*, 2008) and it is unclear whether the viral particles observed by microscopy could lead to productive infection. Therefore, we utilized HCVtcp, which is useful for determining productive entry of the virus without reinfection, and a combination of siRNA knockdown and dominant-negative mutants for analysis of the productive route of infection. Although HCVcc is also utilized in analysis of productive entry, it cannot completely exclude the effects of reinfection by virus produced by infected cells. Reduction of HCVcc infection by knockdown of CHC and Dyn2 was moderate when compared with that of HCVtcp (Fig. 3c, d), thus suggesting slight effects due to reinfection in HCVcc.

The data presented here demonstrate for the first time to our knowledge that HCV is able to enter cells via dynamin-independent endocytosis in addition to the previously described classical clathrin- and dynamin-dependent pathway. First, knockdown of CHC and Dyn2 had no inhibitory effects on HCVtcp and HCVcc entry into Huh7.5.1 cells. Second, overexpression of dominant-negative Dyn2 had no inhibitory effects on HCVtcp in Huh7.5.1 cells. Finally, in addition to Huh7.5.1 cells, Hep3B/miR122 cells were also shown to be infected with HCV via clathrin- and dynamin-independent pathways. We further investigated the role of alternative minor routes of HCV entry into Huh7.5.1 cells; however, the productive endocytosis pathway could not be defined. It should be noted that inhibition of alternative endocytosis routes by siRNA led to an increase of luciferase activity (Figs 3c and 5a, c). This could be explained by the inhibition of a particular endocytosis pathway resulting in a compensatory increase in alternative endocytosis pathways (Damke *et al.*, 1995).

Although we confirmed an alternative endocytosis pathway for the productive entry of HCV, it is not clear why and how the two independent endocytosis pathways operate in different cell lines. SV40 can enter cells via caveolae-dependent (Norkin *et al.*, 2002; Pelkmans *et al.*, 2001) and -independent (Damm *et al.*, 2005) pathways. Influenza virus enters cells via clathrin-mediated endocytosis (Matlin *et al.*, 1981) in addition to non-clathrin-mediated, non-caveola-mediated internalization pathways (Sieczkarski & Whittaker, 2002b). Entry of dengue virus type 2 is clathrin-dependent in HeLa and C6/36 cells (Acosta *et al.*, 2008; Mosso *et al.*, 2008; van der Schaar *et al.*, 2008), and is clathrin-independent in Vero cells (Acosta *et al.*, 2009). Different receptor usage may determine the consequential route of entry. However, we did not observe any differences between Huh-7 and Huh7.5.1 cells in terms of knockdown effects of receptor candidate molecules on HCV infection, as shown in Fig. 1(c), although we cannot exclude the possibility that other undefined receptors are associated with viral entry. Huh7.5.1 cells were established by elimination of the HCV genome from replicon cells derived from Huh-7 cells (Blight *et al.*, 2002; Zhong *et al.*, 2005) and they exhibit more potent replication of HCV than the original Huh-7 cells. Further study showed that the increased permissiveness of cured cells results from a mutation in the retinoic acid-inducible gene I (Sumpter *et al.*, 2005), which impairs IFN signalling. In addition, it has been shown that cured cell lines express higher levels of miR122 than parental cells participating in the efficient propagation of HCVcc (Kambara *et al.*, 2012). As it is unclear whether these changes are the reason for a distinct endocytosis pathway, it will be of interest to explore these associations in further studies.

In conclusion, we confirmed an alternative clathrin-independent endocytosis pathway in HCV-permissive human hepatic-derived cells, in addition to the previously reported clathrin-dependent endocytosis pathway. This paper highlights the fact that clathrin- and dynamin-mediated endocytosis is the main route of HCV entry for Huh-7, HepCD81/miR122 and ORL8c cells, whilst clathrin and dynamin do not play a major role during the productive route of HCV infection in Huh7.5.1 and Hep3B/miR122 cells. Taken together, these studies suggest that different cell entry pathways for HCV infection may be utilized in different cell types, although further studies are necessary in order to understand this phenomenon.

METHODS

Cells. The human hepatocellular carcinoma cell lines Huh-7, Huh7.5.1, Hep3B/miR122 and HepG2/CD81, which overexpressed miR122 (Kambara *et al.*, 2012), were maintained in Dulbecco's modified Eagle's medium (DMEM; Wako Pure Chemical Industries) containing non-essential amino acids, penicillin (100 U ml^{-1}), streptomycin (100 $\mu g\ ml^{-1}$) and 10 % FBS. Li23-derived ORL8c cells (Kato *et al.*, 2009) were maintained in F12 medium and DMEM (1 : 1, v/v) supplemented with 1 % FBS, epidermal growth factor (50 ng ml^{-1}), insulin (10 $\mu g\ ml^{-1}$), hydrocortisone (0.36 $\mu g\ ml^{-1}$), transferrin (5 $\mu g\ ml^{-1}$), linoleic acid (5 $\mu g\ ml^{-1}$), selenium (20 ng ml^{-1}), prolactin (10 ng ml^{-1}), gentamicin (10 $\mu g\ ml^{-1}$), kanamycin monosulfate (0.2 $mg\ ml^{-1}$) and fungizone (0.5 $\mu g\ ml^{-1}$). All cell lines were cultured at 37 °C in a 5 % CO_2 incubator.

Preparation of viruses. HCVtcp and HCVcc derived from JFH-1 with adaptive mutations in E2 (N417S), p7 (N765D) and NS2 (Q1012R) were generated as described previously (Suzuki *et al.*, 2012). For HepCD81/miR122 and ORL8c cells, HCVtcp containing the *Gaussia* luciferase (GLuc) reporter gene were used. To do this, plasmid pHH/SGR-JFH1/GLuc/NS3m carrying the bicistronic subgenomic HCV replicon containing the GLuc reporter gene and the NS3 adaptive mutation was constructed by replacement of the firefly luciferase (FLuc) gene of pHH/SGR-Luc containing the NS3 mutation (N1586D) (Suzuki *et al.*, 2012) with the GLuc gene of pCMV-GLuc (NEB).

Plasmids. HA-tagged Dyn2, a dominant-negative Dyn2 (K44A) in which Lys44 was replaced with Ala, was cloned into pcDNA3.1 as described previously (Kataoka *et al.*, 2012).

Gene silencing by siRNA. siRNAs were purchased from Sigma-Aldrich and were introduced into the cells at a final concentration of