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H. 知的所有権の出願・取得状況

なし

III. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
松本美佐子	免疫応答と回避	下遠野邦忠 瀬谷司	生命科学のためのウイルス学	南江堂	東京	2015	73-104

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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Ⅳ．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

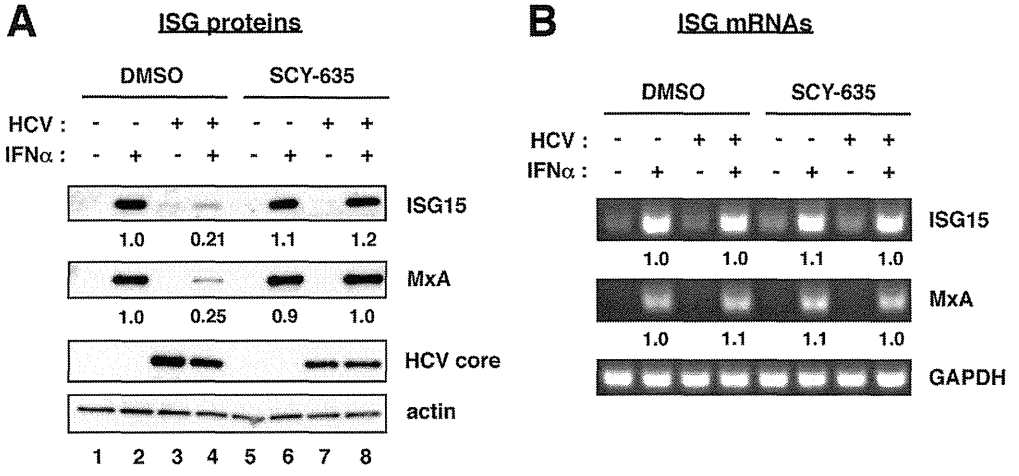


Figure 1. Protein production of ISGs in HCV-infected cells was restored by treatment with a CyP inhibitor, SCY-635. (*A*) HCV-infected (*lanes 3, 4, 7,* and *8*) or uninfected Huh-7 cells (*lanes 1, 2, 5,* and *6*) were pretreated with dimethyl sulfoxide (DMSO), 0.05% (*lanes 1–4*) or SCY-635, 2 μM (*lanes 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

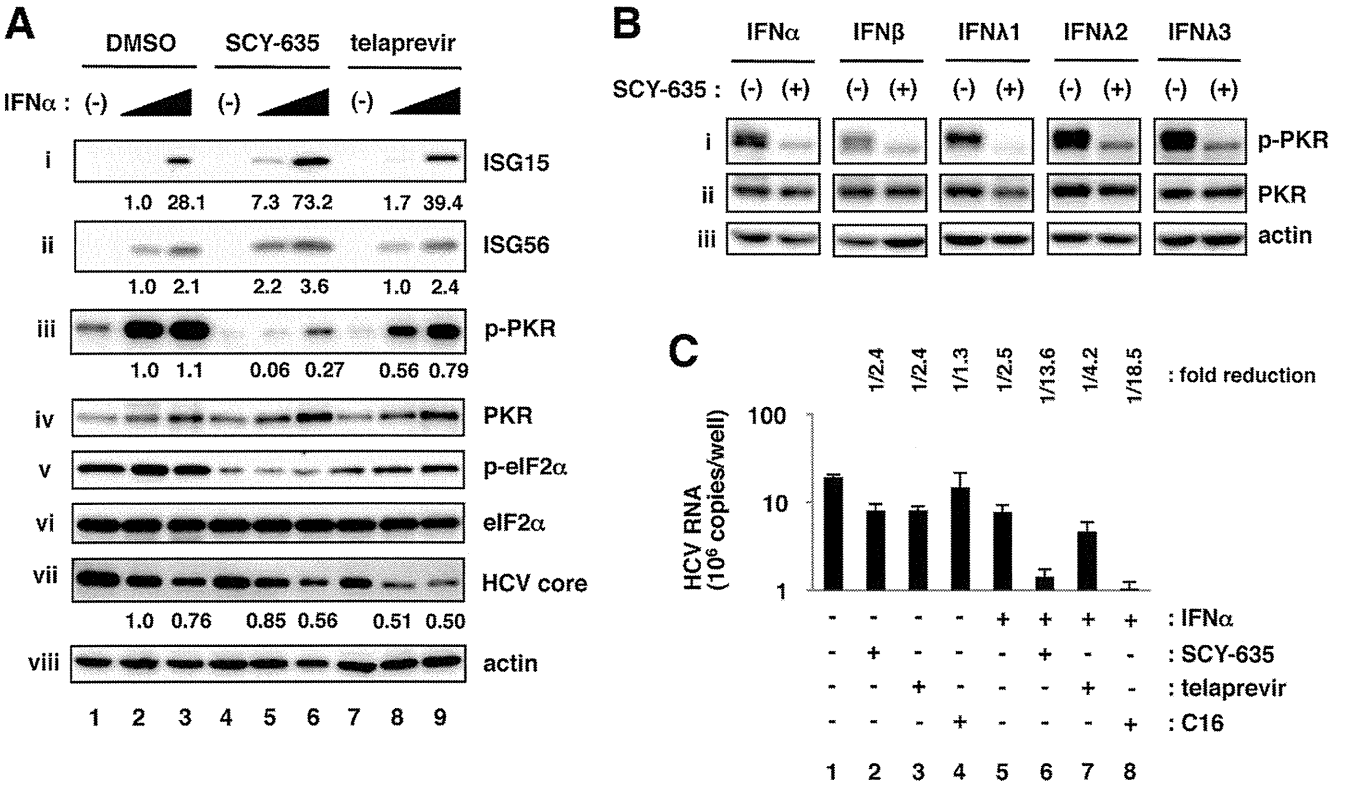
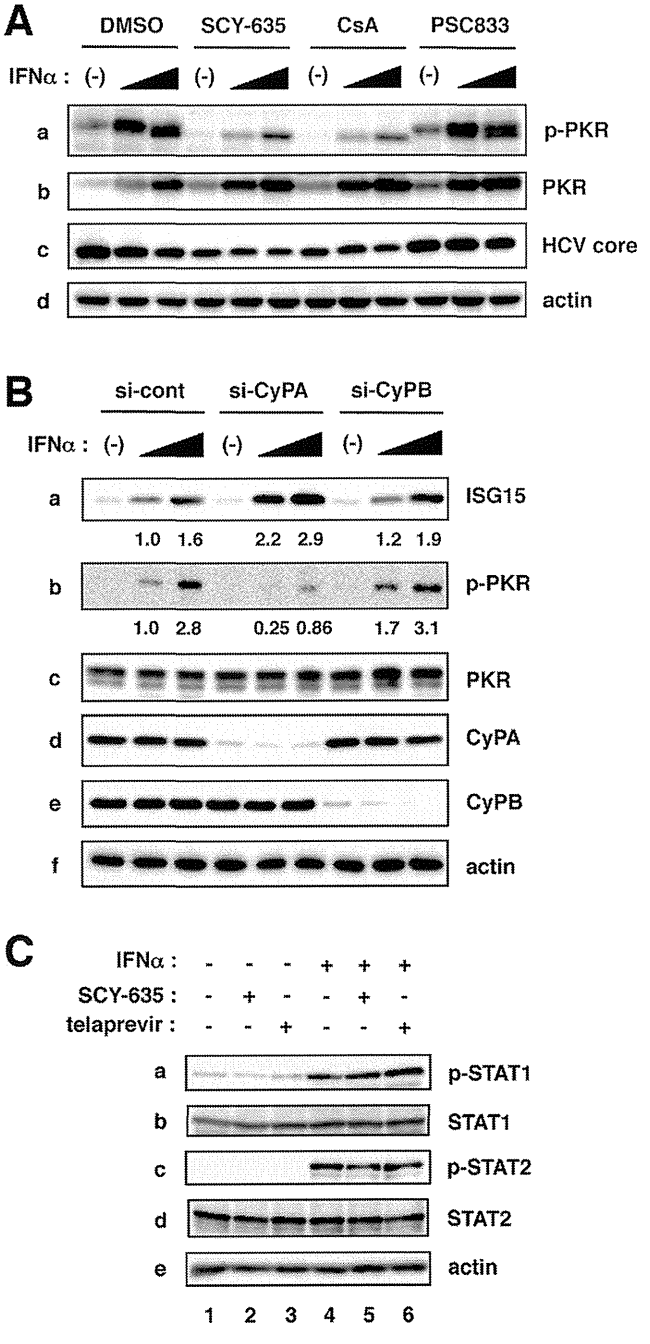


Figure 2. Phosphorylation of PKR was inhibited by SCY-635. (*A*) HCV-infected Huh-7 cells were treated with dimethyl sulfoxide (DMSO), 0.05%; SCY-635, 2 μM; or a protease inhibitor telaprevir, 2 μM for 24 hours, followed by treatment with or without IFNα (10 or 100 IU/mL) for 16 hours. Protein production 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.[29] 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.

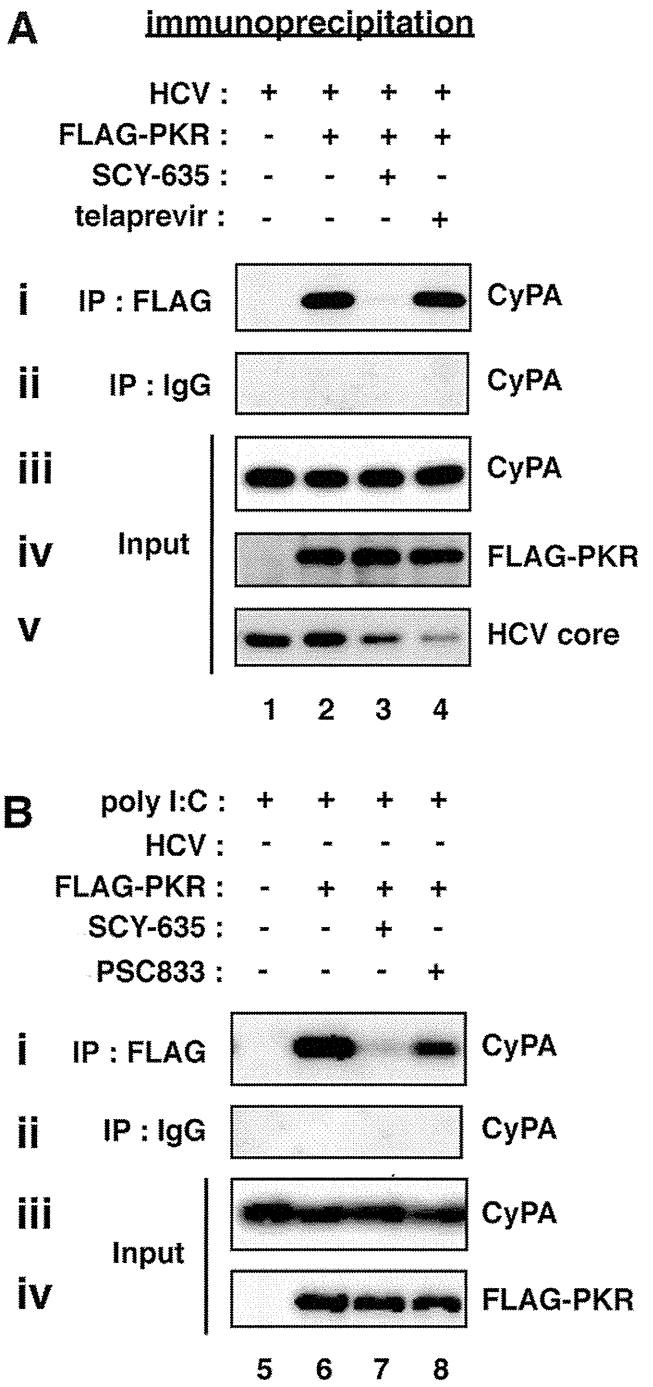


Figure 4. Interaction of PKR and CyPA in HCV-infected and poly I:C–stimulated cells. HCV-infected (*lanes 1–4*) and uninfected Huh7 cells (*lanes 5–8*) transfected with an expression plasmid for FLAG-tagged PKR (*lanes 2–4* and *6–8*) or the empty vector (*lanes 1* and *5*) for 6 hours were pretreated 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

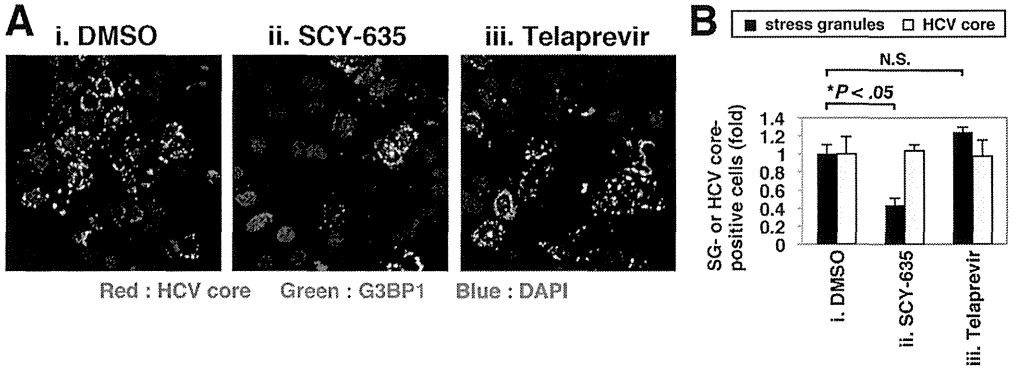


Figure 5. SCY-635 inhibited the formation of SGs. (*A*) HCV-infected Huh-7 cells were treated with dimethyl sulfoxide, 0.05% (*i*); SCY-635, 2 μM (*ii*); or telaprevir, 2 μM (*iii*) together with IFNα 100 IU/mL for 12 hours, and then were detected for G3BP1, an SG marker (*green*), HCV core protein (*red*), 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

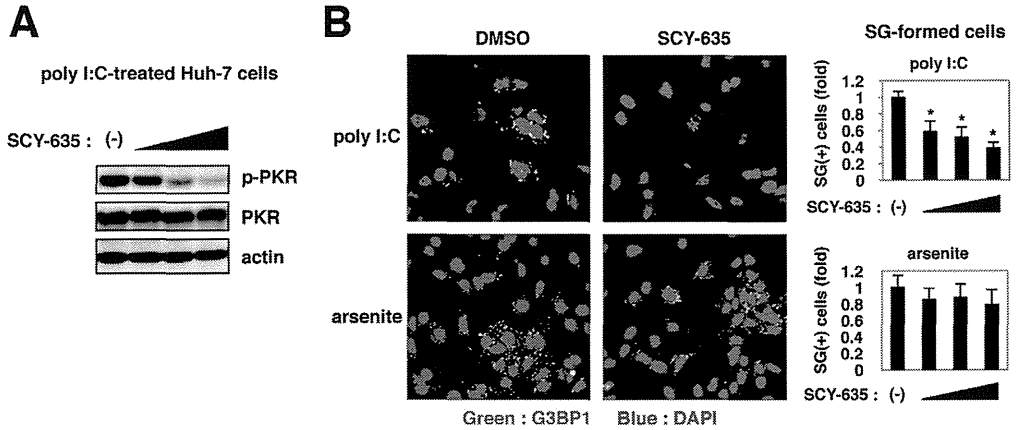


Figure 6. Modulation of PKR and SG formation by SCY-635 in the absence of HCV. (*A*) Huh-7.5.1 cells stimulated with poly I:C 0.5 μg/mL for 6 hours were pretreated with varying concentrations of SCY-635 (0, 2, 4, and 8 μM) for 18 hours. The cells were then treated with IFNα 100 IU/mL. At 24 hours later, 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*).

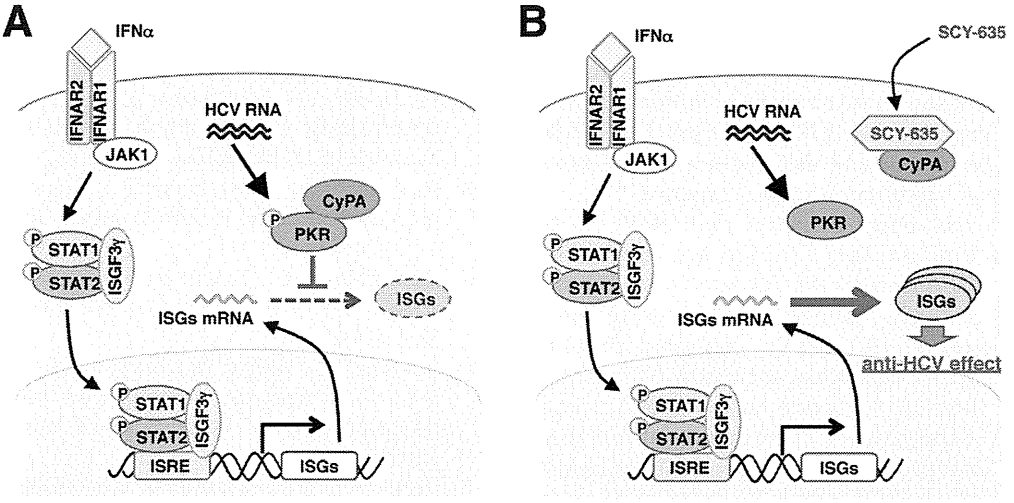


Figure 7. Schematic representation of the IFN signal transduction and the role of CyPA in regulating this pathway. (*A*) In the absence of CyP inhibitors, IFN stimulation triggers the activation of Janus-activated kinase 1, which phosphorylates STAT1 and STAT2, and translocates into the nucleus in association 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
These authors disclose the following: Takuji Daito, Ann Sluder, and Katyna Borroto-Esoda are employees of SCYNEXIS, Inc. The remaining authors disclose no conflicts.

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Competing Interests: HB is an employee of DNAVEC Corporation. M. Hasegawa is a founder and adviser of DNAVEC Corporation. NF was an employee of DNAVEC Corporation until January 2013 but not now. The commercial product developed by DNAVEC Corporation is similar to the vectors described in this paper but the component is different. The patent of the Sendai virus vectors to generate iPS cells that was applied by and of DNAVEC Corporation is pending (WO/2010/008054). NF and HB have waived the right of the patent. These do not alter the authors'

RESEARCH ARTICLE

New Type of Sendai Virus Vector Provides Transgene-Free iPS Cells Derived from Chimpanzee Blood

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Abstract

Induced pluripotent stem cells (iPSCs) are potentially valuable cell sources for disease models and future therapeutic applications; however, inefficient generation and the presence of integrated transgenes remain as problems limiting their current use. Here, we developed a new Sendai virus vector, TS12KOS, which has improved efficiency, does not integrate into the cellular DNA, and can be easily eliminated. TS12KOS carries *KLF4, OCT3/4*, and *SOX2* in a single vector and can easily generate iPSCs from human blood cells. Using TS12KOS, we established iPSC lines from chimpanzee blood, and used DNA array analysis to show that the global gene-expression pattern of chimpanzee iPSCs is similar to those of human embryonic stem cell and iPSC lines. These results demonstrated that our new vector is useful for generating iPSCs from the blood cells of both human and chimpanzee. In addition, the chimpanzee iPSCs are expected to facilitate unique studies into human physiology and disease.