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Cyclophilin A inhibits rotavirus replication by facilitating host IFN-I production

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ABSTRACT

Rotavirus (RV) infection causes serious dehydrating diarrhoea in infants and newborn animals. Our previous study revealed that cyclophilin A (CYPA), a peptidyl-prolyl cis–trans isomerase (PPIase), could be temporarily upregulated in RV-infected MA104 cells in early stage of infection (unpublished data). To find out the possible roles of CYPA in RV infection, we overexpressed and silenced CYPA in various cell lines by gene transfection and shRNA. We found that transfection of CYPA significantly inhibited RV replication, while silencing the expression of CYPA significantly increased RV replication. Accordingly, overexpression of CYPA significantly increased IFN-β production; while silencing CYPA significantly reduced IFN-β production. This effect of CYPA on IFN-β production was independent of its PPIase activity. Moreover, IFN-β secreted by host cells in RV infection had a critical repressive effect on viral replication. Finally, we found that inhibiting JNK pathway by SP600125 and JNK siRNA abrogated the effect of CYPA on IFN-β transcription in RV-infected MA104 cells. Together, our data suggested that CYPA inhibited RV replication by facilitating host IFN-β production, which was independent on the PPIase activity of CYPA but dependent on the activation of JNK signaling pathway.

1. Introduction

Rotavirus (RV) is the primary cause of serious dehydrating diarrhoea in infants and newborn animals [1]. Like most viral infections, RV infection elicits a global shutoff of host protein synthesis. However, several studies revealed that some host proteins such as α2β1 and β2 integrins [2], Grp78 and Grp94 [3,4] were selectively up-regulated in RV infection for efficient virus replication. In our previous study, we found that the expression of CYPA was temporarily upregulated in early stage of RV infection in MA104 cells (unpublished data), indicating that CYPA might also play a role in RV infection. CYPA is one of the main cyclophilins in mammalian cells. It possesses peptidyl-prolyl cis–trans isomerase activity, which enables it to function as a molecular chaperone and a catalyst of conformational changes in several cellular processes [5,6]. Accumulating evidences have shown that CYPA is an important host factor for successful viral infections. CYPA is incorporated into viral particles of many enveloped viruses such as human immunodeficiency virus type 1 (HIV-1) [7] and vaccinia virus [8]. CYPA binds to the CA domain of HIV-1 Gag polyprotein which is necessary for effective production of infectious virions and HIV-1 sensitivity to host restriction factors [9,10]. In HCV infection, CYPA is an essential cofactor and plays a critical role in proper assembly and function of HCV replication complex through its PPIase activity [11]. CYPA is also essential in the replication of vesicular stomatitis virus New Jersey serotype [12]. However, the role of CYPA in RV infection remains unclear.

Viral infection usually triggers host antiviral defenses such as interferon-I (IFN-I)-mediated antiviral response. In RV infection, it was found that the IFN-I response was hampered by RV nonstructural protein 1(NSP1) through the inhibition of IRF3, IRF5, IRF7 and NFκB [13,14]. However, our previous study revealed that RV infection still induced abundant IFN-β, indicating that there might be alternative pathways for IFN-β production in RV infection. It was reported that CYPA conducted IFN-β production in DC cells infected with HIV-1 pseudovirus through its PPIase activity [15]. Thus it is interesting that whether CYPA could also conduct IFN-I response in the infection of natural viruses such as RV.

In this study, we analysed the role and mechanism of CYPA in RV infection with both genomic and chemical methods. We found that CYPA inhibited RV replication by facilitating IFN-β production in RV-infected MA104 cells. This effect of CYPA was independent on its PPIase activity but dependent on the activation of JNK.

Abbreviations: CYPA, cyclophilin A; FACS, flow cytometric analysis; IFN-β, interferon-beta; pAbs, polyclonal antibodies; PPIase, peptidyl-prolyl cis–trans isomerase; qRT-PCR, quantitative RT-PCR; RV, rotavirus; TBS, Tris-buffered saline.

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2. Materials and methods

2.1. Cell culture and viral infection

MA104 cells were provided by Dr. Elschner (Germany). HEK293 and Caco-2 cells were kept in our institute. All the cells were cultured in DMEM (Invitrogen, USA) plus 10% (vol/vol) fetal calf serum (FCS) (Gibco, USA) at 37 °C with 5% CO_2. RV (Wa strain) was from Chinese Center for Disease Control and Prevention (Beijing, China) and propagated in MA104 cells as described [16]. For RV infection, the virus was diluted in DMEM, activated with 10 μg/ml trypsin (Amersco, USA) for 30 min at 37 °C, and then added to confluent target cells previously washed three times with PBS. After 1 h of incubation at 37 °C, the excessive virus was removed; the cells were then washed twice by DMEM and incubated in DMEM at 37 °C till use.

2.2. Flow cytometric analysis

MA104 cells were infected with RV (moi = 0.5), then 10 μg/ml IFN-β and control polyclonal antibodies (pAbs) (Santa cruz, USA) were added into the culture medium. Both the antibodies were pretreated with inactivated RV particles to remove the possible pollution of RV antibodies. After 12 h of incubation at 37 °C, the cells were fixed in 4% paraformaldehyde and permeabilized in 1% Triton-X-100 (Sigma, USA). Then the cells were washed for 3 × 5 min in PBS-1% BSA-50 mM NH_4Cl, incubated with 1:100 dilution of FITC-conjugated goat anti RV pAbs (Virostat, USA) for 1 h at 4 °C, and finally applied to flow cytometer (BD, USA).

2.3. Western blot

Cells were lysed in RIPA buffer (50 mM Tris–Cl (pH 7.4), 150 mM NaCl, 0.1% SDS, 1% Triton-X-100, 0.25% Na-deoxycholate, 1 mM PMSF, EDTA: 1 mM, aprotinin, leupeptin and pepstatin: 1 μg/ml each, Na_3VO_4: 1 mM, NaF: 1 mM). Cell lysates were collected in 1.5 ml EP tubes, clarified by centrifugation at 10,000×*g* for 30 min at 4 °C, and then quantified using a BCA assay kit (Pierce, USA). Clarified lysates (30 μg) were loaded onto precast 4–12% gradient gels (Invitrogen, USA) for SDS–PAGE and then transferred to 0.22 or 0.45 μm PVDF membranes (Millipore, USA) using the XCell *Surelock*™ Mini-Cell and XCell II™ Blot Module system (Invitrogen, USA). Membranes were blocked with 5% defatted milk diluted in 1‰ TBST (TBS (50 mM Tris and 150 mM NaCl, pH 7.6) containing 1‰ Tween 20). The membranes were then incubated with rabbit pAbs to CYPA (Santa Cruz Technology, USA), rabbit pAbs to JNK and phospho-JNK (p-JNK) (Cell Signaling Technology, USA) and goat pAbs to RV (react with RV structural protein VP6 and VP7). GAPDH was detected with rabbit pAbs to GAPDH (Abcam, USA) as control. Afterwards, membranes were incubated with horseradish peroxidase (HRP)–conjugated anti-rabbit or anti-goat IgGs (Santa Cruz Technology, USA). Immunoreactive bands were visualized using enhanced chemiluminescence's substrate (Amersham Pharmacia Biotech, USA) and exposed to films (Kodak, USA) in darkroom.

2.4. Gene over expression and silencing

Wild type of CYPA gene was obtained by RT-PCR from RV-infected Caco-2 cells. The primers were as follows: forward primer 5′-CGGGATCCAAAGATTCTAGGATACTGC-3′ and reverses primer 5′-CTCGAGTATAAAAGGGGCGGGAGG-3′. Then CYPA was subcloned into the retroviral vector pMSCV to generate pMSCV–CYPAwt. The PPIase negative mutant of CYPA (pMSCV–CYPA/R55A) was obtained by point mutation on pMSCV–CYPAwt using site-directed gene mutagenesis kit (Stratagene, USA). The plasmids including pMSCV–CYPAwt, pMSCV–CYPA/R55A and pMSCV empty vector were transfected to confluent target cells with Lipofectamine 2000 (Invitrogen, USA). The stably transfected cells were obtained by puromycin selection (MA104, 10 μg/ml; HEK293, 3 μg/ml and Caco-2, 20 μg/ml) for 4 weeks and confirmed by western blot. To silence the endogenous CYPA, plasmids expressing shRNA specific to CYPA (pSilencer5.1-sh CYPA) (Ambion, USA) and a scrambled sequence (pSilencer5.1-sh scrambled) (Ambion, USA) were transfected into target cells by Lipofectamine 2000 (Invitrogen, USA). The stably transfected cells were also selected by puromycin and confirmed by western blot. All the gene constructions were carried out under the approval of the Ethics Committee of Third Military Medical University. JNK was silenced by cotransfection of synthesized siRNA (40 μM) to JNK1 (GGCAUGGGCUACAAG GAAA) and JNK2 (GAUUGUUUGUGCUGCAUUU) using Lipofectamine 2000. A scrambled sequence (AAGAACGGCAUCAAGGUGAAC) was transfected as control siRNA.

2.5. Immunofluorescence assay (IFA)

MA104 cells on cover slips were infected with RV (moi = 0.3) or mock-infected with DMEM for 12 h, then the cells were fixed in 4% paraformaldehyde for 15 min and permeabilized in 1% Triton-X-100 for 7 min at room temperature. After three washes in PBS-1% BSA-50 mM NH_4Cl, the cells were blocked in 5% BSA for 1 h and incubated with rabbit pAbs to p-JNK (Cell signaling technology, USA) (1:100) overnight at 4 °C. After three washes in PBS-1% BSA, the cells were incubated with a mixture of Dylight 647-conjugated donkey anti rabbit pAbs (Invitrogen, USA) and FITC-conjugated goat anti RV pAbs (Virostat, USA) for 60 min at 37 °C and followed by a 10 min incubation with 5 μg/ml DAPI (Sigma, USA) at room temperature. The triple-stained cells were washed thrice in PBS and examined under a Leica TCS SP5 laser confocal microscope.

2.6. Fluorescence foci assay (FFA)

RV-infected cells together with the culture medium were subjected to three freeze-and-thaw cycles and then clarified by centrifugation at 13,000×*g* for 15 min at 4 °C. The supernatant was collected for detection of total virus production by FFA. Briefly, confluent monolayers of MA104 cells in 96-well tissue culture plates were infected with the supernatant in a series of dilutions (10^3, 10^4 and 10^5). At 20 h p.i., the cells were fixed with 4% paraformaldehyde for 15 min, permeabilized with 1% Triton-X-100 for 7 min and washed for 3 × 5 min in PBS. The cells were then incubated with FITC-conjugated goat anti RV pAbs (Virostat, USA) (1:100) for 1 h at room temperature. The fluorescent foci-forming units were counted under a fluorescent microscope (Olympus, Japan) with the help of a Visiolab semiautomatic system. Viral titer was expressed as focus-forming units (ffu) per millilitre.

2.7. qRT-PCR

The stably transfected (pMSCV-CYPAwt, pMSCV, pSilencer5.1-sh CYPA and pSilencer5.1-sh scrambled) and untransfected (control) MA104 cells were infected with RV at a moi of 3 for 12 h. Then IFN-β mRNA was analysed with a qRT-PCR kit (Takara, Japan) following the manufacturer's instructions. The primers for IFN-β were as follows: forward primer 5′-CTCCTCCAAATTGCTCTCCTG-3′ and reverses primer 5′-GCAAACTG CTCACGAATT TTCC-3′. β-Actin was selected as control with forward primer 5′-CATGTTTG AGACCTTCAA CAC-3′ and reverses primer 5′-CATCTCCTGCTCGAAGTCTAG-3′. The stably transfected and untransfected (control) MA104 cells were infected with RV (moi = 3) or mock-infected by DMEM. Then 10 μM SP600125 (inhibitor of JNK, Sigma, USA) was added into the culture

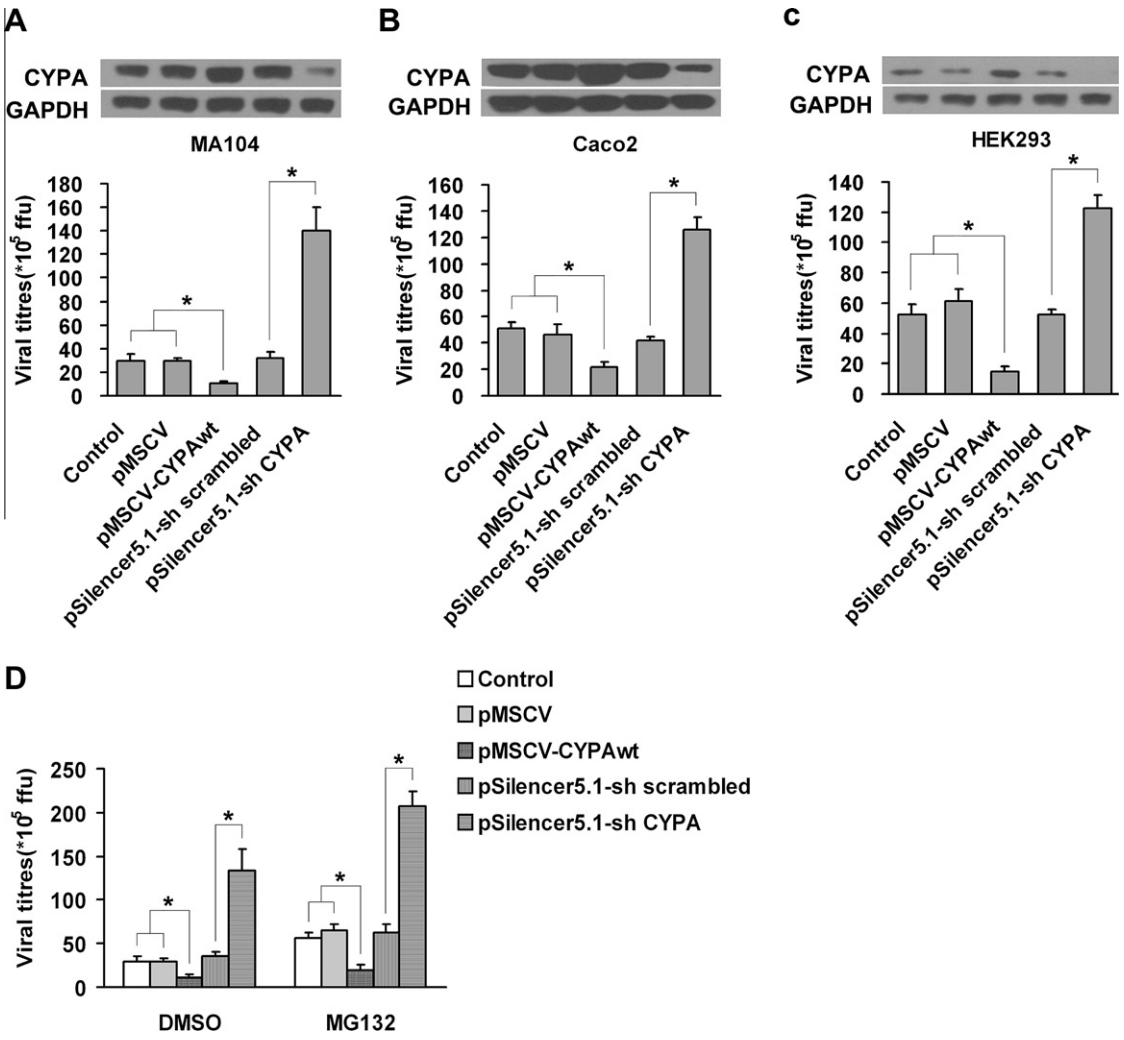

Fig. 1. CYPA inhibited RV replication in host cells. (A–C) MA104, Caco-2 and HEK293 cells stably transfected with plasmids pMSCV-CYPAwt, pMSCV, pSilencer5.1-sh CYPA, pSilencer5.1-sh scrambled and the untransfected cells (control) were infected with RV at a moi of 3 for 12 h and total viral yields were analysed by FFA. (D) The stably transfected and untransfected (control) MA104 cells were infected with RV at a moi of 3 for 8 h. Then 10 μM MG132 and DMSO (control) were added into the culture medium for another 4 h of incubation, then the total viral yields were analysed by FFA. $^{*}p \leqslant 0.05$.

medium for 12 h of incubation. The same amount of DMSO was added as control. The IFN-β mRNA in the cells were analysed by qRT-PCR as above. The stably transfected and untransfected (control) MA104 cells were transfected with 40 μM siRNA to JNK1/2 and control siRNA for 36 h. The same amount of Lipofectamine 2000 was added as control. The cells were then infected with RV (moi = 3) for 12 h. The IFN-β mRNA in the cells was analysed by qRT-PCR as above.

2.8. Chemical administrations

The stably transfected and untransfected (control) MA104 cells were infected with RV (moi = 3) for 8 h. Then 10 μM MG132 (proteosome inhibitor, Sigma, USA) were added into the culture medium and the same amount of DMSO was added as control. After additional 4 h of incubation, total virus yields were analysed by FFA. MA104 cells were infected with RV (moi = 3), then 10 μM CsA (inhibitor of cyclophilins, Sigma, USA), 1 μg/ml FK506 (inhibitor of FKBPs, Sigma, USA), 10 μM Wortmannin (inhibitor of PI3K/Akt, Sigma, USA), 10 μM SP600125 and 1 μM BAY 11-7082 (inhibitor of NF-κB, Sigma, USA) were added into the culture medium for 12 h of incubation. Then IFN-β in the culture medium was analysed by ELISA.

2.9. Elisa

MA104 cells stably transfected with plasmids pMSCV-CYPAwt, pMSCV-CYPA/R55A, pMSCV, pSilencer5.1-sh CYPA, pSilencer5.1-sh scrambled and the untransfected cells (control) were infected with RV (moi = 3). The infected cells were then cultured in 500 μl DMEM for an additional 12 h. At the end of infection, IFN-β in culture medium was detected with an IFN-β ELISA kit (USCNLIFE, USA) following the manufacturer's instructions. MA104 cells were transfected with 40 μM siRNA to JNK1/2 and control siRNA for 36 h, then the cells were infected with RV (moi = 3) for 12 h. At the end of infection, IFN-β in culture medium was analysed by ELISA.

3. Results

3.1. CYPA inhibited RV replication in host cells

To investigate the effect of CYPA in RV infection, we overexpressed wild type of human CYPA and silenced the endogenous CYPA in various host cells. We found that overexpression of CYPA caused a significant decrease in RV replication, while silencing endogenous CYPA significantly increased RV replication (Fig 1A–C). Inhibition

of proteasomes by MG132 did not impair the repressive effect of CYPA on RV replication (Fig 1D), and it also did not significantly alter CYPA expression patterns of these cells (data not shown), either. These results indicated that CYPA in host cells had an inhibitory effect in RV replication, and this effect was independent on host proteosome degradation system.

3.2. CYPA facilitated IFN-β production in RV-infected MA104 cells

IFN-I response is an important host antiviral mechanism. To determine if CYPA played an important role in host IFN-I response to RV infection, we examined the mRNA and protein levels of IFN-β by qRT-PCR and ELISA in RV-infected MA104 cells when CYPA was overexpressed or silenced. Our results showed that in MA104 cells overexpressing wild type of CYPA, IFN-β expression was significantly increased; while in CYPA-silenced MA104 cells, IFN-β expression was remarkably decreased (Fig 2A and B). These results indicated that CYPA facilitated host IFN-β production in RV-infected MA104 cells. To determine whether the PPIase activity of CYPA was necessary for host IFN-I response in RV infection, we inhibited the PPIase activity of CYPA by overexpressing its PPIase negative mutant CYPA/R55A or by administration of chemical inhibitors of PPIase such as CsA and FK506. We found that neither CYPA/R55A nor chemical inhibitors of PPIase impaired IFN-β production (Fig 2C and D), indicating that the PPIase activity of CYPA was not needed for IFN-β production in RV infection.

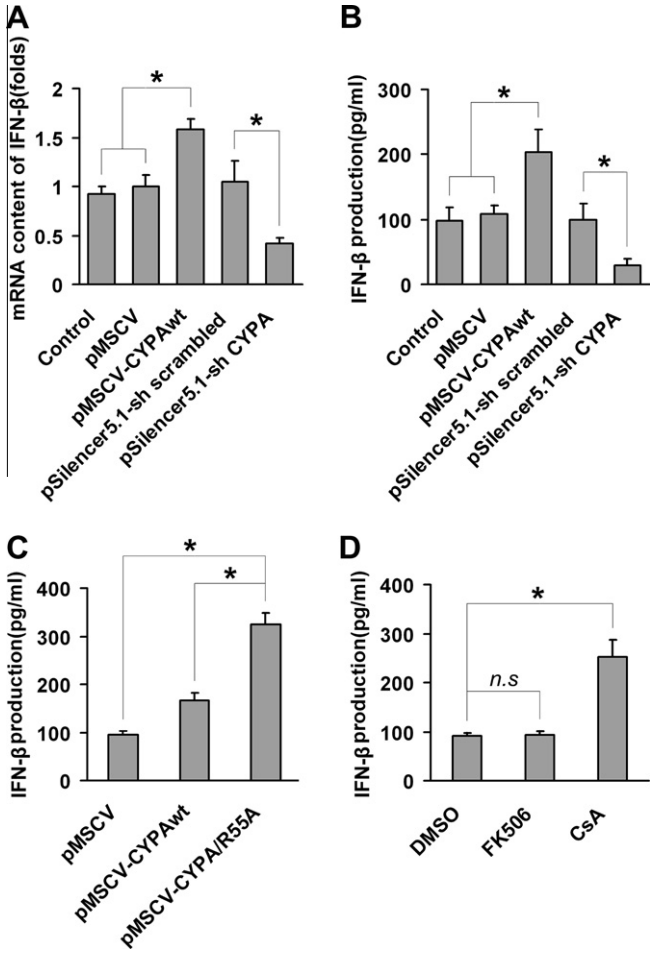

Fig. 2. CYPA facilitated IFN-β production in RV-infected MA104 cells. (A and B) The stably transfected and untransfected (control) MA104 cells were infected with RV at a moi of 3 for 12 h. Then IFN-β in the culture medium and IFN-β mRNA in RV-infected cells were analysed by and ELISA and qRT-PCR, respectively. (C) MA104 cells stably transfected with plasmids pMSCV-CYPAwt, pMSCV-CYPA/R55A and pMSCV plasmids were infected with RV (moi = 3) for 12 h, then IFN-β in the culture medium was analysed by ELISA and (D) MA104 cells were infected with RV (moi = 3), then 10 μM CsA, 1 μg/ml FK506 and equal amount of DMSO (control) were immediately added into the culture medium for additional 12 h of incubation. Then IFN-β in the culture medium was analysed by ELISA. $^{*}p \leqslant 0.05$, n.s: no significance.

3.3. IFN-β produced by host cells inhibited RV infection

It was reported that RV antagonized host IFN-I response by impairing both expression and effect of IFN-β [13,14,17]. However, we found that RV infection still induced abundant IFN-β. To find out whether IFN-β could still play an important role in inhibiting RV infection, we antagonized the effect of IFN-β by adding IFN-β pAbs in the culture medium immediately after RV infection. We found that abrogation of IFN-β by IFN-β pAbs (red) resulted in a slight (about 12%) but significant ($p < 0.05$) increase of RV infection rate compared to control pAbs (black) (Fig 3A). However, there was a more significant increase in RV replication (about 80%, $p < 0.05$) when IFN-β was antagonized (Fig 3B). Accordingly, the expressions of RV structural proteins (such as VP6 and VP7) were remarkably increased when IFN-β was antagonized (Fig 3C). These results indicated that the secreted IFN-β by host cells during RV infection possessed an important repressive effect on viral infection.

3.4. Inhibition of JNK abrogated the facilitative effect of CYPA on IFN-β transcription

By immunofluorescence assay, we found that the activated JNK (p-JNK, red) in RV-infected MA104 cells (green) was significantly more abundant than in mock-infected cells (Fig 4A), especially in the nucleus (blue). Western blot also confirmed the activation of JNK in RV-infected MA104 cells (Fig 4B). IFN-I response in viral infection is usually triggered via activation of several different

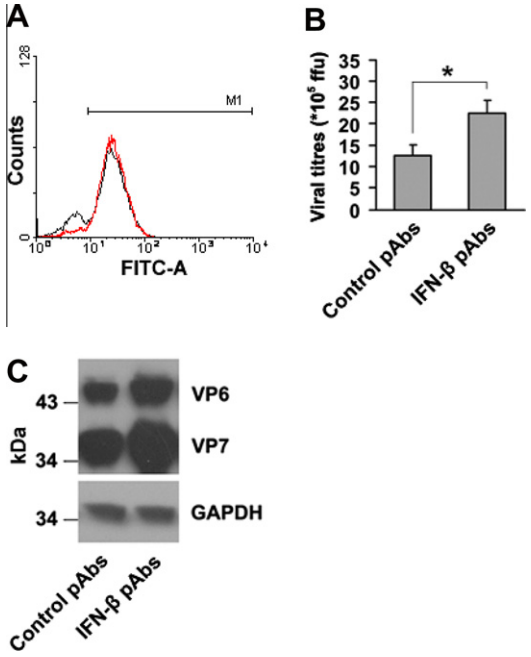

Fig. 3. Host IFN-β inhibited RV infection. MA104 cells were infected with RV (moi = 0.5), then 10 μg/ml IFN-β and control pAbs were immediately added into the culture medium for 12 h of incubation. (A) Flow cytometric analysis of RV infection. Black line: treatment of control pAbs, red line: treatment of IFN-β pAbs. (B) FFA analysis of RV production and (C) Western blot analysis of RV structural proteins. $^{*}p \leqslant 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

signaling pathways, mainly including PI3K/Akt, NF-κB and JNK [18]. To determine whether the JNK signals were critical in IFN-I response in RV-infected cells, chemical inhibitors of these pathways were used. We found that inhibition of PI3K/Akt by Wartmannin or inhibition of NF-κB by BAY11-7082 did not inhibit IFN-β production in RV-infected MA104 cells. However, inhibition of JNK by SP600125 reduced the production of IFN-β to an undetectable level (Fig 4C). Similarly, silencing JNK by siRNA also significantly reduced IFN-β expression in RV-infected MA104 cells (Supplemental Fig. 1, (Fig 4D)). These results indicated that IFN-I response in RV infection mainly depended on JNK signaling pathway. Neither overexpressing wild type of CYPA nor silencing endogenous CYPA had significant effect on JNK activation in RV-infected MA104 cells (Supplemental Fig. 2), however, inhibition of JNK by both SP600125 and JNK siRNA abrogated the facilitative effect of CYPA on IFN-β transcription (Fig 4E and F).

4. Discussion

This study demonstrated that CYPA played an important antiviral role in RV infection, which was remarkably different from its usual roles in many other viral infections where it was exploited for efficient viral replication. However, similar repressive role of CYPA as in RV infection was also found in the infection of Influenza A virus [19]. CYPA is an important PPIase in mammalian cells. Like molecular chaperones, the main role of PPIase is to facilitate conformational changes of proteins for proper folding, which greatly influence protein functions and their ultimate fates. Recent study revealed that in Influenza A infection, CYPA restricted influenza virus replication by accelerating degradation of the M1 protein through the ubiquitin/proteasome-dependent pathway [20]. So it is possible that CYPA might also inhibit RV replication by accelerating degradation of RV structural proteins. However, our results

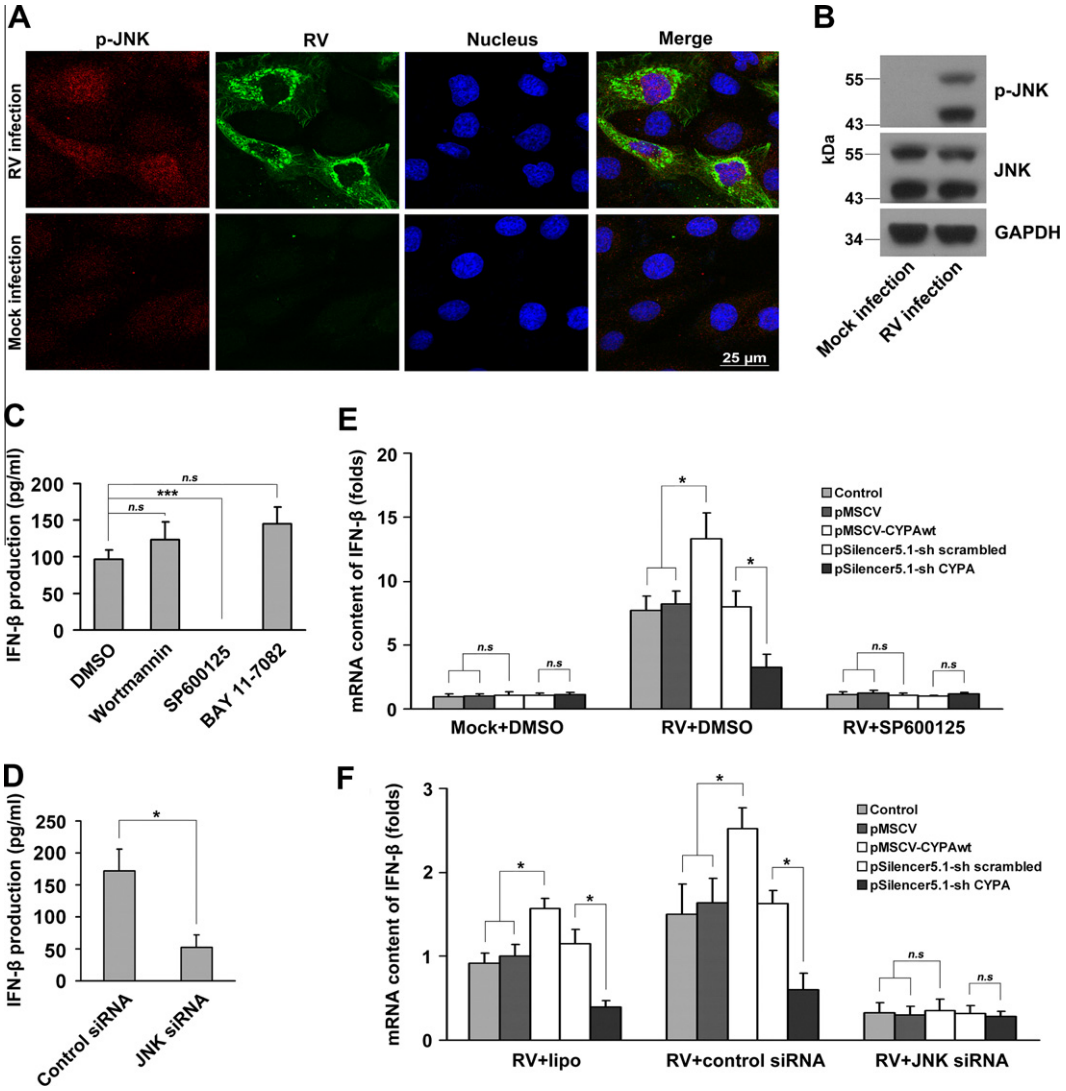

Fig. 4. Inhibition of JNK abrogated the facilitative effect of CYPA on IFN-β transcription. (A) MA104 cells on cover slips were infected with RV (moi = 0.3) or mock-infected with DMEM for 12 h. Then IFA assay was carried out to show the activated JNK (p-JNK, red) in the nucleus (blue) of RV-infected cells (green). Bar, 25 μm. (B) MA104 cells were infected with RV (moi = 3) for 12 h, then total JNK and p-JNK were analysed by western blot. (C) MA104 cells were infected with RV (moi = 3) and then 10 μM Wortmannin, 10 μM SP600125 and 1 μM BAY 11–7082 were added into the culture medium for 12 h of incubation. The same amount of DMSO was added as control. The IFN-β in the culture medium was analysed by ELISA. (D) MA104 cells were transfected with 40 μM siRNA to JNK1/2 and control siRNA for 36 h, then the cells were infected with RV (moi = 3) for 12 h. At the end of infection, IFN-β in culture medium was analysed by ELISA. (E) The stably transfected and untransfected (control) MA104 cells were infected with RV (moi = 3) or mock-infected by DMEM. Then 10 μM SP600125 or the same amount of DMSO was added into the culture medium. After 12 h of incubation, IFN-β mRNA in the infected cells were analysed by qRT-PCR and (F) The stably transfected and untransfected (control) MA104 cells were transfected with 40 μM siRNA to JNK1/2 and control siRNA for 36 h. The same amount of Lipofectamine 2000 (lipo) was added as control. The cells were then infected with RV (moi = 3) for 12 h. The IFN-β mRNA in the cells was analysed by qRT-PCR. $^{*}p \leqslant 0.05$, $^{***}p \leqslant 0.001$, n.s: no significance. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

revealed that the inhibitory role of CYPA on RV replication was independent on proteasomes (Fig 1D). Thus it is probably that there might be a novel way for CYPA to inhibit RV infection.

Viral infection usually triggers host antiviral defense such as IFN-I-mediated response. In DC cells infected with HIV-1 pseudovirus, CYPA recognized the CA domain of HIV Gag protein and induced IFN-β production [21]. In this study we found that CYPA was also critical for IFN-β production in the infection of natural virus like RV. Notably, the PPIase activity of CYPA was not necessary but probably harmful to IFN-β production in RV infection (Fig 2C and D). It is quite different from HIV-1 pseudovirus infection in DC cells where the effect of CYPA on promoting IFN-β production depended on its PPIase activity. Although RV infection could impair both expression and function of IFN-β in host cells, our results revealed that IFN-β produced host cells still played a significant inhibitory role in RV infection (Fig 3). Thus the effect of CYPA on promoting host IFN-β production was probably an important mechanism for CYPA to inhibit RV infection.

In this study, we found that JNK signal pathway played a critical role in host IFN-I response in RV infection. Inhibition of JNK by SP600125 and siRNA significantly impaired IFN-β production in RV infection (Fig 4C and D) but did not impair RV infection (Supplemental Fig. 3) [22]. JNK phosphorylates and activates its downstream transcriptional factors such as ATF-2, c-jun and IRF3 [23], which then facilitate the transcription of target genes including IFN-β [18]. Inhibition of JNK would interrupt these signals and ultimately resulted in an inhibition of IFN-β transcription. CYPA had no significant effect on JNK activation (Supplemental Fig. 2), although it facilitated IFN-β production in RV infection (Fig 2A and B). However, inhibiting JNK abrogated the facilitative effect of CYPA on IFN-β transcription (Fig 4E and F). Thus it was suggested that CYPA might be a downstream effector of JNK in conducting host IFN-β response to RV infection. Further studies are needed to clarify the mechanism how CYPA is involved in JNK signals to facilitate IFN-β production.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.05.050.

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