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# Enterovirus 71 Disrupts Interferon Signaling by Reducing the Level of Interferon Receptor 1

**Jing Lu,[a] Lina Yi,[a] Jin Zhao,[a,d] Jun Yu,[b] Ying Chen,[a] Marie C. Lin,[c] Hsiang-Fu Kung,[a] and Ming-Liang He[a]**

Stanley Ho Center for Emerging Infectious Diseases and Li Ka Shing Institute of Health Sciences, Faculty of Medicine, The Chinese University of Hong Kong, Hong Kong, China[a]; Department of Medicine and Therapeutics, Faculty of Medicine, The Chinese University of Hong Kong, Hong Kong, China[b]; Department of Surgery, Faculty of Medicine, The Chinese University of Hong Kong, Hong Kong, China[c]; and Shenzhen Center for Disease Prevention and Control, Nanshan District, Shenzhen, China[d]

**The recent outbreak of enterovirus 71 (EV71) infected millions of children and caused over 1,000 deaths. To date, neither an effective vaccine nor antiviral treatment is available for EV71 infection. Interferons (IFNs) have been successfully applied to treat patients with hepatitis B and C viral infections for decades but have failed to treat EV71 infections. Here, we provide the evidence that EV71 antagonizes type I IFN signaling by reducing the level of interferon receptor 1 (IFNAR1). We show that the host cells could sense EV71 infection and stimulate IFN-$\beta$ production. However, the induction of downstream IFN-stimulated genes is inhibited by EV71. Also, only a slight interferon response and antiviral effects could be detected in cells treated with recombinant type I IFNs after EV71 infection. Further studies reveal that EV71 blocks the IFN-mediated phosphorylation of STAT1, STAT2, Jak1, and Tyk2 by reducing IFNAR1. Finally, we identified the 2A protease encoded by EV71 as an antagonist of IFNs and show that the protease activity is required for reducing IFNAR1 levels. Taken together, our study for the first time uncovers a mechanism used by EV71 to antagonize type I IFN signaling and provides new targets for future antiviral strategies.**

Enterovirus 71 (EV71) is a typical positive-strand RNA virus which belongs to the *Picornaviridae* family (44). The genome of EV71 is approximately 7.5 kb in length and contains a single open reading frame encoding a polyprotein precursor. This polyprotein is cleaved by two viral proteases, $2A^{pro}$ and $3C^{pro}$, to form four structural proteins (VP1, VP2, VP3, and VP4) and seven nonstructural proteins (2A, 2B, 2C, 3A, 3B, 3C, and 3D) (39). EV71 infection was a major cause of outbreaks of hand, foot, and mouth disease (HFMD) in infants and young children (19, 22). As a typical neurotropic virus, EV71 has a propensity to cause neurological disease during acute infection and may lead to permanent paralysis and even death (1, 42, 45). The outbreaks and severity of EV71 infection have been frequently reported worldwide (60), and recently EV71 has become a major threat to public health in China (56). The Chinese government reported that there were ~1,770,000 cases of HFMD and herpangina with over 900 deaths in 2010 (http://www.moh.gov.cn/publicfiles/business/htmlfiles/zwgkzt/pyq/list.htm). However, to date, no effective vaccine or therapy can be applied to prevent or treat EV71 infection.

Type I interferon (IFN), as the first line of host immune response, is critical in mediating antiviral defense. The host recognizes viral invasion and activates the type I IFN response through the recognition of pathogen-associated molecular patterns (PAMPs) by pattern-recognition receptors (PRRs) (61). Binding with the corresponding receptors, IFN receptor 1 (IFNAR1) and IFNAR2 (11, 50), the secreted IFNs activate the Janus kinases Jak1 and Tyk2 and then phosphorylate signal transducers and activators of transcription STAT1 and STAT2 (23). The phosphorylated STAT1 and STAT2 form heterodimers and bind with IFN regulatory factor 9 to form the transcription factor ISGF3 (IFN-stimulated gene factor 3) (20). ISGF3 translocates to the nucleus and binds a promoter region called the IFN-stimulated response element (ISRE) (31). This interaction initiates activation of hundreds of IFN-stimulated genes (ISGs) that collectively alter the cellular or viral processes and modulate the immune response toward establishing an antiviral state (15, 16). Because of the powerful antiviral activities, IFNs have been used clinically to control and treat viral infections for decades (10, 12, 51). However, conventional IFNs showed limited effects in EV71-infected patients for unknown reasons. Also, the type I IFNs protected mice only when they were administered before EV71 challenge, while little effect could be achieved when IFNs were administered after viral infection (40). In clinical settings, IFNs showed only slight anti-EV71 effects. In cases in which neurological complications occurred in EV71-infected children, IFNs displayed minor, even unobservable, effects in patients. We have recently observed and reported that the conventional type I IFNs can control EV71 infection and replication only at high concentrations (59). These results suggest that EV71 may develop mechanisms to circumvent the type I IFN response.

In this study, we provided the evidence that EV71 efficiently repressed the type I IFN signaling pathway by targeting a subunit of the IFN receptor, IFNAR1. To our knowledge, this was also the first study showing that a picornavirus could evade the host IFN response by blocking IFN signaling. By suppressing IFNAR1 levels, EV71 reduced the IFN-inducible phosphorylation of Jak1, Tyk2, and STAT proteins, thereby inhibiting the activation of ISGs, and established its productive infection. The $2A^{pro}$ was demonstrated to be the viral protein that reduces IFNAR1 levels and interferes with type I IFN signaling.

## MATERIALS AND METHODS

**Cells and viruses.** Rhabdomyosarcoma (RD), HEK293T, and HeLa cells (ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) with 100 U/ml penicillin and 100 $\mu$g/ml streptomycin. EV71 (SHZH98 strain; GenBank ac-

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Address correspondence to Ming-Liang He, mlhe7788@gmail.com.

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cession number AF302996.1) was obtained from the Shenzhen Center for Disease Control and Prevention, Shenzhen, China. To prepare virus stocks, viruses were propagated on 90% confluent monolayer cells in DMEM with 2% FBS as described previously (59).

**Plasmids and site-directed mutation.** All viral proteins expressing vectors were generated with the pcDNA4/HisMax B (Invitrogen) backbone. Primer sequences for the constructions are available upon request. Each cDNA fragment was directionally cloned into the NotI and XbaI sites of the vector. Mutation of $Cys^{110}$ to $Ala^{110}$ of $2A^{Pro}$ (yielding $2A^{C110A}$) was carried out by site-directed mutagenesis with a one-step mutagenesis kit (Invitrogen).

**Cell infection, transfection, and stimulation.** EV71 infection was performed as previously described (59). Briefly, cells were washed twice with PBS and infected with EV71 at the multiplicity of infection (MOI) indicated in the figure legends. After adsorption for 1 h, the inoculum was removed, and cells were washed twice to remove the unattached viruses; thereafter the culture medium was added. EV71-conditioned medium was collected when RD cells were infected with EV71 at an MOI of 10 for 9 h. IFN-$\beta$-specific antibody (ab6979; Abcam) was used for neutralization at 2.7 $\mu$g/ml. HEK293T cells were cotransfected with pAAV-EGFP (where EGFP is enhanced green fluorescent protein) and a plasmid expressing viral proteins (ratio, 1:5) by using Lipofectamine 2000 (Invitrogen) as described previously (43). The transfection efficiency was measured under a fluorescence microscope. The concentration of human IFN-$\alpha$2b or IFN-$\beta$ (PBL) was used for IFN treatment at the time points indicated in the figure legends. Proteins and RNA were extracted and applied for Western blotting and quantitative real-time PCR analysis, respectively.

**Western blotting.** Total cellular proteins were prepared using radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 1× Roche protease inhibitor cocktail) with occasional vortexing. Lysates were then collected by centrifugation at 14,000 rpm for 10 min at 4°C, and protein concentrations were determined by Bradford assay (Bio-Rad). The same amount of total protein for each sample was loaded and separated by 8% to 12% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences). Membranes were blocked with 5% skim milk in TBST (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) for 1 h and incubated with specific antibodies. The unphosphorylated forms of STAT1 and STAT2 were detected by using anti-STAT1 and anti-STAT2 antibodies (sc-346 and sc-476, respectively; Santa Cruz Biotechnology). Antibodies 9171 and 4441 (Cell Signaling Technology) were used to detect the phosphorylated forms of STAT1 and STAT2, respectively. The phosphorylated forms of Jak1 and Tyk2 were detected by using anti-phospho-Jak1 and anti-phospho-Tyk2 antibodies (3331 and 9321; Cell Signaling Technology). The viral structural protein VP1 and IFNAR1 were detected with specific antibodies against VP1 (PAB7631-D01P; Abnova) and IFNAR1 (sc-7391; Santa Cruz Biotechnology). Target proteins were detected with corresponding secondary antibodies (Santa Cruz Biotechnology) and finally visualized by color development with a chemiluminescence detection system (Amersham Biosciences). Each immunoblot assay was carried out at least three times.

**qRT-PCR.** The total RNA was reverse transcribed into cDNA by using a reverse transcription system (Promega). Quantitative reverse transcription-PCR (qRT-PCR) was carried out by using an ABI 7500 Real-Time PCR system with Power SYBR green Master Mix (Applied Biosystems). The PCR was set up under the following thermal cycling conditions: 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. Fluorescence signals were collected by the machine during the extension phase of each PCR cycle. The threshold cycle ($C_T$) value was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), EGFP, and each viral protein (43). The qRT-PCR was performed by using the following primer pairs: for human IFN-$\beta$, GACCAA CAAGTGTCTCCTCCAAA and GAACTGCTGCAGCTGCTTAATC; ISG15, ATGGGCTGGGACCTGACG and GCCAATCTTCTGGGTGAT CTG; ISG56, TCCCCTAAGGCAGGCTGTC and GACATGTTGGCTAG AGCTTCTTC; MxA, GCTTGCTTTCACAGATGTTTCG and AAGGGA TGTGGCTGGAGATG; OAS1, TCCACCTGCTTCACAGAACTACA and TGGGCTGTGTTGAAATGTGTTT; GAPDH, GATTCCACCCATG GCAAATTCCA and TGGTGATGGGATTTCCATTGATGA. The EV71 RNA viral loads were quantified by using the specific forward primer RT-VP1F (GCAGCCCAAAAGAACTTCAC) and reverse primer RT-VP1R (ATTTCAGCAGCTTGGAGTGC). All samples were run in triplicate, and the experiment was repeated three times. The relative mRNA level of each target gene was expressed as fold change relative to the value of corresponding control (set as 1).

**Statistical analysis.** Results were expressed as mean ± standard deviation (SD). All statistical analyses were carried out with SPSS, version 14.0, software (SPSS Inc.). A two-tailed Student *t* test was applied for two-group comparisons. A *P* value of $<0.05$ was considered statistically significant.

## RESULTS

**EV71 infection induced IFN-$\beta$ transcription but inhibited ISG activation.** To assess the effect of EV71 infection on the type I IFN response, the mRNA level of IFN-$\beta$ was investigated at different time points postinfection (p.i.). RD cells were infected with EV71 at an MOI of 1 or 10 or mock infected as described in Materials and Methods. Cellular RNA was isolated at 0, 3, 6, 9, 12, and 24 h p.i., and the mRNA level of IFN-$\beta$ was quantified by qRT-PCR. When cells were infected with EV71 at an MOI of 10, few could be collected at 24 h p.i. because of cytopathic effects (CPE) and cell death. As indicated in Fig. 1A and B, the mRNA level of IFN-$\beta$ increased following the infection. At 6 h, the induction of IFN-$\beta$ was observed, and the mRNA level of IFN-$\beta$ was much higher in cells infected with virus at a higher MOI. In the case of cells infected at an MOI of 1, the mRNA level of IFN-$\beta$ was continuously increased following viral infection and reached a level 25-fold higher than that of mock-infected cells at 24 h. When the cells were infected at an MOI of 10, the induction of IFN-$\beta$ was faster, and the mRNA level of IFN-$\beta$ peaked at 9 h p.i. These results showed that IFN-$\beta$ was actually induced in the process of EV71 replication. To check if the stimulation of IFN-$\beta$ production by EV71 is cell type specific, we also checked the mRNA levels of IFN-$\beta$ in HeLa cells, a cell line derived from a different organ (cervix), and EV71-induced IFN-$\beta$ production was also observed (data not shown).

To further confirm the functional IFN-$\beta$ protein induced by EV71, the secretion of IFN-$\beta$ in EV71-conditioned medium was tested. As shown in Fig. 1C, the expression level of the MxA and ISG56 genes was significantly upregulated by the conditioned medium from EV71-infected cells but not from the mock-infected cells. More importantly, the activation was completely blocked by IFN-$\beta$-specific neutralizing antibody, indicating that EV71 induced functional IFN-$\beta$ production as an innate immune response against EV71 infection.

The produced type I IFN interferes with viral infection and modulates the host immune response by activating the transcription of different ISGs. To dissect the IFN response during EV71 infection, we checked the downstream antiviral effectors, including ISG15, ISG56, MxA, and OAS1. To our surprise, the mRNA levels of ISGs were unchanged and even decreased following EV71 infection compared with levels in the mock-infected cells (Fig. 1D and E). Previous studies have shown that the expression of IFN-induced MxA can efficiently inhibit the replication of enteroviruses, including coxsackievirus B4 (CVB4), coxsackievirus B1 (CVB1), and echovirus 9 (8, 34). However, the MxA mRNA level

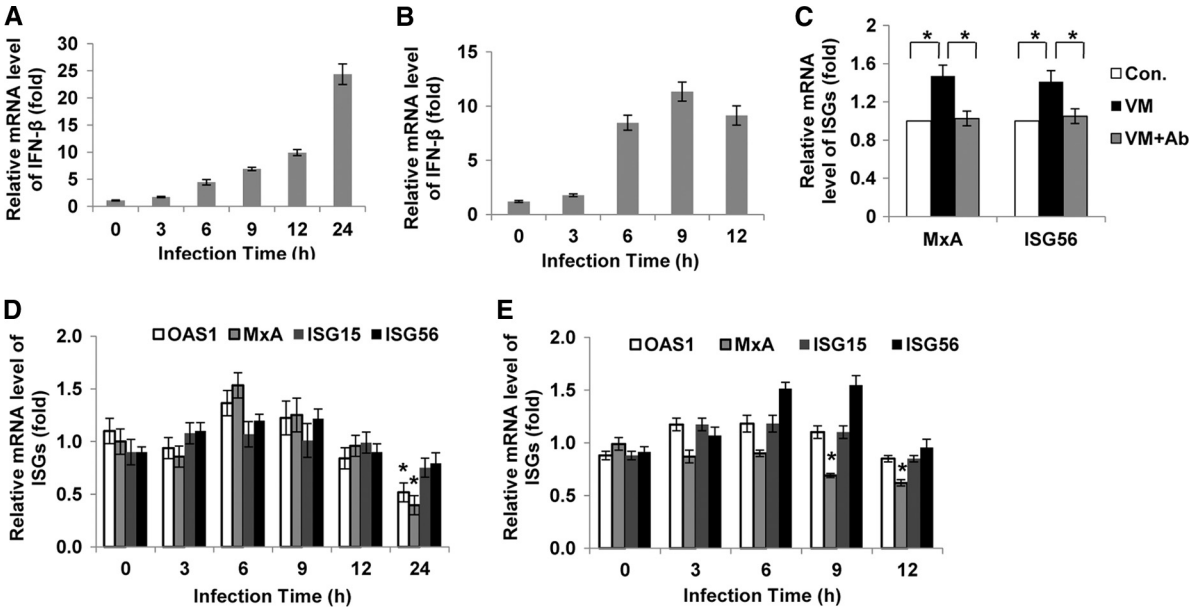


**FIG 1** EV71 infection activated type I IFN production but inhibited ISG activation. RD cells were mock infected or infected with EV71 at an MOI of 1 (A and D) or 10 (B and E). The expression levels of IFN-$\beta$ (A and B) and ISGs (D and E) were measured by qRT-PCR. (C) RD cells were cultured in DMEM with 2% FBS for 24 h, and then the culture medium was replaced with the EV71-conditioned medium. After incubation for 2 h, the cells were harvested to check the mRNA levels of ISGs. IFN-$\beta$-specific antibody was used for the neutralization. Con, treated with control medium; VM, treated with EV71-conditioned medium; VM+Ab, treated with EV71-conditioned medium and IFN-$\beta$ antibody. Data are shown as mean ± SD of three independent experiments; each was done in triplicate. *, $P < 0.05$.

in EV71-infected cells was significantly repressed in the late phase of viral infection compared with the level in mock-infected cells (Fig. 1D and E).

**EV71 antagonized the antiviral effect of IFN treatment.** An interesting question was that whether EV71 also antagonizes the antiviral effects of exogenous IFNs. We have previously demonstrated that IFN-$\beta$ displayed the same anti-EV71 activities as IFN-$\alpha$2b (58). As IFN-$\alpha$2b is more frequently used to treat patients with certain viral infections, IFN-$\alpha$2b was first chosen to address this question. RD cells were infected with EV71 at an MOI of 1 and treated with IFN-$\alpha$2b at a concentration of 100 or 1,000 U/ml at 6 h before infection, at the time of infection, and at 1 h, 3 h, and 6 h p.i. If EV71 could antagonize exogenous interferon, pretreatment would show the best antiviral effects, while the treatments at the time of or after infection would display weaker and weaker antiviral effects as the accumulated viruses or viral components would block or weaken the antiviral effects of the exogenous IFNs. As shown in Fig. 2A, the uninfected cells were healthy and flat (frame a), while the cells became sick and appeared round (frame b) at 20 h p.i. The cells were well protected from CPE when pretreated with 100 U/ml of IFN-$\alpha$2b 6 h before infection (frame c), indicating that strong IFN-signaling-mediated antiviral effects had been achieved at this low concentration. However, little protection was observed when the treatment was carried out at the time of infection and postinfection at this low concentration (frames d to g). With a high dose of IFN-$\alpha$2b treatment (1,000 U/ml), the cells were well protected from CPE when cells had been treated 6 h before infection or upon infection (frames h and i), while the protection became weaker and weaker when the cells were treated at 1, 3, and 6 h p.i. (frames j, k, and l, respectively). These results were further confirmed by measuring the intracellular viral RNA copies 20 h after infection (Fig. 2B). Compared with the mock-treated control, the cellular viral load was reduced by about 40% and 15% when cells were pretreated with 100 U/ml of IFN-$\alpha$2b 6 h before infection and upon infection, respectively. However, similar viral RNA levels were observed only in the cells treated with a high concentration of IFN-$\alpha$2b (1,000 U/ml) at 1 h, 3 h, and 6 h postinfection. In the case of treatment with 1,000 U/ml, the viral load was significantly reduced by approximately 90%, 60%, and 35% when cells were treated at 6 h before infection, upon infection, and at 1 h postinfection. Surprisingly, no significant reduction of viral load was observed even if the cells were treated with this high concentration of IFN-$\alpha$2b at 3 h and 6 h p.i. Similar results were also obtained from IFN-$\beta$ treatments (data not shown).

We further checked the activation of ISGs in EV71-infected cells after IFN treatment. The RD cells were mock or EV71 infected for 9 h, at which time viral replication and viral protein expression reached the peak (41). Thereafter, cells were stimulated with IFN-$\alpha$2b (100 U/ml) for another 2 h. Then the cells were collected, and total cellular RNA was isolated to measure the relative mRNA levels of ISGs. As shown in Fig. 2C, IFN stimulation induced robust expression of all checked ISGs in the mock-infected cells, as expected, but not in the EV71-infected cells. Compared to the mock-infected cells, ISG activation in the EV71-infected cells was dramatically suppressed after IFN-$\alpha$ treatment.

Taken together, these results revealed that EV71 for its survival and replication developed a defense mechanism that could not only eliminate the innate interferon immune response but also weaken the effect of exogenous IFN treatment.

**EV71 inhibited the IFN-signaling-mediated phosphorylation of STAT1 and STAT2.** Antagonizing IFN signaling is a possible mechanism to explain the observed defect in ISG activation above. It is well known that IFN-$\alpha/\beta$ binds to its cognate receptors

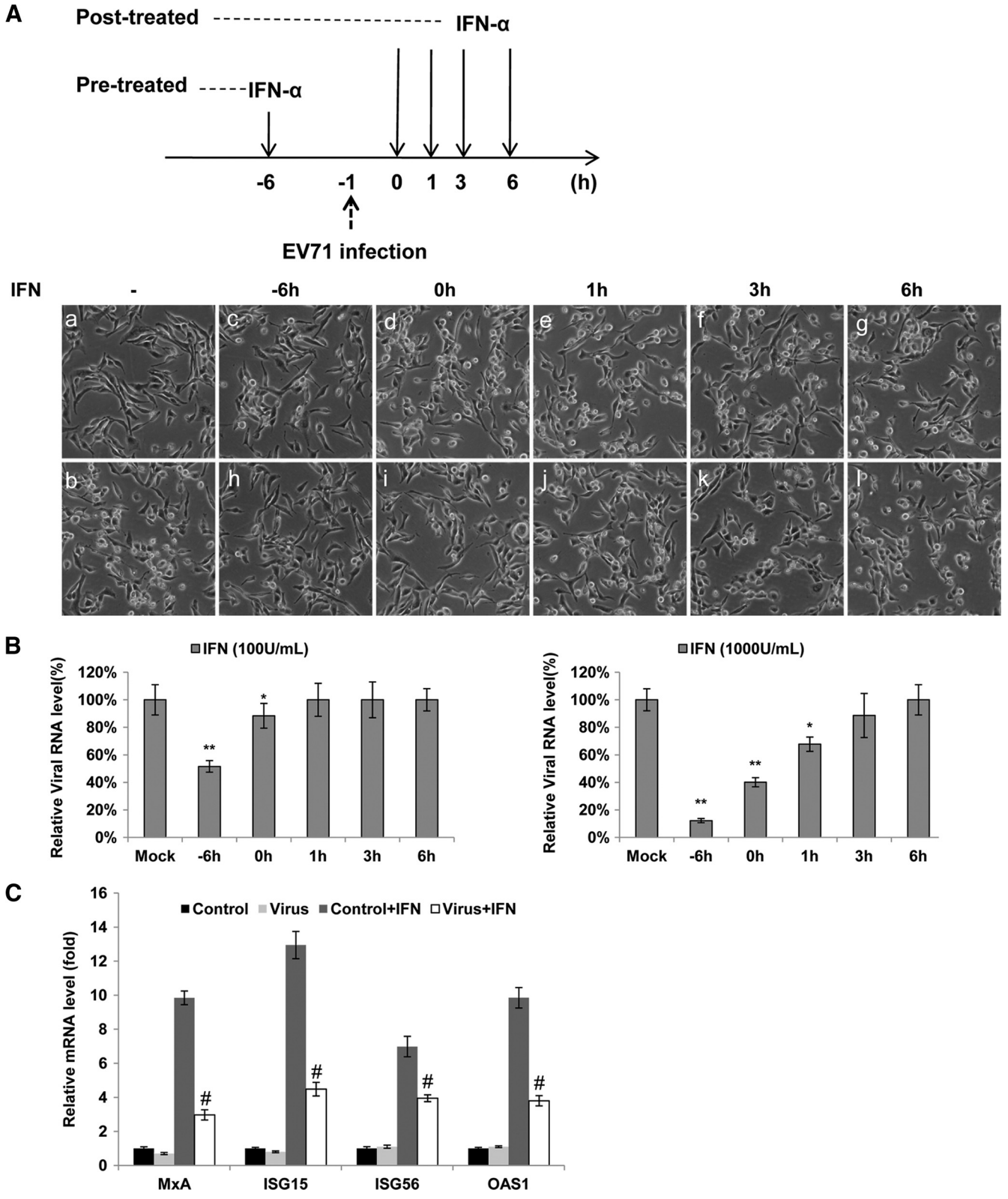


**FIG 2** EV71 antagonized the antiviral effect of IFN-$\alpha$ treatment. (A) RD cells were infected with EV71 at an MOI of 1. IFN-$\alpha$ treatment (100 U/ml, frames c to g; or 1,000 U/ml, frames h to l) was performed before or after viral infection as indicated on the figure. Cells uninfected or infected without IFN treatment are shown in frames a or b, respectively. Photomicrographs were taken at 20 h postinfection (original magnification, ×100). (B) Cellular viral loads were quantified by qRT-PCR at 20 h p.i. The cells infected with EV71 but without IFN treatment were set as controls. (C) RD cells were infected with EV71 at an MOI of 10. At 9 h after infection, cells were stimulated with IFN-$\alpha$2b for another 2 h (Virus+IFN). Cellular RNA was extracted, and expression levels of relative ISGs were measured by qRT-PCR. The expression level of each gene was calculated relative to GAPDH gene expression and normalized to mock-treated cells. Data are shown as mean ± SD of three independent experiments; each was done in triplicate. *, $P < 0.05$, versus the control; **, $P < 0.001$, versus the control; #, $P < 0.001$, Virus+IFN versus Con+IFN.

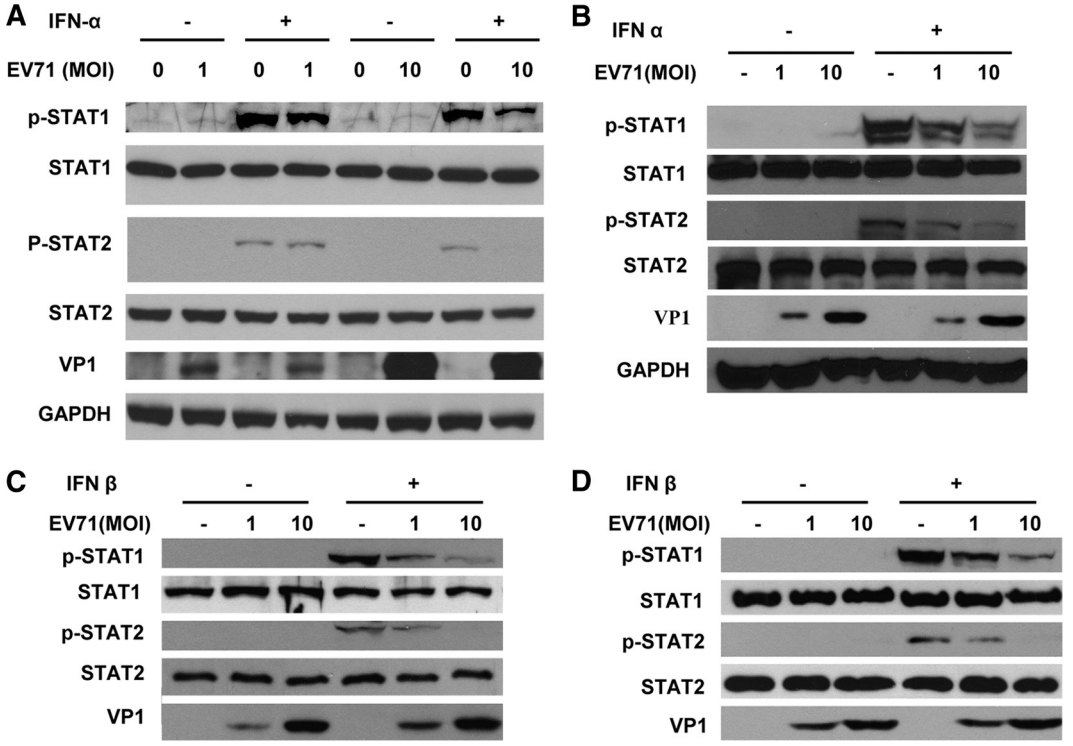


**FIG 3** EV71 inhibited phosphorylation of STAT1/STAT2. RD cells (A and C) or HeLa cells (B and D) were infected with EV71 at an MOI of 1 or 10 for 9 h, and cells were left untreated (−) or treated (+) with IFN-α2b (A and B) or IFN-β (C and D) for another 30 min. Western blotting was performed by detecting STAT1, STAT2, phosphorylated STAT1 (p-STAT1), phosphorylated STAT2 (p-STAT2), and viral structural protein VP1 with relative antibodies. GAPDH was also detected as a loading control.

(IFNAR1 and IFNAR2) on target cells and triggers a signaling cascade that involves Jak1 and Tyk2 phosphorylation. Then the phosphorylated Jak1 and Tyk2 further mediate phosphorylation, heterodimerization, and nuclear translocation of STAT1 and STAT2 to activate ISGs. In this process, STATs play a pivotal role in activation of downstream antiviral effectors (ISGs). However, many viruses have developed variable mechanisms to antagonize the IFN response by interfering with the protein levels or altering the biological activities of STAT1 and STAT2 (4, 7, 49, 53). To dissect how EV71 antagonizes the antiviral effects of the IFNs, we first checked if EV71 would alter the expression levels and phosphorylation patterns of STATs. RD and HeLa cells were either mock or EV71 infected at the MOI indicated in the figure legends. At 9 h p.i., cells were subsequently stimulated with IFN-α2b at 100 U/ml for another 30 min. The levels of total STATs and phosphorylated STATs were assessed. The VP1 protein of EV71 was used as an indicator of the expression level of viral proteins. As shown in Fig. 3, the protein levels of STAT1 and STAT2 were apparently not altered by EV71 infection in either RD cells (Fig. 3A) or HeLa cells (Fig. 3B) when levels were normalized with the level of GAPDH protein. IFN-α treatment induced rapid and easily discernible STAT1 and STAT2 phosphorylation in the mock-infected cells. However, the phosphorylated STAT1 and STAT2 were significantly reduced in the EV71-infected cells. More suppressive effects were observed when more viral proteins were generated by infection at a high MOI (Fig. 3A and B). As expected, EV71 also antagonized IFN-β-mediated antiviral signaling in RD and HeLa cells (Fig. 3C and D). These results demonstrated that EV71 can efficiently block the IFN-induced phosphorylation of STATs and then the activation of antiviral effectors.

**EV71 blocked IFN-mediated Jak/STAT signaling by reducing IFNAR1 protein levels.** We next examined the effect of viral infection on upstream molecules involved in IFN signaling. The phosphorylation of the tyrosine kinases Jak1 and Tyk2 is regarded as the first step in activation of IFN signal transduction. To reveal the effect of EV71 infection on the activation of Jak1 and Tyk2, RD cells were infected with EV71 at an MOI of 10. At 9 h p.i., cells were treated with IFN-α2b (100 U/ml) for 10 min, and the phosphorylation of Jak1 and Tyk2 was analyzed by using specific antibodies. As shown in Fig. 4A, both kinases were phosphorylated in the control cells treated with IFN-α while only background levels of phosphorylation were detected in the infected cells.

Because the phosphorylation of Jak1 and Tyk2 is the first event in the IFN signaling cascade, EV71 was suggested to play an inhibitory role on the IFN-α/β receptor. To prove this hypothesis, we examined the protein levels of interferon-α receptor I (IFNAR1) with mock or EV71 infection at an MOI of 10. Then the RD cells were treated with IFN-α2b for another 30 min at different time points. The total cells were collected to determine the expression levels of phosphorylated STAT1 and IFNAR1 by Western blotting. As shown in Fig. 4B, the inhibition of IFNAR1 started at 6 h p.i. when the viral protein VP1 was expressed at a relatively high level, and more significant reduction of IFNAR1 was observed at 9 h p.i. Accordingly, the IFN-induced STAT phosphorylation also started to be repressed by EV71 at 6 h p.i.

We concluded from these experiments that the EV71 altered

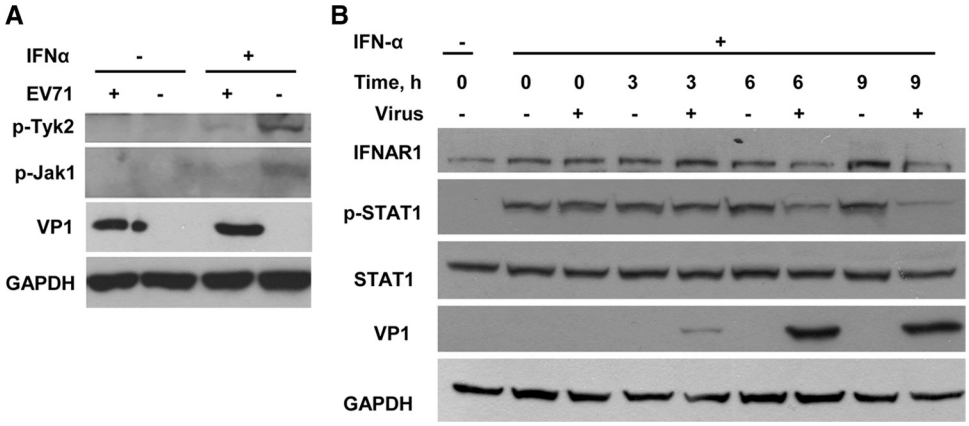


**FIG 4** EV71 inhibited type I IFN-mediated Jak/STAT signaling by reducing IFNAR1 protein levels. (A) RD cells were infected with EV71 at an MOI of 10 for 9 h, and cells were left untreated (−) or treated (+) with IFN-α2b for another 10 min. The phosphorylated Jak1 (p-Jak1), Tyk2 (p-Tyk2), and VP1 were detected with specific antibodies. GAPDH was also detected as a loading control. (B) RD cells were mock infected or infected with EV71 at an MOI of 10. Cells were treated with IFN-α2b for another 30 min at 0, 3, 6, and 9 h after infection. The mock-infected cells without IFN treatment were set as negative controls. The expression levels of IFNAR1, STAT1, VP1, and p-STAT1 were measured with related antibodies.

IFN-α/β signaling by reducing IFNAR1 protein levels in the host cells, which led to disruption of the phosphorylation of Jak1 and Tyk2 as well as the STAT proteins.

**Identification of 2A as an antagonist of IFN signaling.** The above results showed that IFN signaling was inhibited and that this was accompanied by a large accumulation of intracellular viral proteins (Fig. 4B). This observation suggested that viral protein(s) may play an essential role in reducing the IFNAR1 expression level. To identify the potential viral proteins which may function as antagonists to IFN signaling, we constructed plasmids to express all 11 structural and nonstructural viral proteins encoded by EV71. HEK293T cells were transiently transfected with pAAV-EGFP and plasmids expressing individual EV71 proteins. At 24 h after transfection, the cells were stimulated with or without IFN-α2b (100 U/ml) for another 2 h. The mRNA of MxA, one of the ISRE-controlled cellular genes strongly inhibited in EV71-infected cells (Fig. 1E and 2C), was selected and measured by qRT-PCR. The mRNA levels of EGFP and each viral protein were also quantitated by qRT-PCR. After normalization of the mRNA level of each candidate protein, MxA was found significantly inhibited by 2A protein (Fig. 5A). In the case of IFN treatment, 2A protease ($2A^{pro}$) potently reduced the mRNA level of MxA by over 90%, and $3C^{pro}$ slightly inhibited IFN signaling and reduced the mRNA level of MxA by 30%. The other viral proteins, including 2B, 2C, 3A, 3B, 3D, VP1, VP2, VP3, and VP4, had no significant inhibitory effect on MxA transcription under either condition. These results suggested that the $2A^{pro}$ of EV71 functioned as the major antagonist to IFN signaling.

**$2A^{pro}$ blocked type I IFN-mediated phosphorylation of STAT proteins by reducing IFNAR1 protein levels.** To assess the inhibitory role of the $2A^{pro}$ in IFN signaling, HEK293T cells were transfected with a control vector or a plasmid expressing $2A^{pro}$ and treated with IFN-α2b (100 U/ml) 24 h after transfection. The IFNAR1 level and phosphorylation status of the STAT proteins were analyzed 30 min after IFN stimulation. As described in other studies, the expression of viral $2A^{pro}$ was hard to detect directly due to its inhibition of its own translation (32, 35), while the cleavage of eIF4G representing the functional $2A^{pro}$ was expressed

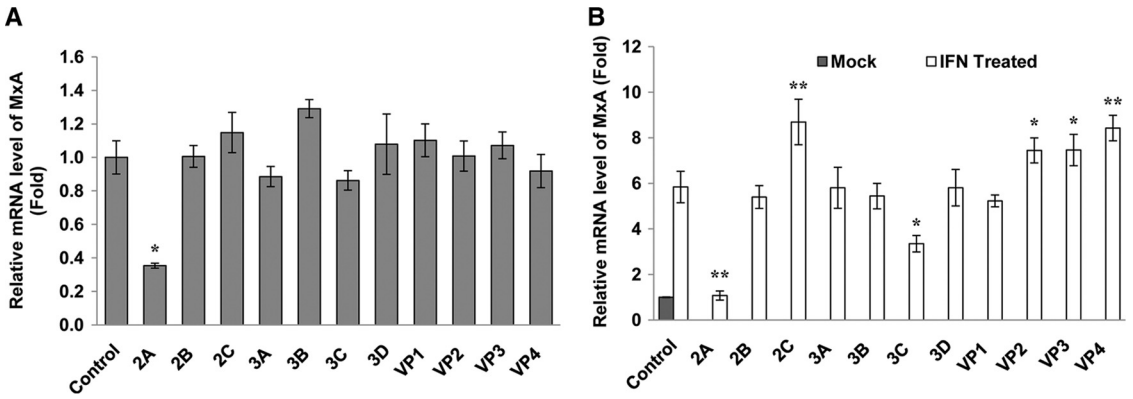


**FIG 5** Identification $2A^{pro}$ as an antagonist of IFN signaling. HEK293T cells were cotransfected with pAAV-EGFP and plasmids expressing individual EV71 proteins. The null vector was used as a control. At 24 h after transfection, cells were mock treated (A) or stimulated with IFN-α2b (B) for another 2 h. Then, total cellular RNA was extracted, and the mRNAs of MxA and EGFP as well as of each viral protein were quantified by qRT-PCR. The mRNA level of MxA was calculated relative to that of each viral protein and normalized to that of the control (set as 1). Data are shown as mean ± SD of three independent experiments; each was done in triplicate. *, $P < 0.05$, versus the control; **, $P < 0.001$, versus the control.

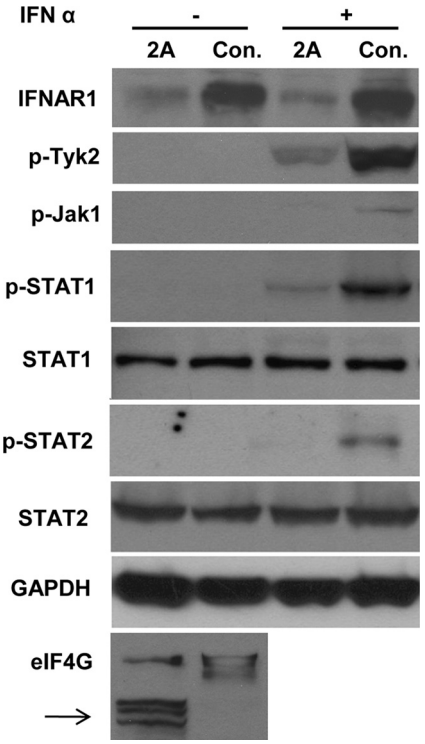


**FIG 6** 2A$^{pro}$ inhibited Jak/STAT signaling by reducing IFNAR1 levels. HEK293T cells were transfected with a plasmid expressing 2A$^{pro}$ or a control vector. At 24 h after transfection, cells were treated with or without IFN-$\alpha$2b. Western blotting was performed to detect IFNAR1, p-Jak1, p-Tyk2, STAT1, STAT2, p-STAT1, p-STAT2, and eIF4G with related antibodies. The arrow indicates the cleaved eIF4G in HEK293T cells. GAPDH was detected as a loading control.

in transfected cells (Fig. 6). Obviously, 2A$^{pro}$ significantly decreased IFNAR1 levels in HEK293T cells (Fig. 6). Repeated experiments showed that the endogenous IFNAR1 was reduced by 2A$^{pro}$ by more than 90% after image quantification in both cases with or without IFN treatment. Consistent with the observations in the EV71-infected cells (Fig. 3 and Fig. 4), 2A$^{pro}$ also potently inhibited the IFN-induced phosphorylation of Janus kinases and STATs without notably changing the total amount of these proteins (Fig. 6).

**The inhibition of type I IFN signaling depended on the protease activity of 2A$^{pro}$.** The 2A$^{pro}$ of EV71 is known as a viral protease. It catalyzes an essential cleavage in the polyprotein in virus replication (5). 2A$^{pro}$ also cleaves several host cellular proteins thus facilitating viral replication and viral pathogenesis (30). Cys$^{110}$ is essential for the protease activity of 2A$^{pro}$ (57, 62). To determine whether the protease activity of 2A$^{pro}$ is critical for IFNAR1 reduction and IFN signaling inhibition, Cys$^{110}$ of 2A$^{pro}$ was mutated to Ala$^{110}$ (2A$^{C110A}$) by site-directed mutagenesis. As shown in Fig. 7A, the wild-type 2A$^{pro}$ efficiently cleaved eIF4G in 293T cells, while 2A$^{C110A}$ exhibited no protease activity as eIF4G was not cleaved. Again, the wild-type 2A$^{pro}$ significantly reduced IFNAR1 levels and suppressed IFN-signaling-mediated STAT1 and STAT2 phosphorylation. Compared with the control, the mutant 2A$^{C110A}$ did not play any inhibitory role in IFNAR1 and IFN signaling (Fig. 7A).

## DISCUSSION

As with many other picornaviruses, the double-stranded RNA (dsRNA) produced in the life cycle of EV71 may activate type I IFN production (21, 29). The type I IFN response functions as the first line of defense against infection by generating an intracellular environment that restricts viral replication and by promoting the presentation of viral antigens to the adaptive immune response (24, 33, 54). We along with others have previously demonstrated

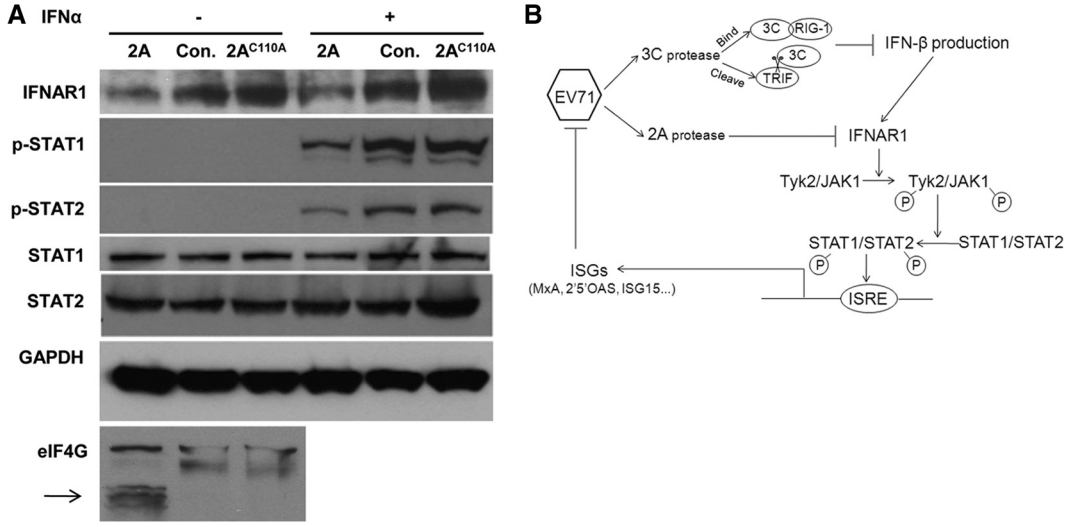


**FIG 7** Protease activity is required for 2A$^{pro}$-mediated inhibition of IFN signaling. (A) HEK293T cells were transfected with a plasmid expressing 2A$^{pro}$ or the mutant 2A$^{C110A}$ or with a control vector. At 24 h after transfection, cells were treated without or with IFN-$\alpha$2b (100 U/ml) for 30 min. Western blotting was performed to detect IFNAR1, STAT1, STAT2, p-STAT1, p-STAT2, and eIF4G with related antibodies. The arrow indicates the cleaved eIF4G. GAPDH was detected as a loading control. (B) A model for EV71 to evade surveillance of type I IFN. During the EV71 replication cycle, the virus encoded two proteases, 2A$^{pro}$ and 3C$^{pro}$. 3C$^{pro}$ interacts with RIG-1 and TRIF to suppress the production of IFN-$\beta$, while 2A$^{pro}$ reduces the IFN receptor IFNAR1 and then blocks the downstream signaling pathway. As a consequence, IFN-induced antiviral effectors like MxA, OAS1, ISG15, and ISG56 are all suppressed during the whole infection process, and high efficient replication and persistent infection could finally be achieved.

that endogenous IFN or IFN treatment generally fails to eliminate EV71 infection in cell or mouse models (40, 59), but the mechanism is not understood. In this study, we showed that EV71 could moderately stimulate IFN production during the infection, but the IFNs induced by EV71 or by exogenous IFN treatment could not effectively trigger the downstream signaling cascade to suppress viral replication. More importantly, our study demonstrated that EV71 interfered with type I IFN signaling by reducing IFNAR1 levels in host cells.

Extensive studies on other viruses have shown that viruses circumvent the IFN signal pathway mainly in two ways: (i) inhibiting IFN induction by minimizing the production of viral pathogen-associated molecular patterns (PAMPs) and/or specifically interfering with cascades in the IFN-inducing signal pathway (6, 25); (ii) blocking the activation of ISGs by interfering with the IFN signal pathway and/or interfering with the normal function of ISGs by direct interaction (9, 37). In the picornavirus family, most members (e.g., poliovirus, rhinoviruses, echovirus, and encephalomyocarditis virus) use strategies to inhibit IFN-$\alpha/\beta$ induction by interfering with melanoma differentiation-associated gene 5 (MDA-5) and retinoid acid-inducible gene I (RIG-I) (2, 3, 52) or by restricting IFN-$\beta$ secretion through repressing the cellular secretory pathway (17). The leader protease of foot and mouth disease virus can inhibit type I IFN production by cleaving p65-RelA (13, 14). Also, the $3C^{pro}$ of hepatitis A virus inhibits type I IFN production by cleaving the mitochondrial antiviral signaling (MAVS) protein (58). More recent studies revealed that the 3C protease of EV71 associated with RIG-I and cleaved TRIF. As a result, IFN-$\beta$ transcription is partially inhibited during viral infection (Fig. 7) (35, 36). To date, no type I IFN-antagonistic mechanism has been described for picornaviruses that acts on the level of IFN signaling rather than IFN induction. This study, for the first time, showed that EV71 inhibited type I IFN-induced ISG activation by reducing IFNAR1 levels. Even though $3C^{pro}$ was reported to suppress IFN production, the IFN-$\beta$ transcriptional activation was not totally inhibited in EV71-infected cells, and the mRNA level of IFN-$\beta$ was moderately increased about 25-fold at 24 h following EV71 infection (Fig. 1A and B). Furthermore, the activation of ISGs by the conditioned medium from EV71-infected cells indicated at least a certain functional level of IFN-$\beta$ protein was secreted by EV71-infected cells (Fig. 1C). Surprisingly, the activation of ISGs by IFN signaling did not correspond with the production of IFN-$\beta$ and even was reduced to below baseline levels at late phases of infection (Fig. 1D and E). The inhibition of the activation of ISGs could weaken the positive-feedback loop of the IFN response. This could partially explain why, unlike other negative-sense single-stranded RNA (ssRNA) viruses, EV71 could not stimulate a high level of IFN-$\beta$ during infection (29, 55). The effect of EV71 on repressing IFN-induced ISG activation was also observed when cells were treated with exogenous IFN-$\alpha$2b (Fig. 2) and IFN-$\beta$ (not shown), providing an explanation for the failure of anti-EV71 treatment.

The phosphorylation of STAT1/STAT2 is regarded as the hallmark of the activation of IFN signaling and antiviral responses. Cells that lost these two effectors were highly susceptible to virus infection (18). Our data showed that IFN-mediated STAT phosphorylation was greatly inhibited by EV71 (Fig. 3) when the cellular viral loads reached their peaks (41). These results suggested that EV71 was able to abolish type I IFN signaling to facilitate its replication and survival and at least partially explained why the viral load of EV71 could reach a high level soon after viral infection in the mouse model or cultured cells (40, 59). The observed inhibitory effects of EV71 on IFN-induced phosphorylation of Janus kinases and STATs led us to discover the interaction between EV71 and type I IFN receptor. We detected the reduction of IFNAR1 protein levels at 6 h p.i., which coincides with the inhibition of IFN-induced STAT1 phosphorylation (Fig. 4B). Actually, IFNAR1 is important for protecting cells from a number of viral infections and other diseases. In mice IFNAR1 has been shown to be essential for the IFN-$\alpha/\beta$ response that is required for survival against most viral infections and for myelopoiesis, as well as B- and T-cell-mediated immune responses (26, 48). The positive correlation between basal levels of IFNAR1 and the IFN response has also been demonstrated in other studies in culture and in clinical investigations (46, 63). Viewed together, the reduction of IFNAR1 levels in host cells is an effective mechanism for EV71 to escape from innate antiviral responses.

There is a question of why EV71 has evolved two cysteine proteases, 2A and 3C, with similar catalytic mechanisms but with different specificities. It has been shown that $3C^{pro}$ could inhibit IFN production by suppressing RIG-1 and TRIF (Fig. 7B). Besides cleavage of viral polyproteins in viral replication, $2A^{pro}$ has also been shown to cleave a variety of host proteins, including the translation proteins eIF4GI (30), eIF4GII (38), and poly(A)-binding protein (27), thereby enhancing viral protein expression managed by its own internal ribosome entry site (IRES) and inhibiting cap-dependent cellular translation shutoff. In addition, the $2A^{pro}$ of poliovirus is also involved in the process of viral RNA replication and viral RNA stability (28). In this study, we revealed a novel function of $2A^{pro}$ that antagonizes IFN signaling by targeting IFNAR1 (Fig. 7B). Our findings could also explain the recent observation that $2A^{pro}$ of poliovirus and EV70 is essential for viral replication in interferon-treated cells (47). However, we found that $2A^{pro}$ could not directly digest IFNAR1 in host cells (data not shown), suggesting an indirect interaction among $2A^{pro}$, IFNAR1, and undefined molecule(s). Although the detailed mechanism used by EV71 or $2A^{pro}$ to regulate IFNAR1 is not completely understood yet, our data indicated that $2A^{pro}$-mediated IFNAR1 reduction depended on its protease activity since the $Cys^{110}$-to-$Ala^{110}$ mutation in the catalytic site completely impaired the process (Fig. 7A).

In conclusion, our study revealed that EV71 was able to inhibit the cellular type I IFN response by reducing IFNAR1 levels. As suggested by our model of how EV71 evades surveillance of type I IFN (Fig. 7B), $2A^{pro}$ plays a crucial role in antagonizing the type I IFN response. Our data provide new knowledge of how EV71 blocks the host innate immune response and may facilitate development of new antiviral therapies for EV71 or other picornavirus infections.

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M.-L.H. designed the study; J.L., L.Y., J.Z., and Y.C. did experiments; J.L., J.Y., M.C.L., and H.-F.K. analyzed data; and J.L., L.Y., and M.-L.H. wrote the paper.

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