Journal of Virology®

VIRUS-CELL INTERACTIONS

Role of Enteroviral RNA-Dependent RNA Polymerase in Regulation of MDA5-Mediated Beta Interferon Activation

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ABSTRACT Infection by enteroviruses can cause severe neurological complications in humans. The interactions between the enteroviral and host proteins may facilitate the virus replication and be involved in the pathogenicity of infected individuals. It has been shown that human enteroviruses possess various mechanisms to suppress host innate immune responses in infected cells. Previous studies showed that infection by enterovirus 71 (EV71) causes the degradation of MDA5, which is a critical cytoplasmic pathogen sensor in the recognition of picornaviruses for initiating transcription of type I interferons. In the present study, we demonstrated that the RNA-dependent RNA polymerase (RdRP; also denoted $3D^{pol}$) encoded by EV71 interacts with the caspase activation and recruitment domains (CARDs) of MDA5 and plays a role in the inhibition of MDA5-mediated beta interferon (IFN-β) promoter activation and mRNA expression. In addition, we found that the $3D^{pol}$ protein encoded by coxsackievirus B3 also interacted with MDA5 and downregulated the antiviral signaling initiated by MDA5. These findings indicate that enteroviral RdRP may function as an antagonist against the host antiviral innate immune response.

IMPORTANCE Infection by enteroviruses causes severe neurological complications in humans. Human enteroviruses possess various mechanisms to suppress the host type I interferon (IFN) response in infected cells to establish viral replication. In the present study, we found that the enteroviral $3D^{pol}$ protein (or RdRP), which is a viral RNA-dependent RNA polymerase for replicating viral RNA, plays a role in the inhibition of MDA5-mediated beta interferon (IFN-β) promoter activation. We further demonstrated that enteroviral $3D^{pol}$ protein interacts with the caspase activation and recruitment domains (CARDs) of MDA5. These findings indicate that enteroviral RdRP functions as an antagonist against the host antiviral response.

KEYWORDS enterovirus 71, beta interferon, MDA5

Infection by enterovirus type 71 (EV71) can result in severe complications that impair the function of the central nervous system in children, such as aseptic meningitis, encephalitis, and neurogenic pulmonary edema (1). The virus belongs to the picornavirus family, which has a single-stranded RNA genome with positive polarity. The positive-sense viral genome serves as viral mRNA for translating a precursor polyprotein that can be processed to functional structural and nonstructural viral proteins by the

Citation Kuo R-L, Chen C-J, Wang RYL, Huang H-I, Lin Y-H, Tam E-H, Tu W-J, Wu S-E, Shih S-R. 2019. Role of enteroviral RNA-dependent RNA polymerase in regulation of MDA5-mediated beta interferon activation. J Virol 93:e00132-19. https://doi.org/10.1128/JVI.00132-19.

Editor Julie K. Pfeiffer, University of Texas Southwestern Medical Center

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Received 26 January 2019
Accepted 21 February 2019
Accepted manuscript posted online 27 February 2019
Published 1 May 2019

virus-encoded proteases 2A and 3C. The genomic EV71 RNA is also the template for generating the complementary viral RNA, which can be further replicated to genomic RNA by viral RNA-dependent RNA polymerase (RdRP; denoted $3D^{pol}$) in the cytoplasm (2). Although the picornaviral $3D^{pol}$ primarily functions as the viral RNA replicase in the cytoplasm, it was reported that the $3D^{pol}$ proteins from several picornaviruses can also enter the nucleus (3, 4) and interfere with cellular pre-mRNA splicing (5).

The induction of type I interferons (IFNs), including alpha and beta interferons (IFN-α and IFN-β, respectively), is a crucial event to activate innate immune responses against RNA virus infection. Two cytoplasmic pathogen recognition receptors, melanoma differentiation-associated gene 5 (MDA5) and retinoic acid-inducible gene I (RIG-I), have been identified as sensors that recognize RNA viruses and stimulate type I IFN expression (6–9). Previous studies have demonstrated that MDA5 is a critical pathogen recognition receptor for activating type I IFN expression in response to picornavirus infection, including EV71 (8, 10–13). MDA5 has N-terminal caspase activation and recruitment domains (CARDs), which can interact with the CARD-containing adaptor protein, mitochondrial antiviral signaling protein (MAVS; also named IPS-1, VISA, or Cardif) (14). The interaction activates the C-terminal catalytic domain of MAVS and initiates the signaling cascade through tumor necrosis factor (TNF) receptor-associated factor 3 (TRAF3). This event promotes the activation of IκB kinase ε (IKKε) and TANK-binding kinase 1 (TBK-1), which phosphorylates interferon regulatory factor 3 (IRF3). The phosphorylated IRF3 forms homodimers, translocates into the nucleus, and then cooperates with the transcriptional factors NFκB and ATF2/c-Jun to bind to the IFN-β promoter, resulting in type I IFN expression (15, 16). It has been demonstrated that MDA5 detects long double-stranded RNA (dsRNA) and that MDA5 forms filamentous oligomers along dsRNA. (17–20). However, several studies have also reported that specific sequences or structures in virus-related RNAs, such as the L region antisense RNA of encephalomyocarditis virus (EMCV), the AU-rich RNAs derived from the measles virus mRNA, and the highly ordered RNA structures derived from EMCV, are important to stimulate MDA5-mediated type I IFN responses (21–23). In addition, it was demonstrated recently that K63-linked ubiquitination is required for activation of MDA5 and that TRIM65 serves as the E3 ubiquitin ligase for the ubiquitination of MDA5 at residue K753 in the helicase domain (24).

Although the type I IFN response elevates the cellular antiviral state against RNA virus, EV71 has evolved strategies to inhibit the IFN activation pathways. For example, infection with EV71 causes MDA5 degradation by cellular caspase activation or the viral protease 2A (12, 25). In addition, EV71 infection may also result in the degradation of the mitochondrial antiviral signaling (MAVS) protein, inhibition of retinoic acid-inducible gene I (RIG-I) activation, and cleavage of interferon regulatory factor 7 (IRF7) to downregulate the type I IFN response (26–28). The aforementioned mechanisms have been correlated to EV71-encoded protease 2A or 3C. Moreover, previous research demonstrated that the EV71 3C protease selectively reduces type I IFN production in mice (29). Nevertheless, infection with EV71 alters host protein expression caused by cleavages of translational initiation factor eIF4G and polyadenylation factor CstF64 and influences splicing factor Prp8 (5, 30, 31), suggesting the biological processes and host responses may be affected by EV71 infection. Furthermore, our previous study profiling the changes of cellular proteins in EV71-infected cells showed that the host protein NEDD4L was upregulated upon EV71 infection and regulated IFN-β expression (32).

In the present study, we found that EV71 $3D^{pol}$ plays a regulatory role in inhibiting MDA5-mediated IFN-β promoter activation and mRNA expression. We further demonstrated that EV71 $3D^{pol}$ interacts with the CARDs of MDA5 and can be colocalized in the cytoplasm. These findings provide a novel mechanism by which EV71 can antagonize the antiviral response in infected cells.

RESULTS

RdRP encoded by EV71 inhibits MDA5-mediated type I IFN activation. Previous studies have shown that activation of IFN-β expression may be blocked during EV71

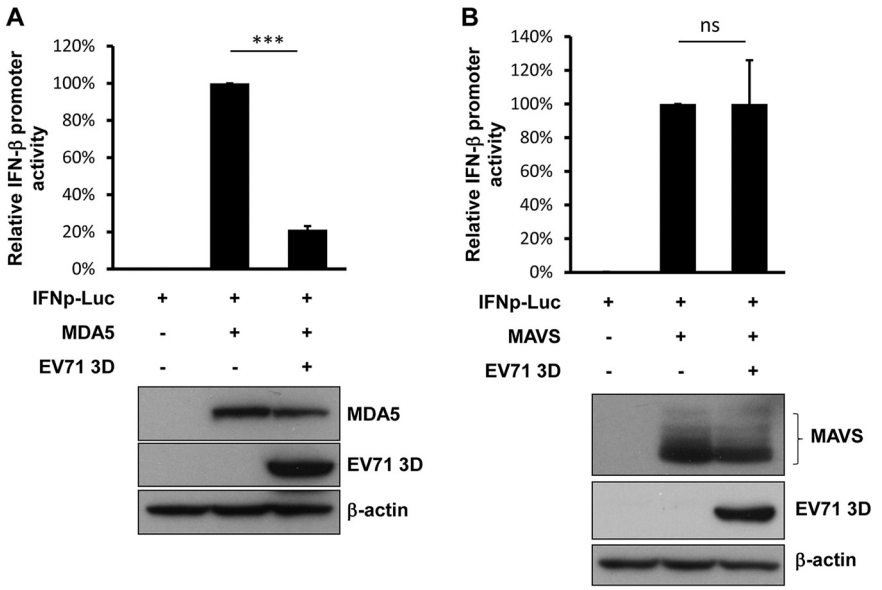

FIG 1 EV71 3D^{pol} inhibits MDA5-mediated IFN-β promoter activation. (A) 293T cells were cotransfected with IFN-β promoter luciferase reporter, FLAG-tagged MDA5-expressing plasmid, and either empty vector or 3×FLAG-tagged EV71 3D-expressing plasmid. (B) 293T cells were cotransfected with IFN-β promoter luciferase reporter, MAVS-expressing plasmid, and either empty vector or EV71 3D-expressing plasmid. At 24 h posttransfection, cell extracts were collected for luciferase activity assays. The protein expression was detected by Western blotting with anti-FLAG, anti-MAVS, and anti-β-actin antibodies. The error bars indicate the standard deviations (SDs). ***, $P < 0.001$; ns, not significant.

infection (12, 26, 27). Although it has been proposed that the EV71-encoded proteases 2A and 3C may be involved in the suppression of type I IFN activation (26, 27), the role of other EV71 nonstructural proteins in the inhibition of type I IFN activation has not been fully elucidated. Since previous studies have demonstrated that EV71 RdRP (i.e., 3D^{pol}) interferes with cellular pre-mRNA maturation, we further investigated the role of 3D^{pol} in the inhibition of antiviral responses. Initially, we found that overexpression of EV71 3D^{pol} strongly inhibited MDA5-initiated IFN-β promoter activation (Fig. 1A). In contrast, the EV71 3D^{pol} protein did not suppress IFN-β promoter activation initiated by overexpression of the MAVS protein (Fig. 1B). This result indicates that EV71 3D^{pol} plays a role in suppressing MDA5-mediated activation of IFN-β transcription and that the signal cascade between MDA5 and MAVS may be interrupted by overexpression of the EV71 3D^{pol} protein.

Because the MDA5-mediated IFN-β promoter activation is initiated by exposure of the CARDs of MDA5 without interference of the Pincer domain and C-terminal domain (CTD), the overexpression of the CARDs alone could strongly induce activation of the promoter. We further determined whether the EV71 3D^{pol} protein could suppress the IFN-β transcription activated by the CARDs of MDA5. We found that the IFN-β promoter activated by overexpression of MDA5 CARDs was inhibited by coexpressed EV71 3D^{pol} (Fig. 2A). In addition to the promoter reporter assay, we compared IFN-β mRNA production in cells transfected with plasmids expressing EV71 3D^{pol} or MDA5 CARDs alone or cotransfected with MDA5 CARD- and EV71 3D^{pol}-expressing plasmids. As shown in Fig. 2B, expression of EV71 3D^{pol} alone did not induce IFN-β mRNA production, and the MDA5 CARD-induced IFN-β mRNA production was reduced by coexpression of EV71 3D^{pol}. Consistently, we found that the expression of antiviral genes regulated by IFN-β, such as interferon-stimulated genes 56 and 54 (ISG56 and ISG54, respectively), was decreased (Fig. 2B). The results imply that EV71 3D^{pol} may suppress MDA5 CARD-induced activation of IFN-β transcription and its downstream antiviral genes.

EV71 3D^{pol} interacts with the CARDs of the cytoplasmic pathogen sensor MDA5. The aforementioned results demonstrate that EV71 3D^{pol} can interrupt MDA5-initiated

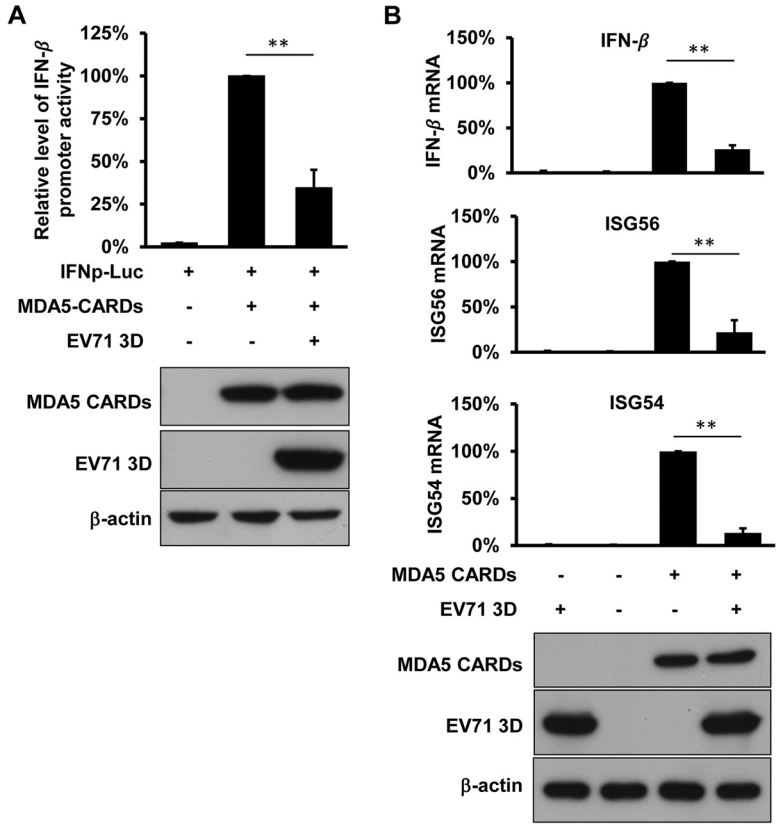

FIG 2 Examination of the role of EV71 $3D^{pol}$ in regulation of MDA5 CARD-activated IFN-β promoter and mRNA expression of IFN-β, ISG56, and ISG54. (A) 293T cells were cotransfected with IFN-β promoter luciferase reporter, FLAG-tagged MDA5 CARD-expressing plasmid, and either empty vector or 3×FLAG-tagged EV71 3D-expressing plasmid. At 24 h posttransfection, cell extracts were collected for luciferase activity assays. (B) 293T cells were cotransfected with FLAG-tagged MDA5 CARD-expressing plasmid, and either empty vector or EV71 3D-expressing plasmid. At 24 h posttransfection, total RNA of transfected cells was collected for RT-qPCR to determine mRNA levels of IFN-β, ISG56, and ISG54. The protein expression was detected by Western blotting with anti-MDA5, anti-FLAG, and anti-β-actin antibodies. The error bars indicate the SDs. **, $P < 0.01$.

signaling for type I IFN activation. We further investigated whether the viral RdRP blocks MDA5-initiated signaling via a protein-protein interaction. To determine whether EV71 $3D^{pol}$ interacts with the cytoplasmic pathogen sensor MDA5, 293T cells were cotransfected with FLAG-tagged MDA5 and EV71 $3D^{pol}$-expressing plasmids. At 24 h posttransfection, lysates of the transfected cells were subjected to immunoprecipitation by anti-FLAG resin. As shown in Fig. 3, EV71 $3D^{pol}$ coprecipitated with FLAG-tagged MDA5 protein, indicating the $3D^{pol}$ interacts with MDA5 in the transfected cells.

As aforementioned, type I IFN activation predicts that the N-terminal CARDs of the MDA5 protein (Fig. 4A) are required for transducing activation signals to downstream MAVS via CARD-CARD interactions between these proteins. We further investigated whether EV71 $3D^{pol}$ could interact with the CARDs of MDA5. First, 293T cells were cotransfected with EV71 $3D^{pol}$-expressing plasmid and the plasmid expressing either the CARDs (residues 1 to 204) or helicase CTDs (residues 205 to 1017) of the MDA5 protein with the 2×FLAG tag. The transfected cell extracts were collected and subjected to anti-FLAG immunoprecipitation. As shown in Fig. 4B, the CARDs of MDA5 interacted with EV71 $3D^{pol}$. To verify the interaction, lysates of 293T cells that were cotransfected with a plasmid that expresses the N terminus, residues 1 to 297, of MDA5 containing a Myc tag and either a 3×FLAG-tagged EV71 $3D^{pol}$-expressing plasmid or an empty vector were subjected to immunoprecipitation by an anti-FLAG antibody. The immunoblot of the precipitates showed that EV71 $3D^{pol}$ associated with the CARDs of MDA5 (Fig. 4C). Moreover, we further examined the localization of EV71 $3D^{pol}$ and

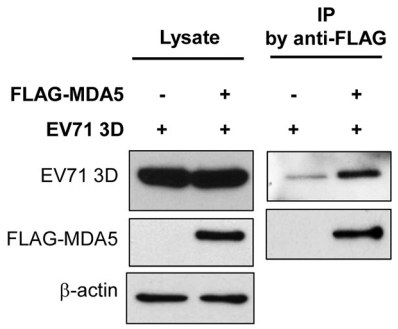

FIG 3 Determination of the interaction of EV71 3D^{pol} and MDA5 proteins. 293T cells were cotransfected with plasmids expressing FLAG-tagged MDA5 and Myc-tagged EV71 3D. At 24 h posttransfection, cell extracts were collected and subjected to immunoprecipitation by anti-FLAG antibody. The cell extracts (left) and precipitated products (right) were analyzed by immunoblotting with anti-FLAG and anti-EV71 3D antibodies.

MDA5 proteins in cells. HeLa cells were cotransfected with FLAG-tagged full-length MDA5 or the N-terminus of MDA5 and Myc-tagged EV71 3D^{pol}-expressing plasmids for 24 h. Confocal microscopy demonstrated that EV71 3D^{pol} colocalized with either the full-length MDA5 or the N terminus (CARDs) of MDA5 in the cytoplasm (Fig. 5C and D). These results indicate that EV71 3D^{pol} interacts with the CARDs of MDA5 and suggest that the suppression of EV71 3D^{pol} in MDA5-mediated type I IFN activation may be caused by interaction of the CARDs of the pathogen recognition receptor MDA5.

EV71 3D^{pol} interacts with MDA5 during infection. In addition, we examined the interaction between MDA5 and the 3D^{pol} protein during EV71 infection. 293T cells were transfected with FLAG-tagged MDA5-expressing plasmid for 24 h and then infected with the EV71 strain 2231/98 at a multiplicity of infection (MOI) of 2. At 12 h postinfection, extracts of the infected cells were collected and subjected to immunoprecipitation with anti-FLAG antibody. We found that EV71 3D^{pol} and its precursor 3CD protein

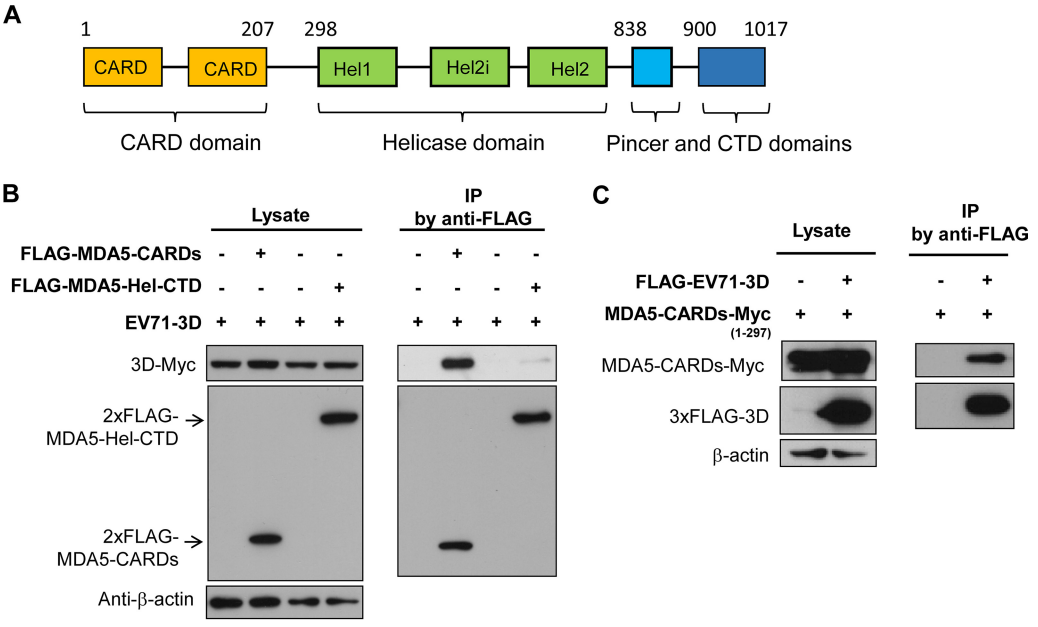

FIG 4 EV71 3D^{pol} interacts with the CARDs of MDA5 protein. (A) Diagram of the domains of MDA5 protein. (B) 293T cells were cotransfected with Myc-tagged EV71 3D-expressing plasmid and a plasmid that expresses either the CARDs or helicase CTDs of MDA5 with 2×FLAG tags. (C) The 293T cells were cotransfected with 3×FLAG-tagged EV71 3D and Myc-tagged CARDs of MDA5-expressing plasmids. At 24 h posttransfection, cell extracts were collected and subjected to immunoprecipitation by anti-FLAG antibody. The cell extracts (left) and precipitated products (right) were analyzed by immunoblotting with anti-FLAG and anti-EV71 3D antibodies.

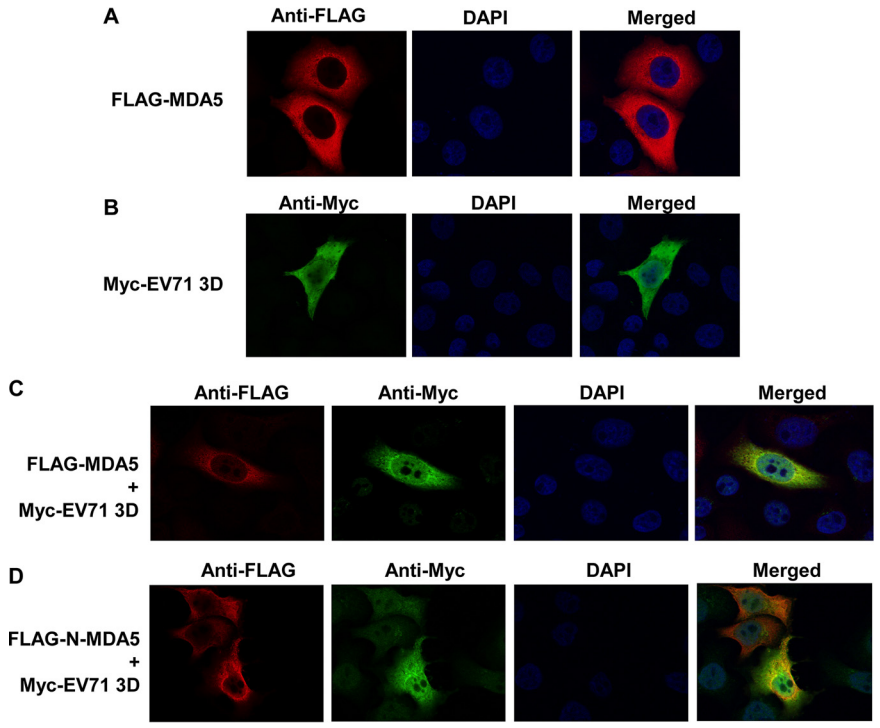

FIG 5 Examination of the localization of EV71 3D^{pol} and MDA5 in cotransfected cells. HeLa cells were transfected with plasmids expressing FLAG-tagged MDA5 (A) or Myc-tagged EV71 3D^{pol} (B), FLAG-tagged MDA5 and Myc-tagged EV71 3D^{pol} (C), or FLAG-tagged N-MDA5 and Myc-tagged EV71 3D^{pol} (D) for 24 h. The transfected cells were fixed and reacted with mouse anti-FLAG (A), rabbit anti-Myc (B), or anti-FLAG and anti-Myc (C and D) antibodies. After incubating with the secondary antibodies conjugated with fluorescent dyes, the cells were examined under confocal microscopy.

were in the precipitates by immunoblotting analysis (Fig. 6A). This result confirmed that EV71 3D^{pol} is able to associate with MDA5 during infection. A similar result was found by using a mouse-adapted strain MP4 of EV71 (Fig. 6B). Interestingly, the immunoprecipitation result might also suggest that MDA5 interacts with 3CD protein more efficiently than 3D^{pol}.

Since our results demonstrated that EV71 3D^{pol} interacts with MDA5, we further examined whether EV71 3D^{pol} can be colocalized with MDA5 during EV71 infection. HeLa cells were transfected with plasmid expressing FLAG-tagged MDA5 for 24 h and then infected with EV71 at an MOI of 80. At 6 h postinfection, the infected cells were fixed, and the localization of EV71 3D^{pol} and the FLAG-tagged MDA5 was monitored. The results showed that EV71 3D^{pol} was partly colocalized with MDA5 in the cytoplasm of infected cells (Fig. 6C). Furthermore, we verified the colocalization of endogenous MDA5 and EV71 3D^{pol} during infection. HeLa cells were transfected with poly(I:C) for 24 h to stimulate MDA5 expression and then infected with EV71 for 6 h. As expected, transfection with poly(I:C) induced endogenous MDA5 expression (Fig. 7A and B), while infection with EV71 alone did not induce MDA5 expression due to the suppression of type I IFN activation by EV71 (Fig. 7C). We then monitored the localization of the poly(I:C)-induced MDA5 and EV71 3D^{pol} during infection. As shown in Fig. 7D, EV71 3D^{pol} partly colocalized with endogenous MDA5 in EV71-infected cells. Collectively, these findings imply that EV71 3D^{pol} may interact with the host cytoplasmic pathogen sensor MDA5 to regulate the activation signal of type I IFN.

The polymerase activity of EV71 3D^{pol} is not required for suppression of type I IFN activation. To investigate whether the RdRP activity of EV71 3D^{pol} is required for inhibiting MDA5-mediated activation of the IFN-β promoter, a plasmid that expresses EV71 3D^{pol} with the D330A mutation, which loses its RNA polymerase activity (33), was

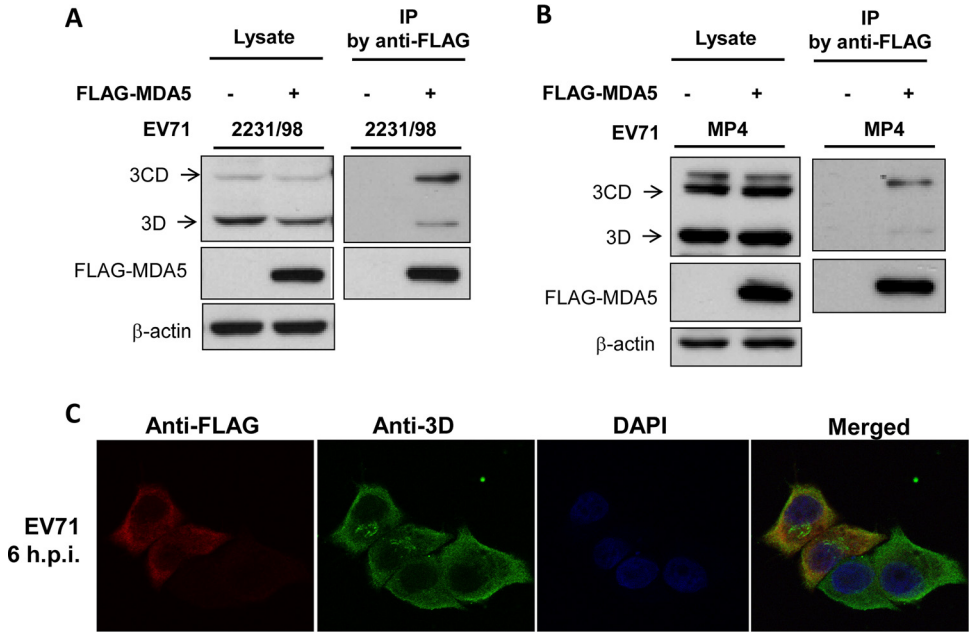

FIG 6 Determination of the interaction and localization of EV71 3D^{pol} and MDA5 proteins in infected cells. 293T cells were transfected with plasmid expressing FLAG-tagged MDA5 for 24 h and then infected with the EV71 strain of 2231/98 (A) or MP4 (B). At 12 h postinfection, cell extracts were collected and subjected to immunoprecipitation by anti-FLAG antibody. The cell extracts and precipitated products were analyzed by immunoblotting with anti-FLAG and anti-EV71 3D antibodies. (C) HeLa cells were transfected with FLAG-tagged MDA5-expressing plasmid for 24 h and then infected with EV71/MP4 at an MOI of 80 for 6 h. The cells were fixed and reacted with rabbit anti-FLAG and mouse anti-EV71 3D antibodies. After incubating with the secondary antibodies conjugated with fluorescent dyes, the cells were examined under confocal microscopy.

applied to the reporter assay for IFN-β promoter activity. 293T cells were cotransfected with an MDA5-expressing plasmid, a luciferase reporter for IFN-β promoter, and either a plasmid expressing EV71 3D^{pol} with the D330A mutation or empty vector for 24 h. Lysates of the transfected cells were collected and subjected to luciferase activity assays. The results showed that the D330A mutant of EV71 3D^{pol} suppressed MDA5-mediated IFN-β activation as did the wild-type EV71 3D^{pol} (Fig. 8). The results demonstrated that the EV71 3D^{pol} mutant that lacks RNA polymerase activity retains the ability to suppress IFN-β expression.

We further applied the 3D^{pol} mutant in an evaluation of the suppression ability in EV71 viral RNA-activated IFN-β expression. HeLa cells were transfected with the EV71 3D^{pol} D330A mutant-expressing plasmid for 14 h and then further transfected with total RNA isolated from either mock- or EV71-infected cells. At 9 h after RNA transfection, total RNA of the transfected cells was extracted and subjected to reverse transcription-quantitative PCR (RT-qPCR) for determining mRNA expression of IFN-β, ISG56, and ISG54. We found that infected cell RNA induced less IFN-β mRNA in the cells overexpressing the EV71 3D^{pol} D330A mutant than in the control cells that were transfected with an empty vector (Fig. 9). The result suggests that EV71 3D^{pol} negatively regulates EV71 RNA-activated IFN-β production.

3D^{pol} encoded by CVB3 interacts with MDA5. To determine whether the 3D^{pol} proteins encoded by other enteroviruses interact with MDA5, extracts of 293T cells that were cotransfected with FLAG-tagged MDA5 and green fluorescent protein (GFP)-fused coxsackievirus B3 (CVB3) 3D^{pol}-expressing plasmids were subjected to anti-FLAG immunoprecipitation. We found that the 3D^{pol} encoded by CVB3 interacted with MDA5 (Fig. 10A). In addition, overexpression of CVB3 3D^{pol} reduced MDA5-initiated IFN-β promoter activity (Fig. 10B). These results imply that interaction of MDA5 and enteroviral 3D^{pol} may be one of the mechanisms for suppressing IFN-β promoter activation during infection with human enteroviruses.

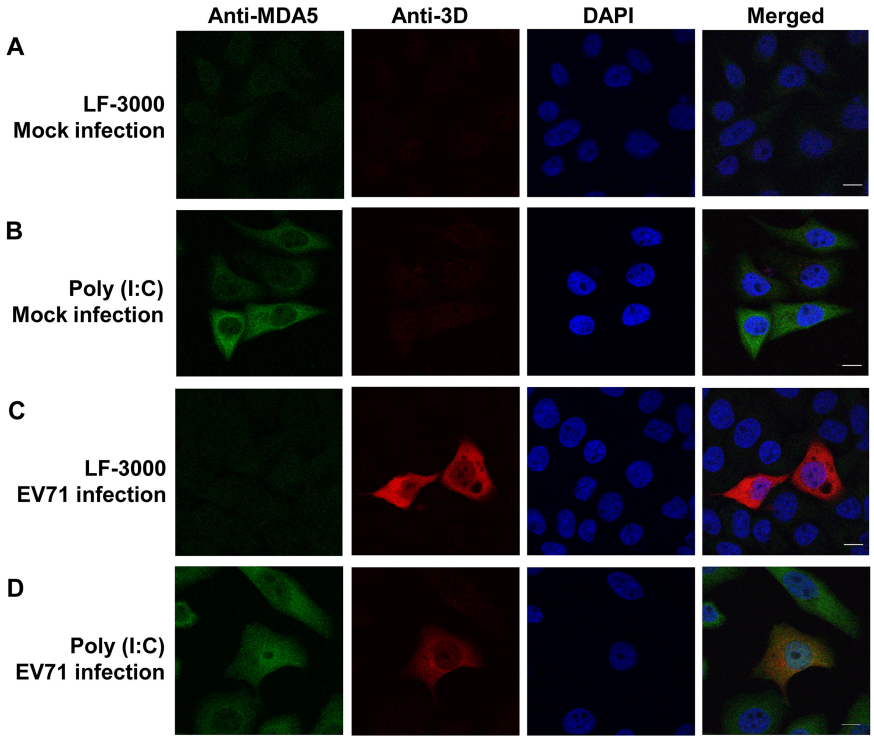

FIG 7 Examination of the localization of EV71 $3D^{pol}$ and endogenous MDA5 in infected cells. HeLa cells were transfected with poly(I:C) (B and D) or treated with Lipofectamine 3000 (LF-3000) transfection reagent alone (A and C) for 24 h and then infected with EV71/MP4 at an MOI of 80 (C and D) for 6 h. The cells were fixed and reacted with rabbit anti-MDA5 and mouse anti-EV71 3D antibodies. After incubating with the secondary antibodies conjugated with fluorescent dyes, the cells were examined under confocal microscopy. Bars, 10 μm.

DISCUSSION

Induction of type I IFNs is a critical step for activating the antiviral state in virus-infected cells. Nevertheless, EV71 has several mechanisms to block type I IFN activation, such as the degradation of MDA5 protein and dysregulation of host protein expression (12, 25, 32). Previous studies have shown that the Smurf1 E3 ubiquitin ligase coordinates with Ndfip1, which is a member of the NEDD4 family, to enhance the ubiquitin-mediated degradation of MAVS (34). We further demonstrated that the host protein NEDD4L reduces the MDA5-stimulated activation of the IFN-β promoter (32). In addition to the identification of the host protein involved in the downregulation of type I IFN activation, in this study, we found that EV71-encoded RdRP ($3D^{pol}$ protein) is able to inhibit the MDA5-mediated IFN-β promoter activation and suppress mRNA production of IFN-β and the downstream antiviral genes. Additionally, we demonstrated that EV71 $3D^{pol}$ interacts with the CARDs of MDA5 and that the $3D^{pol}$ protein can colocalize with MDA5 during EV71 infection. These results suggest that the enteroviral RdRP may block type I IFN activation by disturbing the signal transduction through the CARD-CARD interaction between MDA5 and MAVS. Moreover, we found that the $3D^{pol}$ encoded by coxsackievirus B3 is able to interact with MDA5 and reduce MDA5-mediated activation of the IFN-β promoter, indicating that the interaction of MDA5 and enteroviral RdRP could be a universal mechanism for suppressing IFN-β promoter activation in the cells infected with human enteroviruses. In addition to EV71 $3D^{pol}$, our immunoprecipitation result might also suggest that the precursor, 3CD protein, could efficiently interact with MDA5 in EV71-infected cells. Nevertheless, it requires further investigation to determine whether the 3CD protein of EV71 plays a role in the dysregulation of IFN-β expression during infection.

Previous studies have shown that the RdRP of picornavirus may be involved in the regulation of innate immune responses. Transgenic mice overexpressing RdRP encoded

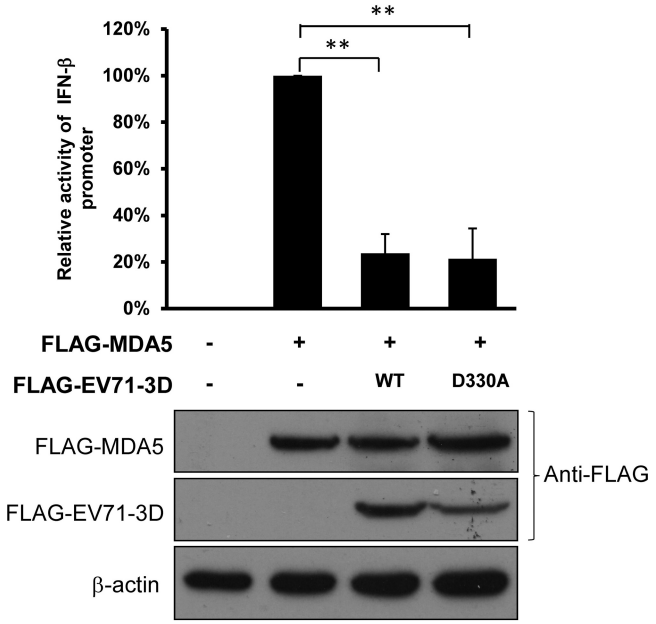

FIG 8 The RdRP activity of EV71 3D^{pol} is not required for inhibition of IFN-β promoter activation. 293T cells were cotransfected with IFN-β promoter luciferase reporter, MDA5-expressing plasmid, and either EV71 wild-type 3D^{pol}- or D330A-mutated 3D^{pol}-expressing plasmid. At 24 h posttransfection, the lysates of the transfected cells were collected and subjected to a luciferase activity assay. The error bars indicate the SDs. **, $P < 0.01$.

by Theiler's murine encephalomyelitis virus (TMEV) had enhanced innate immunity due to the ectopic expression of dsRNA by the RdRP (35). It was also reported that EV71 3D^{pol} associates with the NLRP3-ASC complex and activates IL-1β expression (36). Nevertheless, our study showed that EV71 3D^{pol} interacts with MDA5 and inhibits

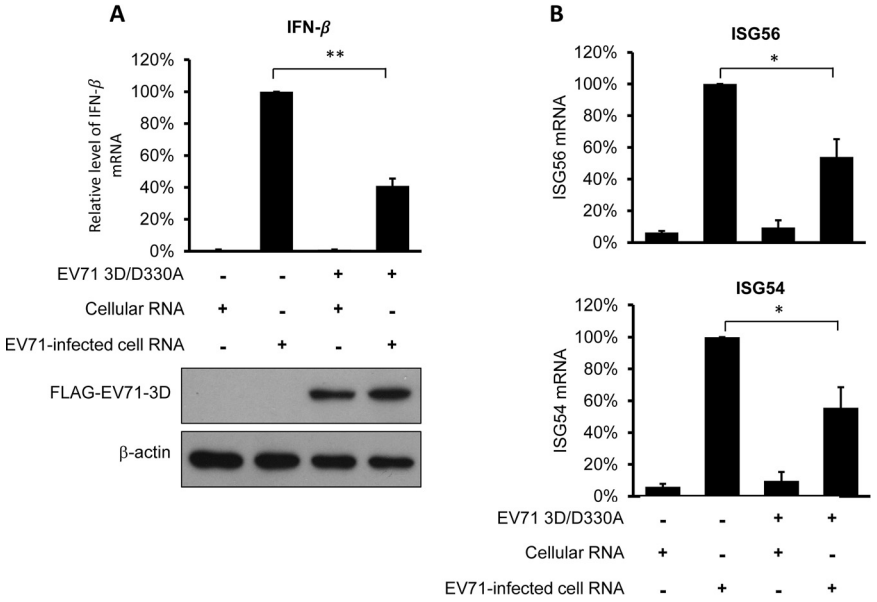

FIG 9 Determination of 3D^{pol} D330A mutant in suppression of the EV71 viral RNA-activated IFN-β signaling pathway. HeLa cells were transfected with either an empty vector or the EV71 3D^{pol} D330A mutant-expressing plasmid for 14 h and then further transfected with total RNA isolated from either mock- or EV71-infected cells, as indicated in the figure. At 9 h after RNA transfection, total RNA of the transfected cells was extracted and subjected to RT-qPCR for determining the mRNA expression of IFN-β (A) or ISG56 and ISG54 (B). The error bars indicate the SDs. *, $P < 0.05$; **, $P < 0.01$.

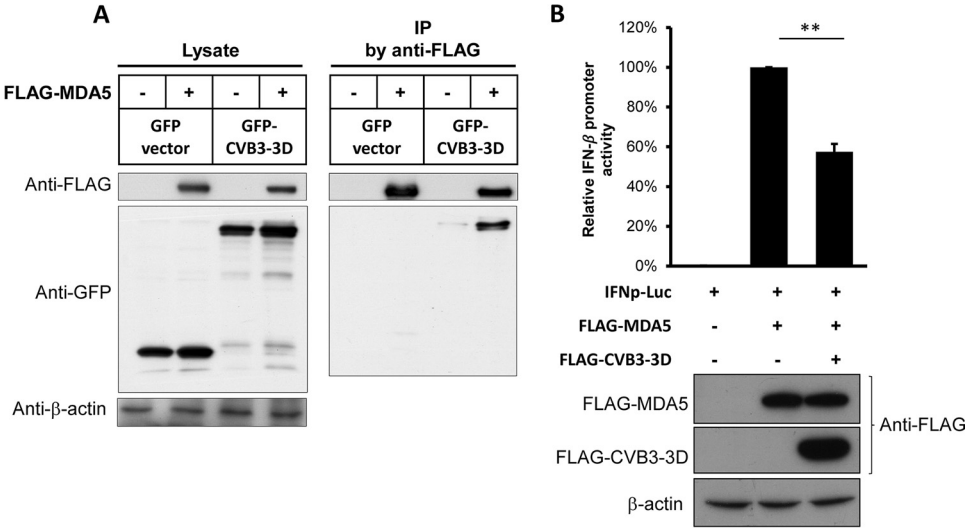

FIG 10 The 3D^{pol} encoded by CVB3 interacts with MDA5 and regulates MDA5-mediated IFN-β promoter activation. (A) 293T cells were cotransfected with plasmids expressing FLAG-tagged MDA5 and GFP-fused CVB3 3D^{pol}. At 24 h posttransfection, cell extracts were collected and subjected to anti-FLAG immunoprecipitation. (B) 293T cells were cotransfected with IFN-β promoter luciferase reporter, MDA5-expressing plasmid, and either CVB3 3D^{pol}-expressing plasmid or empty vector. At 24 h posttransfection, the lysates of the transfected cells were collected and subjected to a luciferase activity assay. The error bars indicate the SDs. **, $P < 0.01$.

MDA5-mediated IFN-β promoter activation. The suppression was also detected by the polymerase activity knockout mutant (D330A) of EV71 3D^{pol}. Because the mutant lacks the activity to replicate viral RNA, we applied the native ligand of EV71-related viral RNA to demonstrate the inhibitory effect in IFN-β induction by EV71 3D^{pol} without the interference of viral RNA replication. Nevertheless, these findings suggest the dual roles of enteroviral RdRP in regulating host innate immunity during infection.

Because the enteroviral 3D^{pol}, which is a viral RNA-dependent RNA polymerase, plays an important role in viral RNA replication, the 3D^{pol} protein is expected to localize to the specialized replication sites of the enterovirus, which are intracellular membrane compartments in the cytoplasm of enterovirus-infected cells, and form the replication complex with viral RNA and other viral and cellular factors (37). The interaction of EV71 3D^{pol} and the endoplasmic reticulum protein UGGT1 has been proved to participate in replication complexes for enhancing viral RNA synthesis (38). Nevertheless, several reports demonstrated that a proportion of EV71 3D^{pol} molecules interacts with cellular proteins and is located in specific cell compartments, such as spliceosomes and inflammasomes (5, 36). We further demonstrated that EV71 3D^{pol} interacts with the cytoplasmic pathogen sensor MDA5 in the activation pathway of type I IFN in this study. Although previous studies showed that EV71 3D^{pol} does not block RIG-I-initiated type I IFN activation (27), we found that EV71 3D^{pol} does suppress MDA5-activated type I IFN expression. Nevertheless, because it has been revealed that MDA5 is relatively critical in detecting EV71 viral RNA compared to RIG-I (12), interruption of MDA5 signaling by the 3D^{pol} may interfere with the activation of type I IFN during EV71 infection. Accordingly, in addition to enterovirus-encoded proteases, we revealed a novel mechanism involving enteroviral RdRP that negatively regulates the host antiviral response.

MATERIALS AND METHODS

Cells, viruses, and plasmids. HEK293T (293T), Vero, RD, and HeLa cells were incubated at 37°C with 5% CO_2 and maintained with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and a 1% penicillin-streptomycin-L-glutamate (PSG) mixture. The enteroviruses EV71/TW2231/98 and coxsackievirus B3 (CVB3) were obtained from the Clinical Virology Laboratory, Department of Pathology, Linkou Chang Gung Memorial Hospital, Taiwan. The mouse-adapted EV71/MP4 strains were provided by Jen-Ren Wang at the National Cheng-Kung University, Taiwan. All of the human enteroviruses used in this study were amplified in Vero cells and titrated with the monolayers of RD cells

cultivated in DMEM containing 2% FBS. The FLAG-tagged full-length MDA5-expressing plasmid was originally provided by Michael Gale, Jr. Plasmids expressing either 2×FLAG-tagged CARDs (residues 1 to 205) or the helicase and C-terminal domains (CTDs) (residues 206 to 1025) of MDA5 constructed with the pEF vector were provided by Helene M. Liu. A plasmid expressing the N terminus of MDA5 (residues 1 to 297) containing a Myc tag was constructed with the pcDNA3.1/myc-His(A) vector (Invitrogen, USA). The cDNA of the 3D region of the EV71 genome was inserted into the p3×FLAG-Myc-CMV25 (Sigma-Aldrich, USA) or pcDNA3.1/myc-His(A) (Invitrogen) vector. The cDNA of 3D of the CVB3 genome was cloned into the pFLAG-CMV2 (Sigma-Aldrich) or pEGFP-C3 (Clontech, USA) vector.

Transfection and virus infection. Cells with 50% to 70% confluence were maintained in DMEM containing 10% FBS. Plasmid transfection was performed using the TransIT-LT1 reagent (Mirus Bio, USA) according to the manufacturer's protocols. Poly(I:C) (Sigma-Aldrich) or total RNA isolated from mock- or EV71-infected Vero cells was transfected using the Lipofectamine 3000 transfection reagent (Invitrogen) according to the manufacturer's instructions. For EV71 infection, cells were added to the virus at the multiplicity of infection (MOI) indicated in Results and in the figure legends. The virus was removed after 1 h adsorption, and the infected cells were maintained at 37°C in DMEM with 2% FBS for the times indicated in Results and in the figure legends.

Immunoprecipitation and immunoblotting. First, 293T cells were cotransfected with the above-described plasmids. At 24 h posttransfection, the cells were lysed and then subjected to immunoprecipitation using anti-FLAG M2 resin (Sigma-Aldrich) or anti-Myc antibody with protein A and protein G Sepharose beads (GE Healthcare Life Sciences, USA). After washing the affinity resin, the proteins that remained on the beads were eluted using a 3×FLAG peptide or the SDS-PAGE sample buffer. Cellular protein extracts and immunoprecipitates were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were analyzed individually by anti-EV71 3D (GeneTex, USA), anti-MAVS (Santa Cruz Biotechnology, USA), anti-c-Myc (Sigma-Aldrich), anti-FLAG (Sigma-Aldrich), anti-GFP (GeneTex, USA), and anti-β-actin (Sigma-Aldrich) primary antibodies, incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (GE Healthcare Life Sciences), and then subjected to a chemiluminescent HRP substrate (Millipore, USA) for detection.

Immunofluorescence. HeLa cells cultivated on glass slides were transfected with plasmids expressing FLAG-tagged MDA5 and Myc-tagged EV71 $3D^{pol}$ for 24 h. For the infection condition, HeLa cells on glass slides were transfected with either poly(I:C) or FLAG-tagged MDA5-expressing plasmid for 24 h and then infected with EV71 at an MOI of 80 for 6 h. The cells were sequentially treated with 3% paraformaldehyde and 0.2% Triton X-100 and then blocked with 5% bovine serum albumin. The treated cells were incubated with mouse or rabbit anti-FLAG, rabbit anti-Myc, or mouse anti-EV71 3D antibodies. After incubation with the secondary antibodies, anti-species-specific IgG conjugated with Alexa Fluor 488 dye or with Alexa Fluor 594 dye (Life Technologies, USA), and washing with phosphate-buffered saline, the cells were mounted by ProLong Gold antifade reagent with DAPI (4′,6-diamidino-2-phenylindole; Thermo Fisher, USA) and observed under a confocal fluorescence microscope.

IFN-β promoter reporter assay. First, 293T cells were transfected with a plasmid expressing firefly luciferase driven by the IFN-β promoter, a renilla luciferase reporter plasmid, a FLAG-tagged MDA5- or V5-tagged MAVS-expressing plasmid, and a 3×FLAG-tagged $3D^{pol}$-expressing plasmid or an empty vector. At 24 h posttransfection, transfected cells were collected and lysed with a passive lysis buffer (Promega, USA). The cell lysates were then subjected to a dual-luciferase reporter assay (Promega) according to the manufacturer's instructions.

RNA extraction and reverse transcription-quantitative PCR. Total RNA of cells transfected with the above-described plasmids or/and RNA was extracted with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Total RNA extracted was subjected to a reverse transcription reaction using SuperScript III reverse transcriptase (Invitrogen) with oligo(dT) or a specific primer annealing to 18S rRNA (5′-CCATCCAATCGGTAGTAGCG-3′). qPCR was performed using Kapa SYBR Fast qPCR kits (Kapa Biosystems, USA) with specific primers for detecting 18S rRNA (forward, 5′-GTAACCCGTTGAACCCCATT-3′; and reverse, 5′-CCATCCAATCGGTAGTAGCG-3′) and mRNA of IFN-β (forward, 5′-CAGTCTGCACCTGA AAAGATATTATG-3′; and reverse, 5′-GATTTCCACTCTGACTATGGTCCAGG-3′) (32), ISG56 (forward, 5′-AAG GCAGGCTGTCCGCTTA-3′; and reverse, 5′-TCCTGTCCTTCATCCTGAAGCT-3′), and ISG54 (forward, 5′-ACG GTATGCTTGGAACGATTG-3′; and reverse, 5′-AACCCAGAGTGTGGCTGATG-3′) (39). The relative levels of mRNA were normalized with the quantification of 18S rRNA.

Statistical analysis. Student's *t* test (via Microsoft Excel and GraphPad Prism softwares) was used for statistical analysis in comparisons of IFN-β promoter activity and mRNA levels between two groups. The differences between two groups with *P* values of <0.05 were considered statistically significant.

ACKNOWLEDGMENTS

We thank Jen-Ren Wang, Michael Gale, Jr., and Helene M. Liu for reagents and helpful discussions.

This research was supported by grants from the Ministry of Science and Technology, Taiwan (MOST 103-2320-B-182-024-MY3, 106-2320-B-182-024-MY3, 106-2632-B-182-001, and 107-3017-F-182-001), and the Chang Gung Memorial Hospital (CMRPD1E0441∼3, CMRPD1G0571∼2, and BMRPC09). This work was also financially supported by the Research Center for Emerging Viral Infections from the Featured Areas Research Center Program within the framework of the Higher Education Sprout Project by the Ministry of Education (MOE) in Taiwan.

REFERENCES

1. Ooi MH, Wong SC, Lewthwaite P, Cardosa MJ, Solomon T. 2010. Clinical features, diagnosis, and management of enterovirus 71. Lancet Neurol 9:1097–1105. https://doi.org/10.1016/S1474-4422(10)70209-X.
2. Racaniello VR. 2007. *Picornaviridae*: the viruses and their replication, p 796–839. *In* Knipe DM, Howley PM (ed), Fields Virology, 5th ed. Lippincott Williams & Wilkins, Philadelphia, PA.
3. Sharma R, Raychaudhuri S, Dasgupta A. 2004. Nuclear entry of poliovirus protease-polymerase precursor 3CD: implications for host cell transcription shut-off. Virology 320:195–205. https://doi.org/10.1016/j.virol.2003.10.020.
4. Sanchez-Aparicio MT, Rosas MF, Sobrino F. 2013. Characterization of a nuclear localization signal in the foot-and-mouth disease virus polymerase. Virology 444:203–210. https://doi.org/10.1016/j.virol.2013.06.011.
5. Liu YC, Kuo RL, Lin JY, Huang PN, Huang Y, Liu H, Arnold JJ, Chen SJ, Wang RY, Cameron CE, Shih SR. 2014. Cytoplasmic viral RNA-dependent RNA polymerase disrupts the intracellular splicing machinery by entering the nucleus and interfering with Prp8. PLoS Pathog 10:e1004199. https://doi.org/10.1371/journal.ppat.1004199.
6. Kang DC, Gopalkrishnan RV, Wu Q, Jankowsky E, Pyle AM, Fisher PB. 2002. mda-5: an interferon-inducible putative RNA helicase with double-stranded RNA-dependent ATPase activity and melanoma growth-suppressive properties. Proc Natl Acad Sci U S A 99:637–642. https://doi.org/10.1073/pnas.022637199.
7. Yoneyama M, Kikuchi M, Natsukawa T, Shinobu N, Imaizumi T, Miyagishi M, Taira K, Akira S, Fujita T. 2004. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. Nat Immunol 5:730–737. https://doi.org/10.1038/ni1087.
8. Kato H, Takeuchi O, Sato S, Yoneyama M, Yamamoto M, Matsui K, Uematsu S, Jung A, Kawai T, Ishii KJ, Yamaguchi O, Otsu K, Tsujimura T, Koh CS, Reis e Sousa C, Matsuura Y, Fujita T, Akira S. 2006. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. Nature 441:101–105. https://doi.org/10.1038/nature04734.
9. Takahasi K, Yoneyama M, Nishihori T, Hirai R, Kumeta H, Narita R, Gale M, Jr, Inagaki F, Fujita T. 2008. Nonself RNA-sensing mechanism of RIG-I helicase and activation of antiviral immune responses. Mol Cell 29:428–440. https://doi.org/10.1016/j.molcel.2007.11.028.
10. Gitlin L, Barchet W, Gilfillan S, Cella M, Beutler B, Flavell RA, Diamond MS, Colonna M. 2006. Essential role of mda-5 in type I IFN responses to polyriboinosinic:polyribocytidylic acid and encephalomyocarditis picornavirus. Proc Natl Acad Sci U S A 103:8459–8464. https://doi.org/10.1073/pnas.0603082103.
11. Wang JP, Cerny A, Asher DR, Kurt-Jones EA, Bronson RT, Finberg RW. 2010. MDA5 and MAVS mediate type I interferon responses to coxsackie B virus. J Virol 84:254–260. https://doi.org/10.1128/JVI.00631-09.
12. Kuo RL, Kao LT, Lin SJ, Wang RY, Shih SR. 2013. MDA5 plays a crucial role in enterovirus 71 RNA-mediated IRF3 activation. PLoS One 8:e63431. https://doi.org/10.1371/journal.pone.0063431.
13. Loo YM, Fornek J, Crochet N, Bajwa G, Perwitasari O, Martinez-Sobrido L, Akira S, Gill MA, Garcia-Sastre A, Katze MG, Gale M, Jr, 2008. Distinct RIG-I and MDA5 signaling by RNA viruses in innate immunity. J Virol 82:335–345. https://doi.org/10.1128/JVI.01080-07.
14. Kawai T, Takahashi K, Sato S, Coban C, Kumar H, Kato H, Ishii KJ, Takeuchi O, Akira S. 2005. IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. Nat Immunol 6:981–988. https://doi.org/10.1038/ni1243.
15. Randall RE, Goodbourn S. 2008. Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures. J Gen Virol 89:1–47. https://doi.org/10.1099/vir.0.83391-0.
16. Honda K, Takaoka A, Taniguchi T. 2006. Type I interferon gene induction by the interferon regulatory factor family of transcription factors. Immunity 25:349–360. https://doi.org/10.1016/j.immuni.2006.08.009.
17. Wu B, Peisley A, Richards C, Yao H, Zeng X, Lin C, Chu F, Walz T, Hur S. 2013. Structural basis for dsRNA recognition, filament formation, and antiviral signal activation by MDA5. Cell 152:276–289. https://doi.org/10.1016/j.cell.2012.11.048.
18. Kato H, Takeuchi O, Mikamo-Satoh E, Hirai R, Kawai T, Matsushita K, Hiiragi A, Dermody TS, Fujita T, Akira S. 2008. Length-dependent recognition of double-stranded ribonucleic acids by retinoic acid-inducible gene-I and melanoma differentiation-associated gene 5. J Exp Med 205:1601–1610. https://doi.org/10.1084/jem.20080091.
19. Triantafilou K, Vakakis E, Kar S, Richer E, Evans GL, Triantafilou M. 2012. Visualisation of direct interaction of MDA5 and the dsRNA replicative intermediate form of positive strand RNA viruses. J Cell Sci 125:4761–4769. https://doi.org/10.1242/jcs.103887.
20. Feng Q, Hato SV, Langereis MA, Zoll J, Virgen-Slane R, Peisley A, Hur S, Semler BL, van Rij RP, van Kuppeveld FJ. 2012. MDA5 detects the double-stranded RNA replicative form in picornavirus-infected cells. Cell Rep 2:1187–1196. https://doi.org/10.1016/j.celrep.2012.10.005.
21. Deddouche S, Goubau D, Rehwinkel J, Chakravarty P, Begum S, Maillard PV, Borg A, Matthews N, Feng Q, van Kuppeveld FJ, Reis e Sousa C. 2014. Identification of an LGP2-associated MDA5 agonist in picornavirus-infected cells. Elife 3:e01535. https://doi.org/10.7554/eLife.01535.
22. Runge S, Sparrer KM, Lassig C, Hembach K, Baum A, Garcia-Sastre A, Soding J, Conzelmann KK, Hopfner KP. 2014. *In vivo* ligands of MDA5 and RIG-I in measles virus-infected cells. PLoS Pathog 10:e1004081. https://doi.org/10.1371/journal.ppat.1004081.
23. Pichlmair A, Schulz O, Tan CP, Rehwinkel J, Kato H, Takeuchi O, Akira S, Way M, Schiavo G, Reis e Sousa C. 2009. Activation of MDA5 requires higher-order RNA structures generated during virus infection. J Virol 83:10761–10769. https://doi.org/10.1128/JVI.00770-09.
24. Lang X, Tang T, Jin T, Ding C, Zhou R, Jiang W. 2017. TRIM65-catalyzed ubiquitination is essential for MDA5-mediated antiviral innate immunity. J Exp Med 214:459–473. https://doi.org/10.1084/jem.20160592.
25. Feng Q, Langereis MA, Lork M, Nguyen M, Hato SV, Lanke K, Emdad L, Bhoopathi P, Fisher PB, Lloyd RE, van Kuppeveld FJ. 2014. Enterovirus 2Apro targets MDA5 and MAVS in infected cells. J Virol 88:3369–3378. https://doi.org/10.1128/JVI.02712-13.
26. Wang B, Xi X, Lei X, Zhang X, Cui S, Wang J, Jin Q, Zhao Z. 2013. Enterovirus 71 protease 2Apro targets MAVS to inhibit anti-viral type I interferon responses. PLoS Pathog 9:e1003231. https://doi.org/10.1371/journal.ppat.1003231.
27. Lei X, Liu X, Ma Y, Sun Z, Yang Y, Jin Q, He B, Wang J. 2010. The 3C protein of enterovirus 71 inhibits retinoid acid-inducible gene I-mediated interferon regulatory factor 3 activation and type I interferon responses. J Virol 84:8051–8061. https://doi.org/10.1128/JVI.02491-09.
28. Lei X, Xiao X, Xue Q, Jin Q, He B, Wang J. 2013. Cleavage of interferon regulatory factor 7 by enterovirus 71 3C suppresses cellular responses. J Virol 87:1690–1698. https://doi.org/10.1128/JVI.01855-12.
29. Lee YP, Wang YF, Wang JR, Huang SW, Yu CK. 2012. Enterovirus 71 blocks selectively type I interferon production through the 3C viral protein in mice. J Med Virol 84:1779–1789. https://doi.org/10.1002/jmv.23377.
30. Kuo RL, Kung SH, Hsu YY, Liu WT. 2002. Infection with enterovirus 71 or expression of its 2A protease induces apoptotic cell death. J Gen Virol 83:1367–1376. https://doi.org/10.1099/0022-1317-83-6-1367.
31. Weng KF, Li ML, Hung CT, Shih SR. 2009. Enterovirus 71 3C protease cleaves a novel target CstF-64 and inhibits cellular polyadenylation. PLoS Pathog 5:e1000593. https://doi.org/10.1371/journal.ppat.1000593.
32. Kuo RL, Lin YH, Wang RY, Hsu CW, Chiu YT, Huang HI, Kao LT, Yu JS, Shih SR, Wu CC. 2015. Proteomics analysis of EV71-infected cells reveals the involvement of host protein NEDD4L in EV71 replication. J Proteome Res 14:1818–1830. https://doi.org/10.1021/pr501199h.
33. Jiang H, Weng L, Zhang N, Arita M, Li R, Chen L, Toyoda T. 2011. Biochemical characterization of enterovirus 71 3D RNA polymerase. Biochim Biophys Acta 1809:211–219. https://doi.org/10.1016/j.bbagrm.2011.01.001.
34. Wang Y, Tong X, Ye X. 2012. Ndfip1 negatively regulates RIG-I-dependent immune signaling by enhancing E3 ligase Smurf1-mediated MAVS degradation. J Immunol 189:5304–5313. https://doi.org/10.4049/jimmunol.1201445.
35. Painter MM, Morrison JH, Zoecklein LJ, Rinkoski TA, Watzlawik JO, Papke LM, Warrington AE, Bieber AJ, Matchett WE, Turkowski KL, Poeschla EM, Rodriguez M. 2015. Antiviral protection via RdRP-mediated stable activation of innate immunity. PLoS Pathog 11:e1005311. https://doi.org/10.1371/journal.ppat.1005311.
36. Wang W, Xiao F, Wan P, Pan P, Zhang Y, Liu F, Wu K, Liu Y, Wu J. 2017. EV71 3D protein binds with NLRP3 and enhances the assembly of inflammasome complex. PLoS Pathog 13:e1006123. https://doi.org/10.1371/journal.ppat.1006123.
37. Hsu NY, Ilnytska O, Belov G, Santiana M, Chen YH, Takvorian PM, Pau C,

van der Schaar H, Kaushik-Basu N, Balla T, Cameron CE, Ehrenfeld E, van Kuppeveld FJ, Altan-Bonnet N. 2010. Viral reorganization of the secretory pathway generates distinct organelles for RNA replication. Cell 141:799–811. https://doi.org/10.1016/j.cell.2010.03.050.

38. Huang PN, Jheng JR, Arnold JJ, Wang JR, Cameron CE, Shih SR. 2017. UGGT1 enhances enterovirus 71 pathogenicity by promoting viral RNA synthesis and viral replication. PLoS Pathog 13:e1006375. https://doi.org/10.1371/journal.ppat.1006375.
39. Orzalli MH, DeLuca NA, Knipe DM. 2012. Nuclear IFI16 induction of IRF-3 signaling during herpesviral infection and degradation of IFI16 by the viral ICP0 protein. Proc Natl Acad Sci U S A 109:E3008–E3017. https://doi.org/10.1073/pnas.1211302109.