Enteroviruses 2A<sup>pro</sup> targets MDA5 and MAVS in infected cells

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ABSTRACT

RIG-I like receptors (RLRs), MDA5 and RIG-I, are key players in the innate antiviral response. Upon recognition of viral RNA, they interact with MAVS, eventually inducing type I interferon production. The interferon induction pathway is commonly targeted by viruses. How enteroviruses suppress interferon production is incompletely understood. MDA5 has been suggested to undergo caspase- and proteasome-mediated degradation during poliovirus infection. Additionally, MAVS is reported to be cleaved during infection of coxsackievirus B3 (CVB3) by its proteinase 3C<sup>pro</sup>, whereas MAVS cleavage by enterovirus71 has been attributed to 2A<sup>pro</sup>. As yet, a detailed examination of the RLR pathway as a whole during any enterovirus infection is lacking. We performed a comprehensive analysis of crucial factors of the RLR pathway including MDA5, RIG-I, LGP2, MAVS, TBK1, and IRF3 during infection of CVB3, a human enterovirus B (HEV-B) species member. We show that CVB3 inhibits the RLR pathway upstream of TBK1 activation, as demonstrated by limited phosphorylation of TBK1 and lack of IRF3 phosphorylation. Furthermore, we show that MDA5, MAVS and RIG-I all undergo proteolytic degradation in CVB3-infected cells through a caspase- and proteasome-independent manner. We convincingly show that MDA5 and MAVS cleavages are both mediated by CVB3 2A<sup>pro</sup>, while RIG-I is cleaved by 3C<sup>pro</sup>. Moreover, we show that proteinases 2A<sup>pro</sup> and 3C<sup>pro</sup> of poliovirus (HEV-C) and enterovirus 71 (HEV-A) exert the same functions. This study identifies a critical role of 2A<sup>pro</sup> by cleaving MDA5 and MAVS and shows that enteroviruses use a common strategy to counteract the interferon response in infected cells.
IMPORTANCE

Human enteroviruses (HEVs) are important pathogens that cause a variety of diseases in humans including poliomyelitis, hand, foot and mouth disease, viral meningitis and cardiomyopathy and more. As many other viruses, enteroviruses target the host immune pathways to gain replication advantage. The MDA5/MAVS pathway is responsible for recognizing enterovirus infections in the host cell, and leads to expression of type I interferons (IFN-I), crucial antiviral signaling molecules. Here we show that three species of HEVs all employ the viral protease 2A (2A<sup>pro</sup>) to proteolytically target MDA5 and MAVS, leading to an efficient blockade upstream of IFN-I transcription. These observations suggest that MDA5/MAVS antagonization is an evolutionarily conserved and beneficial mechanism of enteroviruses. Understanding the molecular mechanisms of enterovirus immune evasion strategies help to develop countermeasures to control infections of these viruses in the future.
INTRODUCTION

Type I interferons (IFN-α/β) are key players in the innate antiviral response against virus infections. Initially produced and secreted by infected cells, IFN-α/β can bind to the type I IFN receptor (IFNAR) in autocrine and paracrine manners, thereby initiating the JAK/STAT pathway. Activation of this pathway leads to the expression of hundreds of interferon-stimulated genes (ISGs), which together induce a so-called antiviral state that restricts virus replication (reviewed in (1)). The initiation of IFN-α/β response relies on specialized pathogen recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs), molecular motifs bearing “non-self” signatures to the host cell. Some members of the Toll-like receptor (TLR) family, such as TLR3 and TLR7/8, are known to play crucial roles in virus recognition. TLRs are mostly expressed in macrophages, dendritic cells and other immune cell types, where they detect PAMPs at the cell surface or in endosomes (2). Another family of PRRs are the RIG-I-like receptors including RIG-I (3), MDA5 (4–6) and LGP2 (3, 7). These receptors are ubiquitously expressed, and monitor the cytoplasm of virtually all nucleated cells. Upon activation by viral RNA, RIG-I and MDA5 interact with MAVS, an adaptor molecule localized at the outer membrane of mitochondria (8–11). MAVS then initiates signaling cascades via TBK1 and IKK complexes, leading to activation of IRF3 and NF-kB, transcription factors required for transcription activation of IFN-α/β and other proinflammatory cytokine genes (2). RIG-I and MDA5 have non-redundant roles in detecting invading viruses. While RIG-I is crucial in detecting many negative-strand RNA viruses (e.g. VSV and influenza virus) and some Flaviviruses (e.g. hepatitis C virus and Japanese encephalitis virus) (12–15), MDA5 is important for the recognition of members of the Picornavirus, Coronavirus and Calicivirus families (16–18). The molecular motifs that activate RIG-I and MDA5 also vary. RIG-I is activated by 5’
triphosphate (5′ppp)-containing dsRNAs as well as double-stranded regions within ssRNA molecules, such as the panhandle structure formed by genomic RNAs of negative-strand RNA viruses (2, 19, 20). MDA5 requires long dsRNA duplexes for potent activation, as exemplified by the replicative form of picornaviruses (16, 21).

Picornaviridae is a large family of non-enveloped, positive-strand RNA viruses. This family includes many important human and animal pathogens, and members of the Enterovirus genus are particularly important. Poliovirus (PV), the causative agent of poliomyelitis, is the subject of a multi-billion dollar eradication campaign from the World Health Organization. Enterovirus 71 (EV71) continues to cause outbreaks of hand, foot and mouth disease associated with neurological complications in south-east Asia. Coxsackieviruses (CV) and echoviruses are the leading causes of viral meningitis and cardiomypathy. Rhinoviruses cause common colds and are frequently associated with asthma exacerbations and chronic obstructive pulmonary disease. Upon entry, the viral genome is immediately translated into a single polyprotein, which is proteolytically processed into mature peptides by viral proteinases 2A\textsuperscript{pro} and 3C\textsuperscript{pro}. These viral proteins then facilitate viral RNA replication and eventually production of progeny virion particles. Like most viruses, picornaviruses have evolved to actively suppress host IFN-α/β response to gain replication advantage. In fact, many reports have demonstrated that picornaviruses efficiently suppress IFN-α/β at the transcription level in cultured cells (16, 22, 23). However, the mechanism by which picornaviruses interfere with IFN-α/β induction is not completely understood.
Many studies have been performed to investigate how picornaviruses interfere with the RLR-mediated IFN-α/β induction pathway. MDA5, the receptor responsible for recognizing picornavirus RNA, is reported to be degraded during infection of PV (24) and EV71 (25). MDA5 degradation was shown to be dependent on caspases, and in the case of PV, also proteasome activities (24). Surprisingly, other closely related enteroviruses such as HRV16 and echovirus 1 did not induce MDA5 degradation (24), suggesting that there may be variations among enteroviruses in their strategies to suppress IFN-α/β induction. Besides MDA5, RIG-I is also targeted by several enteroviruses such as PV, echovirus, and HRV 16 and 1A, most likely via their 3C<sup>pro</sup> (26), though it remains to be elucidated why these viruses would target a RNA sensor that does not participate in their recognition. In addition, the downstream adaptor molecule MAVS is targeted by several enteroviruses including HRV1A (27), CVB3 (23) and EV71 (28). However, different mechanisms have been proposed regarding how these viruses accomplish MAVS inactivation. MAVS is reported to be cleaved by 2A<sup>pro</sup> during EV71 infection (28), and 3C<sup>pro</sup> during CVB3 infection (23), whereas both of these viral proteinases, as well as caspase 3, were implicated in HRV1A-induced MAVS cleavage (27). This diversity in MAVS inactivation mechanisms is rather uncommon for enteroviruses as they often utilize the same strategies to target a particular host factor. For instance, eIF4G is cleaved by 2A<sup>pro</sup> of various enteroviruses and rhinoviruses (29, 30) while G3BP is cleaved by 3C<sup>pro</sup> of PV (31) as well as CVB3 (this study). Importantly, each of the studies focused on a single factor (MDA5, MAVS or RIG-I), and used a different group of viruses. Hence, it is yet impossible to paint a complete picture of the IFN-α/β antagonization strategy of any individual virus, and it is challenging to conclude with confidence whether different enteroviruses truly employ diverse strategies to inactivate these
host factors, or the differences merely result from the use of different reagents and/or assays in different studies.

To gain a comprehensive overview of the fate of the important components of the RLR signaling pathway during enterovirus infection, we examined multiple factors along the signaling cascade during infection of a model virus, CVB3. We show that RIG-I is targeted by 3Cpro of CVB3, PV and EV71, resulting in similar cleavage products previously reported for PV (26). Importantly, we report that cleavage of MDA5 occurs in a caspase- and proteasome-independent manner, but instead, relies on viral proteinase 2Apro. Furthermore, in contrast to a previous report (23), we demonstrate that MAVS cleavage during CVB3 infection is also (primarily) mediated by 2Apro, and not 3Cpro. Moreover, we show that 2Apro from other enteroviruses, namely PV and EV71, also target MDA5 and MAVS for cleavage, suggesting that Enteroviruses likely share common strategies to target the RLR-mediated IFN-α/β induction pathway.
**MATERIALS AND METHODS**

**Cells and viruses.** HeLa R19 cells were maintained in DMEM supplemented with 10% FCS and 100 U/ml penicillin-streptomycin, in a humidified incubator in the presence of 5% CO₂. Coxsackievirus B3, mengovirus and mengo-Zn have been described previously (16). cDNAs of M-2A(CVB3/PV/EV71) and M-3C(CVB3/PV/EV71) viruses were generated by cloning the 2A_pro- or 3C_pro-coding region of the indicated viruses upstream of Leader-coding region in the mengovirus infectious clone pM16.1. To ensure proper maturation of the 2A_pro or 3C_pro from the mengovirus polyprotein, we also added Glutamine-Glycine codons after the inserted sequences, to allow cleavage by mengovirus 3C_pro during polyprotein processing. Viruses were produced by directly transfecting *in vitro* transcribed RNAs from the infectious clones of 2A(CVB3/PV/EV71) and M-3C(CVB3/PV/EV71) in BHK-21 or HeLa R19 cells.

**Plasmids.** pcDNA3-FLAG-MAVS-HA was generated by inserting the HA tag in pcDNA3-FLAG-MAVS provided by Prof. Z. Chen (University of Texas Southwestern Medical Center, Texas). Q148A mutation was generated by site-directed mutagenesis.

**Reagents.** Antibodies against MDA5 has been previously described (32). Antibodies against RIG-I was purchased from Abgent, MAVS from Alexis-biochemicals, IRF3 from Santa Cruz Biotechnology, p-IRF3 (S396),TBK1 and p-TBK1 (S172) from Cell Signaling Technology, LGP2 from Abcam, actin and FLAG tag from SIGMA-Aldrich, eIF4G from Bethyl Laboratories, HA tag from Covance, G3BP from BD Biosciences, and PARP from Roche Applied Sciences. Q-VD-OPh (QVD) was purchased from BioVision, MG132 from SIGMA-Aldrich, and Staurosporine from Roche Applied Sciences. Recombinant CVB3 2A_pro has been
previously described (33), and recombinant CVB3 3C\textsuperscript{pro} was a kind gift from Rolf Hilgenfeld (University of Luebeck, Luebeck).

**Quantitative real-time PCR** was performed as previously described (16).

**Immunoblotting.** Cells were lysed in TEN lysis buffer (40 mM Tris-HCl, 150 mM NaCl, 10 mM EDTA and 1% NP40), and lysate was cleared by centrifugation at 16,000 \( x \) g at 4\( ^\circ \)C for 5 min. Total protein concentrations were determined using Bio-Rad Protein Assay Dye Reagent Concentrate according to manufacturer’s protocols. Lysates were subjected to SDS-PAGE and immunoblotting using indicated antibodies. Native PAGE (for IRF3 dimerization assay) was performed as described previously (22).

**In vitro cleavage with recombinant 2A\textsuperscript{pro} or 3C\textsuperscript{pro}**. Cells were resuspended in 2x volume of the cell pellet of hypotonic buffer (20mM HEPES pH7.4, 10mM KCl, 1.5mM Mg(CH\textsubscript{3}COO)\textsubscript{2}, 1mM DTT), incubated on ice for 10 min, and lysed by passing through a thin needle repeatedly at 4\( ^\circ \)C. 0.1 x volume of Buffer A (200mM HEPES pH7.4, 1.2M KCH\textsubscript{3}COO, 40mM Mg(CH\textsubscript{3}COO)\textsubscript{2}, 50mM DTT) was added to the cell lysate, and this was cleared by centrifugation at 2,500x g for 5min at 4\( ^\circ \)C. Unless indicated otherwise, 0.5 \( \mu g \) 2A\textsuperscript{pro} or 0.75 \( \mu g \) 3C\textsuperscript{pro} were incubated with each 150 \( \mu g \) lysates at room temperature overnight. Reaction was terminated by addition of Laemmli sample buffer, and heat treatment at 95\( ^\circ \)C for 10 min.
RESULTS

CVB3 interrupts the RLR pathway upstream of TBK1 phosphorylation.

It is known that picornaviruses induce little IFN-α/β response in infected cells (23, 28, 34). In cells infected with wildtype (wt) CVB3 or mengovirus (a strain of EMCV that was used as a control), little IFN-β mRNA was detected, and only at very late stages of infection (i.e. at 9 & 12 hours post infection (h.p.i.)) (Fig. 1A). In contrast, infection with a mutant mengovirus, which has lost its IFN-suppressing activities due to substitutions in the Zn finger domain of Leader protein (Mengo-Zn) (22), already induced strong IFN-β mRNA upregulation at 6 h.p.i., and this further dramatically increased, and eventually reached plateau at 9 h.p.i. (Fig. 1A). This increased IFN-α/β response induced by mengo-Zn was not due to differences in virus replication as viral RNA accumulated with similar kinetics, and to similar levels as wt mengovirus and CVB3 (Fig. 1B).

To gain a first insight in how the viruses interfere with IFN-α/β induction pathways, we first asked whether the key transcription factor required for IFN-α/β transcription activation, namely IRF3, is activated in infected cells. HeLa R19 cells were infected with CVB3, and harvested at 6, 9 and 12 h.p.i.. Cell lysates were subjected to immunoblotting to determine IRF3 expression level, as well as the activation status of IRF3 as indicated by phosphorylation at serine 396 and dimerization of this transcription factor. We also performed parallel experiments with mengovirus or mengo-Zn as negative and positive controls, respectively. Infection with mengo-Zn, but not wt mengovirus, induced efficient IRF3 phosphorylation and dimerization (Fig. 1C). CVB3 infection did not induce any detectable IRF3 phosphorylation or dimerization (Fig. 1C), consistent with the lack of a significant IFN-β mRNA induction (Fig. 1A). The absence of IRF3
activation was not due to changes in IRF3 expression levels since this remained unchanged through CVB3 (and mengovirus) infection (Fig. 1C). Our data clearly indicate that IFN-α/β induction pathway is severely inhibited upstream of IRF3 phosphorylation during wt CVB3 infection.

It is well established that IRF3 phosphorylation relies on the activity of TBK1 (TANK-binding kinase 1), which is itself activated by phosphorylation at serine 172 (35). We therefore asked whether TBK1 expression and/or activation are affected in CVB3-infected cells. Cells were infected with CVB3, wt mengovirus and mengo-Zn for 9 hours and cell lysates were analyzed by immunoblotting using a polyclonal antibody against TBK1 and a monoclonal antibody against S172-phosphorylated TBK1. As shown in Fig. 1D, basal expression level of TBK1 did not change during infection with any of the three viruses. As expected, S172 phosphorylation of TBK1 was readily detectable in mengo-Zn-infected cells. Wt mengovirus infection also resulted in TBK1 phosphorylation, to the same extent as mengo-Zn, but this was significantly reduced in CVB3-infected cells (Fig. 1D). These results indicate that CVB3 targets the RLR pathway upstream of TBK1 phosphorylation, while mengovirus does so between TBK1 activation and IRF3 phosphorylation.

CVB3 induces MDA5, MAVS and RIG-I cleavage in infected cells.

To further investigate where CVB3 interferes with the RLR pathway, we examined the crucial factors involved in this signaling cascade by immunoblotting during a course of infection. From 9 h.p.i. onwards we observed a decrease in full-length MDA5 signal accompanied by the appearance of a smaller product of about 100 kDa (Fig. 2), suggesting that MDA5 is cleaved
during CVB3 infection. Also RIG-I and MAVS were cleaved from 9 h.p.i., but not TBK1 or LGP2, another member of the RLR family (Fig. 2). RIG-I cleavage was accompanied by the appearance of a band around 70 kDa, similar to the cleavage product observed in PV-infected cells (26). We also observed a putative cleavage product (< 37 kDa) of MAVS (Fig. 2), which is significantly smaller than the reported cleavage product (~ 50 kDa) produced by CVB3 3Cpro (23), suggesting an alternative MAVS cleavage. None of these factors were cleaved during infection of mengovirus or mengo-Zn (Fig. 2), in agreement with our observation that the RLR pathway upstream of TBK1 phosphorylation remains intact in cells infected with these viruses (Fig. 1).

**CVB3-induced MDA5, MAVS and RIG-I cleavages are independent of caspase or proteasome activities.**

We noticed that the cleavage of RIG-I, MDA5 and MAVS in CVB3-infected cells coincided with PARP cleavage resulting in an 89kDa cleavage product (Fig. 2), the hallmark of apoptosis. It has been suggested that poliovirus and enterovirus 71 induce caspase-dependent MDA5 degradation (24, 25). Therefore, we asked whether caspases could be responsible for cleaving MDA5, MAVS and/or RIG-I in CVB3-infected cells. To address this question, we chemically induced apoptosis in mock-infected cells using staurosporin (STS), a general kinase inhibitor. MDA5, MAVS and RIG-I were analyzed by immunoblotting in mock- or STS-treated cells.

STS induced potent caspase activation, as shown by PARP cleavage, as well as the appearance of the known caspase 3-mediated MAVS cleavage product of around 50 kDa (27). However, it did not lead to any detectable cleavage of MDA5, MAVS or RIG-I as observed in CVB3-infected cells (Fig. 3A), suggesting that caspases or the proteasome may not catalyze the
infection-induced cleavage events reported here. To further demonstrate that MDA5 is not cleaved by caspases, we infected cells with CVB3 in the presence of a broad-spectrum caspase inhibitor, Q-VD-OPh (QVD). We also included a proteasome inhibitor, MG132, in a parallel experiment because an efficient caspase activation, as the one we observed in CVB3 infected cells (Fig. 2), often leads to protein degradation via the proteasome system. QVD and MG132 both inhibited apoptosis as demonstrated by significantly decreased PARP cleavage in treated cells (Fig. 3A). Under these conditions, CVB3 infection still induced MDA5, MAVS and RIG-I cleavage to similar extent as in non-treated cells (Fig. 3A). Also in an independent experiment where apoptosis was completely inhibited by QVD, efficient MDA5 cleavage induced by CVB3 infection was observed (Fig. 3B). Together with our STS data, these results indicate that the cleavage events of MDA5, MAVS and RIG-I during CVB3 infection are not mediated by caspases or the proteasome.

Recombinant CVB3 proteinase 2A\textsuperscript{pro} induces MDA5 and MAVS cleavage, and 3C\textsuperscript{pro} induces RIG-I cleavage.

Next, we asked whether the two viral proteinases 2A\textsuperscript{pro} and 3C\textsuperscript{pro} could be responsible for the cleavage events we observed during CVB3 infection. Since these proteinases efficiently shut off of host mRNA translation by cleaving eIF4G (29, 36, 37), their overexpression results in poor expression levels and severe cytotoxicity. Therefore, we chose to address this question using recombinant 2A\textsuperscript{pro} and 3C\textsuperscript{pro}. Native cell lysate was treated with recombinant 2A\textsuperscript{pro} or 3C\textsuperscript{pro}, and subjected to immunoblotting analysis using antibodies against MDA5, MAVS and RIG-I. To control for the activities of 2A\textsuperscript{pro} and 3C\textsuperscript{pro}, we also probed for eIF4G, which is known to be cleaved by 2A\textsuperscript{pro} and 3C\textsuperscript{pro}-activated caspase 3 (29, 37, 38), and G3BP, which is cleaved by 3C\textsuperscript{pro}
only (31). As shown clearly in Fig. 4, both proteinases were very active in our in vitro cleavage assay. Under these conditions, MDA5 and MAVS cleavages were observed in 2A\textsuperscript{pro}-treated samples, whereas RIG-I was cleaved in 3C\textsuperscript{pro}-treated lysate (Fig. 4). The cleavage products seen in the in vitro cleavage assay showed identical electrophoretic mobility as those from CVB3-infected cells (Fig. 4), demonstrating the relevance of our in vitro findings. In 3C\textsuperscript{pro}-treated samples, a putative MDA5 cleavage product of approximately 100 kDa was detected; however, this protein fragment was not consistently detected in in vitro cleavage assays with 3C\textsuperscript{pro}. More importantly, we never observed this cleavage product in CVB3-infected cells. These results suggest that the CVB3-induced cleavage of MDA5 and MAVS resulted from 2A\textsuperscript{pro} activity, whereas that of RIG-I from 3C\textsuperscript{pro}.

As mentioned before, it has been reported that CVB3 3C\textsuperscript{pro} cleaves MAVS, resulting in a N-terminal cleavage product of 50 kDa, and a single amino acid substitution at position 148 (Q148A) was sufficient to prevent this cleavage (23). We also observed a 3C\textsuperscript{pro}-induced cleavage product of approximately 50 kDa when we prepared lysates from cells overexpressing a FLAG- and HA-tagged MAVS protein (FLAG-MAVS-HA) (Fig. 5A). This cleavage product was also detectable using an antibody against the N terminal FLAG tag, but not the C-terminal HA tag, confirming that it is an N-terminal fragment of MAVS. When the reported cleavage-resistant mutant (Q148A) was overexpressed, the 3C\textsuperscript{pro}-mediated cleavage was no longer detectable (Fig. 5B), indicating that it is likely the same cleavage product reported by Mukherjee et al. However, we did not observe this cleavage fragment from endogenous MAVS during CVB3 infection (Fig. 2-4), even when using the same antibody as in the published study (data not shown).
The 2A<sup>pro</sup>-mediated cleavage was readily detectable from both overexpressed (Fig. 5) as well as endogenous MAVS (Fig. 2-4), releasing two cleavage products (approximately 30 and 40 kDa) (Fig. 5). Using antibodies against tags at either terminus of overexpressed MAVS, we saw that the smaller cleavage product represented an N-terminal fragment whereas the larger product is a C-terminal fragment (Fig. 5). These data suggest that MAVS is primarily cleaved by 2A<sup>pro</sup> in CVB3-infected cells.

**2A<sup>pro</sup> mediates cleavage of MDA5 and MAVS while 3C<sup>pro</sup> cleaves RIG-I in infected cells.**

We further pursued experiments to confirm these results in the context of an infection. As 2A<sup>pro</sup> and 3C<sup>pro</sup> are both essential for CVB3 polyprotein processing, deletion mutants of these proteinases are lethal. Instead, we took advantage of the fact that wt mengovirus does not induce cleavage of RIG-I, MDA5 or MAVS (Fig. 2). By inserting CVB3 2A<sup>pro</sup> or 3C<sup>pro</sup> in front of the Leader-coding region (i.e. at the extreme 5' terminus of the polyprotein-coding region) in mengovirus genome, we could study the role of the CVB3 proteinases in the cleavage events during a normal picornavirus infection. These viruses, named M-2A(CVB3) and M-3C(CVB3), were used to infect cells, and RIG-I, MDA5 and MAVS cleavages were examined by immunoblotting. As shown before, wt mengovirus infection did not induce any changes in the integrity of these factors, whereas M-2A(CVB3) induced MDA5 and MAVS cleavage, and M-3C(CVB3) induced RIG-I cleavage (Fig. 6A). The cleavage products observed in M-2A(CVB3)- and M-3C(CVB3)-infected cells were exactly the same as seen in CVB3-infected cells (Fig. 6A). We observed an additional band around 90 kDa with the anti-RIG-I antibody in M-2A(CVB3)-infected cells (Fig. 6A). We never observed a cleavage product of this electrophoretic mobility in wt CVB3-infected cells, and this 90 kDa band was also absent in M-2A(CVB3)-infected cells in...
other experiments (Fig. 6B). As additional controls to confirm that the cleavages were results of the proteinase activities of 2A\textsuperscript{pro} and 3C\textsuperscript{pro}, we also produced mutant mengoviruses carrying the catalytically inactive forms of 2A\textsuperscript{pro} (2A-C109A) and 3C\textsuperscript{pro} (3C-C147A). Infection with these viruses did not lead to cleavage of targets of 2A\textsuperscript{pro} or 3C\textsuperscript{pro} (Fig. 6B). These results, combined with our \textit{in vitro} cleavage data, convincingly show that CVB3 induces MDA5 and MAVS cleavage via 2A\textsuperscript{pro} activity and RIG-I via 3C\textsuperscript{pro}.

\textbf{Other enteroviruses also target MDA5 and MAVS by their 2A\textsuperscript{pro}, and RIG-I by 3C\textsuperscript{pro}}

We further asked whether the cleavage events we observed here were unique to CVB3, a Human Enterovirus B (HEV B) member, or common to other enterovirus species as well. To this end, we produced mengoviruses carrying the 2A\textsuperscript{pro} or 3C\textsuperscript{pro} from enterovirus 71 (EV71) and poliovirus (PV), belonging to species HEV A and C, respectively. Infections with these mutant viruses revealed that the 2A\textsuperscript{pro} and 3C\textsuperscript{pro} from both viruses induced MDA5 and RIG-I cleavage, respectively (Fig. 7A). The cleavage products found with these viruses were identical to the corresponding cleavage products observed in CVB3-infected cells (Fig. 6A). M-2A(EV71) and M-2A(PV) infections also induced MAVS cleavage, though, M-2A(PV) resulted in a less prominent MAVS cleavage and a slightly different MAVS cleavage product pattern than that of CVB3. The larger cleavage product (~ 40 kDa) seems identical to that from M-2A(CVB3)- and M-2A(EV71)-infected cells, but the smaller product (~ 35 kDa) had a slower electrophoretic mobility than that from CVB3-infected cells (~ 30 kDa) (Fig. 7A).

To investigate whether these cleavages also occur during a normal infection of enteroviruses, we infected cells with EV71 (strain BrCr), and analyzed MDA5, MAVS and RIG-I by...
imunoblotting. Infection of EV71 induced similar cleavages of all three factors as seen in
CVB3-infected cells (Fig. 7B). Together with data from the M-2A viruses, our results suggest
that cleavage of RIG-I, MDA5 and MAVS may be a common phenomenon among enteroviruses.
DISCUSSION

The RLR signaling pathway is a crucial part of the innate antiviral response. Consequently, this pathway is targeted by numerous viruses from various families, including picornaviruses. However, our knowledge on how picornaviruses interfere with this pathway remains somewhat fragmented, since the reported studies each used a different panel of viruses, and focused on only one factor of the pathway at a time. Here we performed the first comprehensive analysis of key factors of the RLR signaling pathway during enterovirus infections. Our data suggest that enteroviruses utilize their 2A\textsuperscript{pro} to target MDA5 and MAVS, and 3C\textsuperscript{pro} to cleave RIG-I.

Consistent with these results, several studies have reported that IRF3 activation is severely inhibited during enterovirus infection (Fig. 1C, (23, 25, 27)). Thus, cleavage of upstream factors of the RLR pathway is likely an evolutionarily conserved and advantageous mechanism to suppress IFN-I gene transcription. Meanwhile, it is worth noting that RLR pathway antagonization may not be the only evasion mechanism of enteroviruses. It is known that enteroviruses induce a shutoff of host mRNA transcription and translation, which likely further limits the production of, among others, virus-induced cytokines such as IFNs. To what extent the host shutoff contributes to suppressing IFN production is difficult to investigate since enterovirus-induced host shutoff is also mediated by 2A\textsuperscript{pro} and 3C\textsuperscript{pro} (38–40) – the same proteases that cleave factors of the RLR-mediated IFN induction pathway, making it difficult to separately investigate the role of these phenomena.

MDA5, the receptor responsible for sensing picornavirus infection (16, 21), is cleaved during infection of CVB3 and EV71. Using recombinant CVB3 2A\textsuperscript{pro} and our M-2A viruses carrying
2A^pro of CVB3, EV71 or PV, we demonstrate that this cleavage can be mediated by all these 2A^pro, indicating that this may be a common strategy of enteroviruses to inactivate MDA5. This seems to contradict previous reports that PV and EV71 induce caspase- (and in the case of PV also proteasome-) mediated MDA5 degradation. However, in those studies cells were first transfected with either poly(I:C) (24) or viral RNA (25) in order to upregulate MDA5 expression to a detectable level before infections were carried out. Pretreatments with these triggers induce IFN-α/β production, which, in turn, upregulate expression of interferon stimulated genes including MDA5. It is important to realize that IFN-α/β itself is known to potentiate cells to virus-induced apoptosis (41). Therefore, it is likely that in IFN-α/β-primed virus-infected cells, MDA5 is degraded in a caspase-dependent manner. In this study, we investigated the fate of endogenous MDA5 during enterovirus infection of naïve cells. We show convincingly that MDA5 cleavage during infection is not attributed to caspases or the proteasome, but the viral proteinase 2A^pro.

We also set out to identify the 2A^pro cleavage site in MDA5 by infecting cells overexpressing MDA5 mutants carrying substitutions at potential 2A^pro recognition sites. However, infection following protein overexpression proved to be very inefficient in our hands (i.e. viruses preferably infect non-transfected cells) (data not shown). Another approach to test potential cleavage-resistant MDA5 mutants is to use lysates from cells overexpressing tagged MDA5 mutants in our in vitro cleavage assay. However, under conditions where we did observe cleavage of endogenous MDA5 or MAVS or overexpressed MAVS (e.g. the Q148A mutant), we did not find any cleavage product of the overexpressed MDA5 (as detected by antibodies against terminal epitope tags) (data not shown). In addition, no cleavage of recombinant MDA5 purified
from mammalian cells was observed (data not shown). It eludes us why overexpressed MDA5 is so poorly cleaved by recombinant 2Apro, and unfortunately, it did not allow us to further investigate and identify the 2Apro cleavage site in MDA5.

In addition to MDA5, the downstream adaptor molecule MAVS is also cleaved in a 2Apro-dependent manner during enterovirus infection. CVB3 and EV71 induced identical MAVS cleavage products. These products are also reasonably similar to what was previously observed when in vitro translated MAVS was treated with CVB3 2Apro (27), further supporting our conclusion that this is a 2Apro-mediated cleavage. PV 2Apro also led to MAVS cleavage, suggesting a similar cleavage mechanism, though the cleavage products were slightly different from those induced by CVB3. While this paper was in preparation, it was shown in another study that EV71 2Apro cleaves MAVS at three distinct positions, namely Q209, Q251 and Q265, leading to the production of two cleavage products of 30–40 kDa (28). We show here that both CVB3 and EV71 induce identical MAVS cleavage patterns, yielding two cleavage products similar in size as reported in that study, leading us to conclude that CVB3 2Apro likely also cleaves MAVS at these positions. Furthermore, 2Apro of PV also targeted MAVS, though the size of one of the cleavage products was slightly different from that observed in CVB3- or EV71-infected cells. It is not at odds that 2Apro from different enteroviruses cleave the same target protein at various positions. It has been previously shown that 2Apro of human rhinoviruses cleave nucleoporins at different sites, most likely due to sequence diversity in 2Apro (42). Moreover, caspase-mediated MAVS cleavage has been suggested for HRV1A (27). We did not observe the caspase-mediate MAVS cleavage product during CVB3 infection in HeLa R19 cells or other human cell lines such as HeLa Kyoto and Huh7 (data not shown). Together, our data
show that 2A<sub>pro</sub> from three different enterovirus species all target not only MDA5, but also MAVS, during infection. The evolutionary conservation of these activities suggests that this is probably highly advantageous for enterovirus replication.

Although RIG-I is dispensable in sensing picornaviruses (16), it has been reported to be cleaved during infection of a few enteroviruses such as PV, echorvirus, and HRV 16, mostly likely via their 3C<sub>pro</sub>. Here we provide data that two additional enteroviruses, namely CVB3 and EV71, also cleave RIG-I via their 3C<sub>pro</sub>. Unlike the varying 2A<sub>pro</sub> cleavage sites in MAVS, the yet unidentified cleavage site in RIG-I by 3C<sub>pro</sub> seems to be well conserved across this panel of different enteroviruses, each yielding a putative cleavage product of approximately 70 kDa (26).

Cleavage of RIG-I, MDA5 and MAVS does not appear to be common to all picornaviruses. In this study, we also examined the fate of factors involved in the RLR pathway during mengovirus infection, a species of EMCV of the Cardiovirus genus. We show that MDA5, MAVS, RIG-I, LGP2 and TBK1 all remained intact in cells infected with either wt mengovirus, which effectively suppresses IFN-α/β response, or mengo-Zn, which induces high levels of IFN-α/β. Furthermore, equal levels of TBK1 activation were observed in cells infected with both wt mengovirus and mengo-Zn, indicating that factors upstream of TBK1 are not targeted by mengovirus. These results may seem to contradict earlier reports that MDA5 and RIG-I are also cleaved in mengovirus-infected cells (24). However, MDA5 cleavage was only observed in cells pretreated with poly(I:C) (24), which, as mentioned before, potentiates cells for virus-induced apoptosis, and likely results in caspase-mediated MDA5 cleavage. In agreement with our observation that MDA5 is not cleaved in mengovirus-infected cells, MDA5 also remained intact.
during EMCV infection without poly(I:C) pretreatment in an independent study (43). Two reports have shown that RIG-I, which is dispensable for EMCV RNA recognition (16), is targeted by EMCV. Barral et al. reported that RIG-I is cleaved in EMCV-infected cells, and implicated 3Cpro as the responsible proteinase since the cleavage product (of approximately 70 kDa) was similar to the cleavage product released by 3Cpro of PV (26). Another study showed a gradual decrease of RIG-I during EMCV infection, which could be prevented by a caspase inhibitor, indicating that this is caspase-mediated degradation of RIG-I (43). These authors also demonstrated that recombinant EMCV 3Cpro can cleave RIG-I in vitro, resulting in a putative cleavage product of approximately 50 kDa. However, this RIG-I fragment was not observed during a normal EMCV infection (43), suggesting that it may be an artifact of in vitro study, similar to the MDA5 cleavage we observed with CVB3 3Cpro in our own in vitro cleavage experiments.

The exact mechanism of EMCV to suppress IFN-α/β response remains to be clarified. As indicated above, this blockade likely lies between TBK1 activation and IRF3 phosphorylation. It is known that TBK1 must form a complex with other kinases such as the non-canonical IkB kinase IKK-epsilon in order to phosphorylate its substrate IRF3 (44–46). The assembly of a functional TBK1 complex requires not only phosphorylation of TBK1 at serine 172, but also other post translational modifications of IKK-epsilon and the assistance of scaffold proteins such as NAP1 and SINTBAD (47). It is possible that mengovirus interferes with the correct assembly of the TBK1 complex to terminate the signal transduction. Alternatively, IFN-α/β shutdown may be a secondary effect of the so-called nuclear-cytoplasmic trafficking disorder that is induced by mengovirus Leader protein (48). This causes an unregulated cargo transport between nucleus and...
cytoplasm, and possibly interferes with IRF3 activation since this transcription factor must 
shuttle between these two compartments. Intriguingly, the same mutations in Leader (e.g. those 
in the Zn finger domain) simultaneously inactivate its activities in both IFN-α/β suppression (16, 
22, 34) as well as the induction of nuclear-cytoplasmic trafficking disorder (48), suggesting that 
there may be a link between these two phenomena. Future research is called upon to investigate 
the exact mechanism of IFN antagonization by mengovirus.

In short, our data show that several enteroviruses all target MDA5, MAVS and RIG-I in infected 
cells, and all via the same mechanisms (i.e. 2Apro targeting MDA5 and MAVS, and 3Cpro RIG-I). 
These cleavage events likely account for the lack of IFN-α/β response in enterovirus-infected 
cells. Our data that TBK1 phosphorylations, and thus activation, is inhibited in CVB3-infected 
cells indicates that the pathway is shut down upstream of TBK1. The fact that the RLR pathway 
is targeted at multiple steps during infection makes it technically challenging to demonstrate the 
biological consequence of these cleavage events. One would have to simultaneously reconstitute 
minimally three factors, MDA5, MAVS and RIG-I, to hopefully study the effect of an intact 
RLR signaling pathway on virus replication. Additionally, enteroviruses may also employ 
additional mechanisms to ensure effective IFN-α/β suppression. For instance, enterovirus 2Apro is 
also known to cause nuclear-cytoplasmic trafficking disorder (49), possibly interfering with IRF3 
activation, and thereby, further assure a total shut off of IFN-α/β response in infected cells. 
Although a clear contribution of these phenomena to IFN-α/β inhibition remains to be 
established, this study provides important new insights in potential roles of enterovirus 
proteinases in suppressing the RLR-mediated antiviral pathway.
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REFERENCES


FIGURE LEGENDS

Figure 1. CVB3 antagonizes RLR pathway upstream of TBK1, while mengovirus does so downstream of TBK1 activation. HeLa R19 cells were mock-infected or infected with CVB3, mengovirus (mengo) or mengo-Zn (M-Zn) at MOI of 50, and cells were harvested at indicated hours post infection (h.p.i.). Total RNA was isolated and subjected to RT-qPCR analysis for IFN-β mRNA (A) and viral RNA (vRNA) (B). Data were first normalized against actin mRNA, then calculated as fold of induction as compared to mock-infected sample, and presented as mean ± SD. (C) Infection was carried out as in A, and cells were lysed at indicated times p.i.. Lysates were then subjected to SDS-PAGE followed by immunoblotting using indicated antibodies. IRF3 dimerization analysis was carried out under native conditions. (D) Infection was carried out as in A, and cells were lysed at 9 h.p.i.. Cell lysates were analyzed by SDS-PAGE followed by immunoblotting using indicated antibodies. Data representative of at least 3 independent experiments.

Figure 2. Fate of multiple factors of the RLR signaling pathway during infection. HeLa R19 cells were mock-infected or infected with CVB3, mengovirus (mengo) or mengo-Zn (M-Zn) at MOI of 50, and cells were lysed at indicated hours post infection (h.p.i.). Cell lysates were analyzed by SDS-PAGE followed by immunoblotting using indicated antibodies. Marker bands of indicated size in kDa are indicated on the right side. Open arrow, full-length protein. Filled arrowhead, putative cleavage products. Filled arrow, known MAVS cleavage product from caspase-mediated cleavage. Data representative of at least 3 independent experiments.
Figure 3. Caspases and proteasomal proteases are not responsible for CVB3-induced MDA5, MAVS or RIG-I cleavage. (A) HeLa R19 cells were mock-infected, infected with CVB3 at MOI of 50 for 9 hours, or treated with 2 μM staurosporine (STS) for 6 hours, in the presence or absence of 10 μM QVD or 10 μM MG132. Cells were lysed and the indicated proteins were analyzed by SDS-PAGE followed by immunoblotting. Open arrow, full-length protein. Filled arrowhead, putative cleavage products. Filled arrow, known MAVS cleavage product from caspase-mediated cleavage. (B) HeLa R19 cells were mock-treated, infected with CVB3 at MOI of 50 for 9 hours, or treated with 2 μM STS for 6 hours, in the presence or absence of 10 μM QVD or 10 μM MG132. Cells were lysed and MDA5, PARP and actin were analyzed by SDS-PAGE followed by immunoblotting. Open arrow, full-length protein. Filled arrowhead, putative cleavage products. Filled arrow, additional PARP-derived fragment that is observed in CVB3-infected, but not STS-treated cells, which persists throughout CVB3 infection. Data representative of at least 5 experiments.

Figure 4. Recombinant CVB3 2Apro cleaves MDA5 and MAVS while 3Cpro cleaves RIG-I. Native lysate of HeLa R19 cells was treated with recombinant CVB3 2Apro or 3Cpro at room temperature overnight. The reaction mixtures were subjected to SDS-PAGE followed by immunoblotting using antibodies against indicated proteins. As controls, lysates from mock- and CVB3-infected (MOI 50, 8 hrs) cells were also included. Open arrow, full-length protein. Filled arrowhead, putative cleavage products. Filled diamond, MDA5 cleavage product by 3Cpro that is not seen in CVB3-infected cells. Data representative of at least 3 experiments.
Figure 5. Both 2A_pro and 3C_pro of CVB3 can cleave MAVS, but at different sites. Plasmids encoding for FLAG-MAVS-HA (A) or FLAG-MAVS Q148A-HA (B) were transfected into cells, and lysed 24 hours later under native conditions. The native lysates were treated with recombinant 2A_pro or 3C_pro at room temperature overnight, and analyzed by SDS-PAGE and immunoblotting using the indicated antibodies. Open arrow, full-length protein. Filled arrowhead, putative cleavage product. Data representative of at least 2 experiments.

Figure 6. MDA5, MAVS and RIG-I cleavage in the context of an infection. (A) HeLa R19 cells were mock-infected or infected with CVB3, mengovirus expressing CVB3 2A_pro or 3C_pro (M-2A and M-3C, respectively), or wt mengovirus at MOI of 50 for 8 hours. Cells were lysed and the indicated proteins were analyzed by SDS-PAGE followed by immunoblotting. Open arrow, full-length protein. Filled arrowhead, putative cleavage products. Filled diamond, RIG-I cleavage product by 2A_pro that is not seen in CVB3-infected cells. (B) HeLa R19 cells were mock-infected or infected with CVB3, M-2A(CVB3), M-3C(CVB3), or M-2A-C109A(CVB3), or M-3C-C147A(CVB3) at MOI of 50 for 8 hours. Cells were lysed and the indicated proteins were analyzed by SDS-PAGE followed by immunoblotting. Open arrow, full-length protein. Filled arrowhead, putative cleavage products. Data representative of at least 3 experiments.

Figure 7. MDA5, MAVS and RIG-I cleavage by other enteroviruses. (A) HeLa R19 cells were mock-infected or infected with CVB3, M-2A(CVB3), M-2A(EV71), M-2A(PV), M-3C(CVB3), M-3C(EV71) or M-3C(PV) at MOI of 50 for 8 hours. Cell lysates were analyzed by SDS-PAGE followed by immunoblotting using antibodies against the indicated proteins. (B) HeLa R19 cells were mock-infected or infected with CVB3 or EV71 at MOI of 50 for 8 hours.
Cells lysates were analyzed as in A. Open arrow, full-length protein. Filled arrowhead, putative cleavage product. Data representative of at least 2 experiments.
Fig. 1
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Fig. 7