Roles of RIG-I N-terminal tandem CARD and splice variant in TRIM25-mediated antiviral signal transduction

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The caspase recruitment domain (CARD) of intracellular adaptors and sensors plays a critical role in the assembly of signaling complexes involved in innate host defense against pathogens and in the regulation of inflammatory responses. The cytosolic receptor retinoic acid-inducible gene-1 (RIG-I) recognizes viral RNA in a 5′-triphosphate-dependent manner and initiates an antiviral signaling cascade. Upon viral infection, the N-terminal CARDs of RIG-I undergo the K48-linked ubiquitination induced by tripartite motif protein 25 (TRIM25), critical for the interaction of RIG-I with its downstream signaling partner MAVS/VISA/IPS-1/Cardif. Here, we demonstrate the distinct roles of RIG-I first and second CARD in TRIM25-mediated RIG-I ubiquitination: TRIM25 binds the RIG-I first CARD and subsequently ubiquitinates its second CARD. The T55I mutation in RIG-I first CARD abolishes TRIM25 interaction, whereas the K177R mutation in the second CARD eliminates polyubiquitin attachment. The necessity of the intact tandem CARD for RIG-I function is further evidenced by a RIG-I splice variant (SV) whose expression is robustly up-regulated upon viral infection. The RIG-I SV carries a short deletion (amino acids 36–80) within the first CARD and thereby loses TRIM25 binding, CARD ubiquitination, and downstream signaling ability. Furthermore, because of its robust inhibition of virus-induced RIG-I multimerization and RIG-I-MAVS signaling complex formation, this SV efficiently suppresses the RIG-I-mediated IFN-β production. This study not only elucidates the vital role of the intact tandem CARD for TRIM25-mediated RIG-I activation but also identifies the RIG-I SV as an off-switch regulator of its own signaling pathway.

alternative splicing | innate immunity | interferon

The caspase recruitment domain (CARD)-containing intracellular sensors, RIG-I, melanoma differentiation-associated gene 5 (MDA5), and nucleotide-binding and oligomerization domain (NOD), play important roles in the detection of conserved molecules to ultimately initiate a signaling cascade, resulting in the production of IFN-α/β and inflammatory cytokines to limit viral or bacterial proliferation (9, 10).

RIG-I and MDA5 consist of two N-terminal CARDs, a central DECH box ATPase domain, and a C-terminal regulatory/repressor domain (RD) (11, 12). Whereas the C-terminal RD of RIG-I binds viral RNA in a 5′-triphosphate-dependent manner and activates the central ATPase by RNA-dependent dimerization (13, 14), the CARDs of RIG-I trigger the interaction with its downstream partner MAVS/VISA/IPS-1/Cardif (15–18). The crucial role of the tandem CARD for RIG-I downstream signaling is further evidenced by the finding that the hepatocyte cell line HuH7.5, which carries the T55I mutation in the RIG-I first CARD, strongly supports hepatitis C virus (HCV) replication (19). The T55I mutation disrupts the signaling function of the RIG-I CARDs to induce antiviral IFN production, leading to a high permissiveness to HCV replication. Furthermore, we have recently shown that the CARDs of RIG-I interact with the C-terminal SPRY domain of tripartite motif 25 (TRIM25) E3 ligase, and this interaction effectively delivers the K63-linked ubiquitin moieties to the RIG-I second CARD, resulting in a marked increase of RIG-I downstream signaling activity (20). RIG-I Lys-172 (K172) is critical for TRIM25-mediated ubiquitination and MAVS/VISA/IPS-1/Cardif binding, as well as the ability of RIG-I to induce antiviral signal transduction.

Unbalanced, continuous production of IFNs and inflammatory cytokines could lead to deleterious effects on host immunity. To tightly regulate the on/off switch of RIG-I-mediated innate immunity, RIG-I activity is negatively regulated by several mechanisms, including K48-linked ubiquitination leading to RIG-I degradation (21) and the LGP2 helicase protein, which lacks the N-terminal CARDs (22, 23). In addition, alternative splicing has been identified as an important cellular regulatory mechanism in fine-tuning host IFN signaling activity. For instance, the alternatively spliced variants of NOD2 and Mdx8 function as dominant-negative inhibitors of NOD2 and TLR-induced signal transduction, respectively (24–26).

Here, we describe the distinct roles of the CARDs of RIG-I for TRIM25-binding and TRIM25-mediated ubiquitination and identify an alternatively spliced variant of RIG-I as a potential feedback inhibitor of its signal transduction, thereby unveiling the intricate regulation of RIG-I-mediated antiviral innate immunity.

Results

Distinct Roles of the RIG-I First and Second CARD in TRIM25-Mediated RIG-I Ubiquitination. To define the functions of the RIG-I first and second CARD in TRIM25-RIG-I-complex formation and TRIM25-mediated RIG-I activation, GST-RIG-I first CARD, GST-RIG-I second CARD, and GST-RIG-I 2CARD mammalian


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fusion constructs were tested for TRIM25 binding. GST-pulldown indicated that GST-RIG-I first CARD and GST-RIG-I 2CARD strongly bound full-length TRIM25 and TRIM25-SPRY, whereas GST and GST-RIG-I second CARD showed no interactions (Fig. 1A and B). Regardless of TRIM25 binding, however, neither GST-RIG-I first CARD nor GST-RIG-I second CARD showed any detectable level of ubiquitination and MAVS-CARD-proline-rich-domain (PRD) interaction, whereas GST-RIG-I 2CARD underwent robust ubiquitination and efficiently interacted with MAVS-CARD-PRD under the same conditions [Fig. 1C and supporting information (SI) Fig. S1A]. In correlation with their lack of ubiquitination and MAVS binding, each CARD of RIG-I was incapable of activating the IFN-β and NF-κB promoters (Fig. 1D and Fig. S1B). These results indicate that, whereas the first CARD is responsible for TRIM25 binding, the intact tandem CARD of RIG-I is necessary for TRIM25-mediated ubiquitination, ultimately allowing efficient MAVS interaction and downstream signal transduction.

**Threonine 55 Residue of the RIG-I First CARD Is Critical for TRIM25 Binding.** The T55I mutation of RIG-I first CARD abolishes RIG-I-mediated antiviral activity, leading to a high permissiveness to HCV replication (19). However, the molecular mechanism by which the T55I mutation abrogates RIG-I-mediated antiviral signal transduction has not yet been illustrated. We found that the RIG-I T55I mutation abolished its TRIM25 binding ability, leading to the loss of TRIM25-induced RIG-I ubiquitination (Fig. 2A and B and Fig. S2A). Consistently, ectopic expression of TRIM25 efficiently enhanced the activity of GST-RIG-I 2CARD WT but not GST-RIG-I 2CARD T55I in inducing IFN-β promoter activation (Fig. 2C). To examine the capability of RIG-I 2CARD T55I for interacting with MAVS, several previously described (20) GST-RIG-I 2CARD mutants were included in this assay: GST-RIG-I 2CARD K172R, in which the main ubiquitination site is mutated, GST-RIG-I 2CARD K99/169/172/181/190/193R, in which all six ubiquitination sites are mutated, and GST-RIG-I 2CARD K172only containing five K → R substitutions while leaving K172 intact (Fig. 2D). GST-RIG-I 2CARD T55I, K172R, and K99/169/172/181/190/193R mutants that did not undergo ubiquitination bound poorly to MAVS-CARD-PRD, whereas GST-RIG-I 2CARD WT and K172only, which were heavily ubiquitinated, interacted strongly with MAVS-CARD-PRD (Fig. 2D). This indicates that TRIM25-mediated ubiquitination of RIG-I is necessary for efficient interaction with MAVS. Indeed, MAVS-CARD-PRD displayed a higher binding affinity to the ubiquitinated form of the RIG-I 2CARD than the unmodified form (Fig. S2B). Moreover, shRNA-mediated TRIM25 knockdown markedly suppressed the interaction between RIG-I 2CARD and MAVS-CARD-PRD (Fig. S2C). These results demonstrate that the T55 residue of the RIG-I first CARD is critical for TRIM25 binding and subsequently TRIM25-mediated ubiquitination, which is ultimately necessary for efficient RIG-I-MAVS interaction.

Because the T55 or S55 residue of RIG-I is highly conserved among various species, the potential phosphorylation of this residue might trigger TRIM25 interaction. To test this hypothesis, we introduced a series of point mutations in place of T55: T55E to mimic constitutive phosphorylation and T55I, T55A, and T55Q to mimic nonphosphorylation. The GST-RIG-I 2CARD mutants were then tested for TRIM25 binding, ubiquitination, and downstream signaling activity (Fig. 2E and F). As shown in Fig. S2E, GST-RIG-I 2CARD T55Q and T55E exhibited an apparent reduction of TRIM25 binding and ubiquitination compared with GST-RIG-I 2CARD WT, whereas GST-RIG-I 2CARD T55A showed a similar extent of TRIM25 binding and ubiquitination compared with GST-RIG-I 2CARD WT. Furthermore, mass spectrometry analysis of purified GST-RIG-I 2CARD WT in the presence of phosphatase inhibitors showed no detectable phosphorylation or other known modifications at the T55 residue (data not shown). In addition, the levels of TRIM25 binding and ubiquitination of these mutants directly correlated with their ability to induce IFN-β and NF-κB promoter activation (Fig. 2F).

Given that mutation of the RIG-I T55 residue to the hydrophobic amino acid isoleucine abolished TRIM25 interaction, CARD ubiqui-
detected in IFN-CARDs sequence (735 bp), a band with a smaller size (603 bp) was to the band corresponding to the expected size for the RIG-I deletion of exon 2 (Fig. 3 identified the alternatively spliced variant of RIG-I carrying the 36–80 of RIG-I. Additional RT-PCR analysis using primers to amplified the RIG-I CARDs (exons 1–3) (Fig. 3 and S4). These results collectively indicate that the lack of antiviral activity of the RIG-I T55I mutant is due to the loss of its ability to bind TRIM25.

Identification of RIG-I SV. Given that the RIG-I first and second CARD are essential for TRIM25 binding and TRIM25-mediated ubiquitination and downstream signaling, respectively, we postulated that alternative splicing in the N-terminal tandem CARD of RIG-I might affect its ubiquitination-dependent signaling function. To test this hypothesis, we isolated total RNAs from mock- or IFN-β-treated HEK293T cells and performed RT-PCR using primers that specifically amplified the RIG-I CARDs (exons 1–3) (Fig. 3B Upper). In addition to the band corresponding to the expected size for the RIG-I CARDs sequence (735 bp), a band with a smaller size (603 bp) was detected in IFN-β-treated, but not in mock-treated HEK293T cells (Fig. 3B). Cloning and sequence analysis of the smaller amplicon identified a RIG-I SV that lacked the exon 2, coding for amino acids 36–80 of RIG-I. Additional RT-PCR analysis using primers to specifically amplify the complete ORF of RIG-I (exons 1–18) also identified the alternatively spliced variant of RIG-I carrying the deletion of exon 2 (Fig. 3A).

We further tested the expression patterns of full-length RIG-I and the SV in mock-treated versus IFN-β-treated or Sendai virus (SeV)-infected HEK293T cells, using primers specific for full-length RIG-I or the SV (Fig. 3B and Fig. S4A). Whereas a low level of full-length RIG-I mRNA was detected in mock-treated cells, the RIG-I SV was undetectable under the same conditions (Fig. 3B and Fig. S4A). Furthermore, the transcript levels of both full-length RIG-I and the SV significantly increased upon IFN-β treatment or SeV infection. Additionally, the RIG-I SV was detectable in a number of cell lines upon IFN-β treatment, including lymphatic endothelial cells (LECs), HeLa, HCT116, Huh7, LnCap, and NHLF lung fibroblast cells, but not after treatment with all-trans retinoic acid (Figs. S4B and S4A), indicating an IFN-stimulation-specific expression of the RIG-I SV. In line with this, the RIG-I SV was readily detectable in 2TGH WT human fibroblasts but not in STAT1-deficient (U3A) or STAT2-deficient (U6A) cells upon treatment with IFN-α or IFN-β (Fig. S5B). Furthermore, a monoclinal RIG-I antibody that reacted with the central helicase domain readily detected a 100-kDa band in addition to the 116-kDa band corresponding to full-length RIG-I in IFN-β-treated or SeV-infected HEK293T cells but not in mock-treated cells (Fig. 3B and Fig. S4A). This 100-kDa band comigrated with exogenously expressed Flag-tagged RIG-I SV (Fig. S4A). Furthermore, a RIG-I antibody generated by the peptide containing residues 37–55 detected the 116-kDa full-length RIG-I but not the 100-kDa SV (Fig. S4C). These results indicate that RIG-I undergoes alternative splicing upon IFN stimulation, resulting in an isoform that lacks the short sequence of the first CARD containing the T55 residue.

Lack of TRIM25 Binding, Ubiquitination, and Signaling Activity of RIG-I SV. To study the potential role of the RIG-I SV in antiviral signal transduction, GST-RIG-I 2CARD SV was tested for a series of biochemical activities: TRIM25 binding, ubiquitination, MAVS binding, and downstream signal transducing ability. Like the GST-RIG-I 2CARD T55I mutant (Fig. 2), GST-RIG-I 2CARD SV was unable to bind TRIM25 full-length and TRIM25-SPRY at detect-
able levels and did not undergo TRIM25-mediated ubiquitination (Fig. 3C and Fig. S6A). Consistently, full-length RIG-I SV showed no detectable TRIM25 binding and extremely low K73-linked ubiquitination (Fig. S6 B and C). Furthermore, whereas GST-RIG-I-2CARD T83I, K127R, or K9/169/172/318/193/194(R) mutants bound poorly to MAVS-CARD-PRD compared with GST-RIG-I-2CARD WT, GST-RIG-I-2CARD SV showed no interaction with MAVS-CARD-PRD (Fig. 3D). Finally, GST-RIG-I-2CARD SV was incapable of inducing IFN-β or NF-κB promoter activation in the presence or absence of exogenous TRIM25 (Fig. 3E and Fig. S6D).

To further test the antiviral activity of the RIG-I SV upon viral infection, we tested the replication of enhanced-GFP-containing vesicular stomatitis virus (VSV-eGFP) in RIG-I−/− mouse embryonic fibroblasts (MEFs) expressing vector, RIG-I WT, RIG-I SV, or RIG-I T55I mutant. RIG-I WT expression drastically suppressed VSV-eGFP replication: VSV-eGFP titer was ~200-fold lower in RIG-I WT-expressing cells than in vector-containing cells (Fig. 3F). In contrast, MEFs expressing the RIG-I SV or RIG-I T55I mutant had similar viral titters to MEFs expressing vector only (Fig. 3F). Thus, the RIG-I SV, lacking a critical part of the CARD, loses TRIM25 binding, which subsequently abolishes TRIM25-mediated ubiquitination and MAVS binding and, thereby, antiviral activity.

RIG-I SV Acts as a Dominant Inhibitor of RIG-I-Mediated Antiviral IFN Response. To explore the effects of the RIG-I SV on RIG-I signal transduction, HEK293T cells were transfected with IFN-β or NF-κB promoter luciferase together with increasing amounts of Flag-RIG-I SV, followed by SeV infection (Fig. 4A and Fig. S7).

RIG-I T55I mutant was included as a control. Although both RIG-I SV and RIG-I T55I mutant markedly suppressed the SeV-induced IFN-β or NF-κB promoter activation, the level of suppression induced by RIG-I SV was markedly stronger than that induced by RIG-I T55I mutant (Fig. 4A and Fig. S7). In addition, the exogenously expressed RIG-I SV potently inhibited SeV-induced IFN-β promoter activation in HCT116, HuH7, and HeLa cells (Fig. S8).

To further delineate the inhibitory effect of the RIG-I SV on the RIG-I-mediated downstream signaling cascade, we examined virus-induced phosphorylation, dimerization, and nuclear translocation of IFN regulatory factor 3 (IRF3). HEK293T cells were cotransfected with Myc-tagged RIG-I WT together with vector, Flag-RIG-I SV or RIG-I T55I mutant followed by SeV infection. This showed that SeV infection led to a considerable shift of endogenous IRF3 to the slow-migrating phosphorylated forms that were also readily detected by anti-S396 phospho-specific IRF3 antibody (Fig. 4B). In contrast, ectopic expression of RIG-I SV and RIG-I T55I mutant strongly suppressed the SeV-induced phosphorylation of IRF3 (Fig. 4B). In addition, RIG-I SV and RIG-I T55I mutant almost completely blocked SeV-induced IRF3 dimerization (Fig. 4C). We further tested the nuclear translocation of IRF3-eGFP induced by SeV infection in HEK293T stably expressing vector, RIG-I WT, RIG-I SV, or RIG-I T55I mutant. Although the nuclear translocation of IRF3-eGFP was apparently detected in HEK293T cells expressing vector or RIG-I WT upon SeV infection, it was not observed in cells expressing RIG-I SV or RIG-I T55I mutant (Fig. 4D). Finally, RIG-I SV and RIG-I T55I mutant expression detectably increased VSV-eGFP replication compared with vector expression. In contrast, RIG-I WT expression markedly decreased VSV-eGFP replication (Fig. 4E). Furthermore, HEK293T cells
expressing RIG-I SV or RIG-I T55I mutant showed reduced amounts of secreted IFN-β upon SeV infection compared with cells expressing vector or RIG-I WT (Fig. 4F). These results collectively indicate that the RIG-I SV acts as a dominant inhibitor of the RIG-I-mediated antiviral response, possibly serving as a negative-feedback mechanism.

**Inhibition of RIG-I Multimerization and RIG-I-MAVS Signaling Complex Formation by RIG-I SV.** To decipher the molecular mechanism of how the SV inhibits RIG-I-mediated IFN signal transduction, we tested RIG-I SV mutants in their ability to suppress the SeV-induced IFN induction: RIG-I SV K270A mutant with the loss of ATPase activity (11) and RIG-I SV C810,813A with the abolished RD structure (13). Although RIG-I SV and RIG-I SV K270A strongly inhibited the SeV-induced IFN-β promoter activation, RIG-I SV C810,813A did not show any significant inhibitory effect (Fig. 5A), indicating that the intact structure of the C-terminal RD is critical for the inhibitory effect of RIG-I SV. The C-terminal RD of RIG-I specifically binds 5′-triphosphate viral RNA, and this interaction triggers RNA-dependent RIG-I dimerization. Therefore, the RIG-I SV was tested for its ability to bind 5′-triphosphate viral RNA and to interact with RIG-I WT. Fluorescence anisotropy analysis showed that RIG-I WT and RIG-I SV had similar binding affinities to in vitro-transcribed 5′-triphosphate-containing rabies virus leader RNA (5′pppRVL): WT ($K_d = 246.41$ nM) and SV ($K_d = 233.39$ nM) (Fig. S9A). Furthermore, RIG-I SV readily interacted with RIG-I WT in the presence and absence of viral infection, and this interaction detectably inhibited RIG-I multimerization in a dose-dependent manner (Fig. 5B and C and Fig. S9B). Finally, ectopic expression of the RIG-I SV and RIG-I T55I led to a considerable reduction of RIG-I WT interaction with MAVS-CARD-PRD or full-length MAVS induced by SeV infection (Fig. 5D and Fig. S9C). These results indicate that the RIG-I SV interacts with RIG-I WT, and this interaction efficiently inhibits RIG-I multimerization and RIG-I-MAVS-interaction, resulting in a potent inhibition of virus-induced IFN signal transduction.

**Discussion**

A previous study (19) has shown that the naturally occurring T55I mutation of the RIG-I first CARD in the hepatocyte Huh7.5 cells disrupts the signaling activity of the CARDs, resulting in a high permissiveness to HCV replication. Our data revealed that the T55I mutation of the RIG-I first CARD completely abolished its ability to bind TRIM25, leading to the loss of RIG-I CARD ubiquitination and signaling activity.

The essence of the intact tandem CARD for RIG-I ubiquitination-dependent activity is further evidenced by the identification of a novel RIG-I SV whose expression is induced upon viral infection or IFN stimulation. Because of a short deletion in its first CARD, the RIG-I SV is unable to carry out TRIM25 interaction, ubiquitination, and, ultimately, antiviral signal transduction. Besides its lack of signaling activity, this SV acts as an endogenous inhibitor of
RIG-I signal transduction, possibly providing a negative-feedback inhibition or fine-tuning mechanism.

The RIG-I C-terminal RD contains the highly conserved Cys616, Cys649, and Cys685-comprising zinc-coordination site as a key structural element for its functional activities (13). Our data showed that the structural integrity of the RD is critical for the inhibitory activity of the RIG-I SV, because the C810,813A mutant no longer inhibited virus-induced IFN signal transduction. Upon binding to viral RNA, the RIG-I RD triggers a structural switch to induce the RNA-dependent RIG-I dimerization (13). Because RIG-I WT and the RIG-I SV exhibited similar binding affinities to 5′-triphosphate RNA, viral RNA-sequestration may not serve as the main mechanism of the inhibitory effect of the RIG-I SV on virus-induced IFN signal transduction. The RIG-I SV, instead, efficiently interacted with RIG-I WT to form a RIG-I WT-RIG-I SV heterocomplex, and this interaction suppressed RIG-I WT multimerization as well as RIG-I-MAVS interaction that are critical for RIG-I signaling.

Cui et al. (13) have suggested a potential model for the C-terminal RD-dependent RIG-I activation. In the absence of viral infection, RIG-I is monomeric and inactive by masking the central DECH domain with its N-terminal CARDs. Upon viral 5′-pppRNA interaction, the RD undergoes a conformational change and dimerizes, displacing the CARDs. Our study also suggests the multiple steps of the N-terminal CARD-dependent RIG-I activation. Upon viral RNA binding, the RIG-I first CARD is exposed and binds TRIM25 that subsequently ubiquitinates the lysine residues of the RIG-I second CARD. The ubiquinated RIG-I CARDs effectively interact with MAVS, eliciting downstream antiviral signal transduction to induce IFN-α/β production.

Materials and Methods

Native PAGE. Native PAGE was performed by using a 7.5% acrylamide gel (Bio-Rad). The gel was prerun with 25 mM Tris and 192 mM glycine (pH 8.4) with or without 0.7% deoxycholate in the cathode and anode chamber, respectively, for 30 min at 30 mA. Samples in the native sample buffer (62.5 mM Tris-HCl (pH 6.8), 15% glycerol) were applied on the gel and electrophoresed for 40–80 min at 10 mA, followed by immunoblotting.

Confocal Immunofluorescence Microscopy. Cell preparation and confocal microscopy analysis were performed as described (20).

Other methods and materials are provided as SI Text.

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Supporting Information

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SI Text

Cell Culture, Transfection, and Reagents. HEK293T, MEF, Vero, EcoPack2-293 (BD Biosciences), HeLa, HCT116, Huh7, NHLF, 2TGH wild-type, U3A, and U6A cells (provided by George Stark, Cleveland Clinic Foundation, Cleveland, OH) were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% FBS, 2 mM l-glutamine, and 1% penicillin-streptomycin (GIBCO–BRL). LnCap human prostate cancer cells were cultured in RPMI medium supplemented with 10% FBS, 2 mM l-glutamine, and 1% penicillin-streptomycin. Lymphatic endothelial cells (LECs) were cultured in endothelial growth medium (EGM-2) supplemented with the microvascular supplement pack (Clonetics). Transient transfections were performed with Lipofectamine 2000 (Invitrogen), FuGENE 6 (Roche) or calcium phosphate (Clontech) according to the manufacturer’s instructions. RIG-I WT and knockout MEFs were immortalized as described in ref. 1. To obtain stable RIG-I MEF cells, pBabe-Puro vector, pBabe-Puro-RIG-I WT, pBabe-Puro-RIG-I T55I, or pBabe-Puro-RIG-I SV retrovirus was produced in Ecopack2-293. After retroviral infection of RIG-I knockout MEFs, cells were selected by using 1 μg/ml puromycin.

Plasmid Construction. All constructs for transient and stable expression in mammalian cells were derived from pEBG GST fusion vector, pEF-IREs-Puro, or pBabe-Puro expression vector. DNA fragments corresponding to the coding sequence of the RIG-I and TRIM25 genes were amplified from template DNA tor. DNA fragments corresponding to the coding sequence of the RIG-I CARDs, the original sequence.

RT-PCR. HEK293T cells were treated with IFN-β (1,000 units/ml) for 24 h. Total RNA was extracted by using the RNeasy Plus mini kit (Qiagen) and reverse transcribed by using Long Range Step RT-PCR kit (Qiagen). From the resulting cDNAs, RIG-I WT and splice variant were amplified by using the following primers: forward 5'-(AGTTTGAACACGCGTACAGTGC)-3' and reverse 3'-(CTACTTTGGGAGGATTCGAGC)-3'. The resulting amplicon of ~27 kb was digested with SacI to specifically digest RIG-I WT but not RIG-I splice variant transcripts. After cloning into the pGEM-TEasy vector (Promega), clones were subjected to sequence analysis using an ABI PRISM 377 automatic DNA sequencer.

GST Pulldown Assay, Immunoprecipitation, and Immunoblot Analysis. HEK293T cells were lysed in Nonidet P-40 buffer [50 mM Heps (pH 7.4), 150 mM NaCl, 1% (vol/vol) Nonidet P-40, and protease inhibitor mixture (Roche)]. GST pulldown and immunoprecipitations were performed as described in ref 20. For immunoblotting, proteins were resolved by SDS/PAGE and transferred onto a PVDF membrane. The following primary antibodies were used: anti-V5 (1:5,000) (Invitrogen), anti-Flag (1:5,000) (Sigma), anti-HA (1:5,000) (Sigma), anti-Myc (1:5,000) (Convance), anti-GST (1:10,000) (Sigma), anti-actin (1:10,000) (Abcam), anti-ubiquitin (P4D1; Santa Cruz Biotechnology), anti-TRIM25 (BD Biosciences), monoclonal anti-RIG-I (1:1,000) (Alexis), polyclonal anti-RIG-I (1:200) (IBL), anti-IRF3 (1:500) (Santa Cruz Biotechnology), and anti-phospho-Ser30-IRF3 (1:200) (Upstate Biotechnology). The proteins were visualized by an enhanced chemiluminescence reagent (Pierce) and detected by a phospho imager (Fuji LAS-4000).

Protein Expression, Fluorescence and Anisotropy Measurement. Protein expressions of RIG-I and RIG-I splice variant in insect cells were performed as described in ref. 2. Proteins were purified to homogeneity by using metal affinity (Qiagen), anion exchange and gel filtration chromatography with standard protocols. Fluorescence anisotropy experiments were performed with a FluoroMax-P fluorimeter (Horiba Jobin Yvon), equipped with a Glan–Thompson prism polarizer. Typically, 1.2 ml of buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM DTT, and 10 μM ZnCl2] and 50 nM RNA (in vitro-transcribed pppRVL with incorporated Alexa Fluor 488–5–UTP) were prequillibrated in a quartz cuvette at 12°C. Protein samples were added in a stepwise manner and briefly mixed by magnetic stirring. After 3 min of reequilibration, the anisotropy data were measured in triplets for each titration step by using an excitation wavelength of 492 nm and monitoring the emission 516 nm. The band pass was 5 nm for excitation and 5 nm for emission.

Luciferase Reporter Assay. HEK293T, HCT116, Huh7, and HeLa cells were seeded into six-well plates. At 24 h, the cells were transfected with an IFN-β or NF-κB luciferase construct together with constitutive β-gal-expressing PGK-β-gal. At 24 h after transfection, the cells were mock-treated or infected with SeV (50 HA units/ml) for 16 h. WCLs were prepared and subjected to a luciferase assay (Promega). Luciferase values were normalized to β-galactosidase to measure transfection efficiency.

IFN-β ELISA and VSV replication. HEK293T cells were seeded into a 12-well plate and mock-infected or infected with SeV (50 HA
units/ml) for 20 h. The supernatants were collected and analyzed for IFN-β production by using enzyme-linked immunosorbent assays (PBL Biomedical Laboratories). For viral replication assays, stable HEK293T or RIG-I knockout MEFs were seeded into six-well plates and infected with VSV-eGFP at MOI 0.5. At 24–48 h after infection, the culture medium was harvested and the virus yield determined by plaque assay on Vero cells.

Fig. S1. The intact tandem CARD of RIG-I is required for TRIM25-mediated RIG-I ubiquitination and downstream signaling. (A) Both CARDs are required for TRIM25-mediated RIG-I ubiquitination. HEK293T cells were transfected with GST-RIG-I 2CARD, GST-RIG-I first CARD, or GST-RIG-I second CARD with or without V5-tagged TRIM25. WCLs were used for GST pull down (GST-PD), followed by IB with α-GST (Top) or α-Ub (Middle) to show the ubiquitination of RIG-I constructs. WCLs were used for immunoblotting (IB) with α-V5 (Bottom). Arrows indicate the ubiquitinated bands. (B) Both CARDs are necessary for TRIM25-induced RIG-I NF-κB activation. HEK293T were transfected with GST, GST-RIG-I 2CARD, GST-RIG-I first CARD, or GST-RIG-I second CARD with or without TRIM25 together with NF-κB luciferase and constitutive β-gal-expressing pGK-β-gal reporter. Luciferase values were determined and normalized to β-galactosidase. Data represent the mean ± SD (n = 3).
Fig. S2. TRIM25-mediated RIG-I ubiquitination is critical for RIG-I-MAVS interaction. (A) T55I mutation of RIG-I abolishes TRIM25 binding. At 48 h after transfection with GST, GST-RIG-I 2CARD WT, or GST-RIG-I 2CARD T55I mutant, WCLs were used for GST-PD, followed by IB with α-TRIM25 (Top) or α-GST (Middle). WCLs were immunoblotted with α-TRIM25 (Bottom). Arrows indicate the ubiquitinated bands. (B) MAVS preferentially interacts with ubiquitinated RIG-I. (Upper) At 48 h after transfection with GST or GST-MAVS-CARD-PRD together with Flag-tagged RIG-I 2CARD, HEK293T WCLs were used for GST-PD, followed by IB with α-Flag (Top) or α-GST (Middle). WCLs were used for IB with α-Flag to determine the expression of RIG-I 2CARD (Bottom). Arrows indicate the ubiquitinated bands. (Lower) The quantitation of ubiquitinated and nonubiquitinated RIG-I bands in the WCLs and in the GST-MAVS-CARD-PRD complex by using a Fuji phospho imager. (C) TRIM25-mediated ubiquitination is necessary for efficient RIG-I-MAVS interaction. At 48 h after transfection with GST or GST-RIG-I 2CARD together with MAVS-CARD-PRD-Flag and increasing amount of TRIM25 shRNA-specific retroviral pSuper vector, HEK293T WCLs were used for GST-PD, followed by IB with α-Flag, α-GST, or α-Ub. Arrows indicate the ubiquitinated bands. WCLs were used for IB with α-TRIM25 or α-Flag to show the expression of TRIM25 and MAVS-CARD-PRD, respectively. Loading control was determined by using an α-actin antibody.
Fig. S3. Mutation of T55 to hydrophobic residues abolishes RIG-I CARD ubiquitination, TRIM25 binding, and RIG-I signaling. (A) Mutation of T55 to hydrophobic residues abolishes RIG-I CARD ubiquitination and TRIM25 binding. At 48 h after transfection with GST, GST-RIG-I 2CARD WT, GST-RIG-I 2CARD T55S, GST-RIG-I 2CARD T55W, or GST-RIG-I 2CARD T55V, HEK293T WCLs were subjected to GST-PD, followed by IB with α-GST, α-Ub or α-TRIM25. To determine endogenous TRIM25 expression, WCLs were subjected to IB with α-TRIM25. Arrows indicate the ubiquitinated bands. (B) RIG-I T55W and T55V mutants show a near complete loss of downstream signaling activity. GST, GST-RIG-I 2CARD WT, GST-RIG-I 2CARD T55S, GST-RIG-I 2CARD T55W, or GST-RIG-I 2CARD T55V was expressed in HEK293T together with IFN-β or NF-κB luciferase and pGK-β-gal. At 36 h after transfection, the cells were harvested and the luciferase and β-galactosidase values determined. Luciferase values, normalized to β-galactosidase activity, are presented as fold induction. Data represent the mean ± SD (n = 3).
Fig. S4. RIG-I splice variant transcript and protein. (A) RIG-I WT and splice variant transcripts and proteins in HEK293T upon SeV infection. HEK293T were mock-infected or infected with 25 HA units/ml SeV for the indicated number of hours. To identify potential splice variants of the RIG-I CARDs, total cellular RNA was isolated and subjected to RT-PCR to amplify the RIG-I CARDs (exon 1–3) (Top). Transcript levels of RIG-I WT and the splice variant (SV) were determined using primers specific for each isoform (Second and Third images). Actin transcripts were determined as control. Endogenous protein levels of RIG-I WT and SV upon SeV infection were evaluated by IP with α-RIG-I followed by IB with α-RIG-I (Bottom). Flag-tagged RIG-I WT and SV were loaded as controls. (B) RIG-I WT and splice variant transcript levels in LECs. LECs were mock-treated, treated with 1,000 units/ml IFN-β, or 10 μM all-trans retinoic acid (RA) for the indicated hours. Total cellular RNA was isolated and subjected to RT-PCR with primers specific for RIG-I WT or SV, respectively. Actin transcripts were determined as control. (C) Control IP for the RIG-I splice variant. WCLs of HEK293T cells, treated with IFN-β (1,000 U/ml) for 24 h, were used for immunoprecipitation with an α-RIG-I antibody recognizing the central helicase domain (residues 201–713). Precipitated endogenous RIG-I WT and splice variant were determined by IB with RIG-I antibodies, recognizing the helicase domain (residues 201–713) or first CARD (residues 37–55), respectively. Arrows indicate RIG-I WT or the splice variant.
Fig. S5. IFN-dependent expression of RIG-I splice variant in various cell lines. (A) HeLa, HCT116, Huh7, LnCap, and NHLF cells were mock-treated or stimulated with 1,000 units/ml IFN-β for 24 h. Total cellular RNA was isolated and subjected to RT-PCR with primers specific for RIG-I WT or SV, respectively. Actin transcripts were determined as control. (B) The 2fTGH wild-type, STAT1-deficient (U3A), and STAT2-deficient (U6A) human fibroblasts were mock-treated or treated with 1,000 units/ml IFN-β or IFN-α for 24 h. Total cellular RNA was isolated and subjected to RT-PCR with primers specific for RIG-I WT or SV, respectively. Actin transcripts were determined as control.
Fig. S6. Abolished TRIM25 binding, ubiquitination, and signaling activity of RIG-I splice variant. (A) RIG-I WT but not RIG-I splice variant interacts with TRIM25-SPRY. V5-tagged TRIM25-SPRY together with GST, GST-RIG-I 2CARD WT, or GST-RIG 2CARD SV were expressed in HEK293T cells. At 48 h after transfection, cells were lysed, and WCLs were subjected to GST-PD, followed by IB with α-V5 (Top) or α-GST (Middle). WCLs were used for IB with α-V5 to determine the expression of TRIM25-SPRY (Bottom). (B) RIG-I splice variant and RIG-I T55I do not interact with TRIM25. After transfection of V5-tagged TRIM25 together with vector, Flag-tagged RIG-I WT, RIG-I T55I, RIG-I SV, or RIG-I K172R, HEK293T WCLs were subjected to IP with α-Flag, followed by IB with α-V5 (Top) or α-Flag (Middle). WCLs were used for IB with α-V5 to test the TRIM25 expression (Bottom). (C) RIG-I splice variant exhibits a near-complete loss of K63-linked ubiquitination. Flag-tagged RIG-I WT, RIG-I T55I, RIG-I SV or RIG-I K172R together with HA-tagged K63only ubiquitin were expressed in HEK293T cells. At 24 h after transfection, the cells were infected with SeV (25 HA units/ml) for 10 h. WCLs were used for IP with α-Flag, followed by IB with α-HA (Top) or α-Flag (second). WCLs were subjected to IB with α-HA to determine HA-ubiquitin expression (third). Loading control was determined by using an α-actin antibody (fourth). (D) Lack of signaling activity of RIG-I splice variant. HEK293T were transfected with GST, GST-RIG-I 2CARD WT, or GST-RIG-I 2CARD SV with or without V5-tagged TRIM25 together with NF-κB luciferase and pGK-β-gal. At 36 h after transfection, the cells were harvested, and luciferase and β-galactosidase values were determined. Luciferase values were normalized to β-galactosidase activity and are presented as fold induction. Data represent the mean ± SD (n = 3).
**Fig. S7.** RIG-I splice variant inhibits SeV-induced NF-κB promoter activation. HEK293T were transfected with vector or increasing amount of RIG-I T55I or RIG-I splice variant (SV) together with NF-κB luciferase and pGK-β-gal. At 24 h after transfection, the cells were mock-infected or infected with SeV for 16 h. Luciferase and β-galactosidase values were determined and NF-κB luciferase values normalized to β-galactosidase activity for transfection efficiency control. Data represent the mean ± SD (n = 3).
**Fig. S8.** RIG-I splice variant inhibits SeV-induced IFN-β promoter activation in various cell lines. HCT116, Huh7, and HeLa cells were transfected with vector or increasing amount of RIG-I splice variant (SV) together with IFN-β luciferase and pGK-β-gal. At 24 h after transfection, the cells were mock-infected or infected with SeV for 16 h. Luciferase and β-galactosidase values were determined and IFN-β luciferase values normalized to β-galactosidase activity for transfection efficiency control. Data represent the mean ± SD (n = 3).
Fig. 59. (A) Fluorescence anisotropy changes of fluorescently labeled pppRVL in response to titration with RIG-I SV (filled square $k_d = 233.39 \pm 35.47$ nM) and RIG-I WT (open circles, $k_d = 246.41 \pm 42.33$ nM). The plot was non-linearly fitted according to a one-site binding model $\Delta A = B_{max} \times \text{[Protein]} / (k_d + \text{[Protein]})$. (B) RIG-I splice variant interferes with virus-induced RIG-I multimerization. HEK293T cells were transfected with vector, Myc-tagged RIG-I WT, Flag-tagged RIG-I WT and increasing amount of V5-tagged RIG-I SV. At 24 h posttransfection, the cells were infected with SeV for 16 h. Co-immunoprecipitation of Myc-RIG-I WT with Flag-RIG-I WT was determined by IP using α-Flag followed by IB with α-Myc or α-Flag. WCLs were subjected to IB with α-Myc or α-V5. (C) RIG-I splice variant inhibits the virus-induced RIG-I-MAVS-complex formation. HEK293T were cotransfected with Flag-tagged full-length MAVS and Myc-tagged RIG-I WT together with vector, V5-tagged RIG-I T55I, or splice variant (SV) and subsequently either mock-treated or infected with SeV (50 HA units/ml) for 16 h. WCLs were subjected to IP with α-Flag, followed by IB with α-Myc or α-Flag. WCLs were subjected to IB with α-Myc or α-V5.