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The RNA Helicase Lgp2 Inhibits TLR-Independent Sensing of Viral Replication by Retinoic Acid-Inducible Gene-I[1]

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The paramyxovirus Sendai (SV), is a well-established inducer of IFN-$\alpha\beta$ gene expression. In this study we show that SV induces IFN-$\alpha\beta$ gene expression normally in cells from mice with targeted deletions of the Toll-IL-1 resistance domain containing adapters MyD88, Mal, Toll/IL-1R domain-containing adaptor inducing IFN-β (TRIF), and TRIF-related adaptor molecule TLR3, or the E3 ubiquitin ligase, TNFR-associated factor 6. This TLR-independent induction of IFN-$\alpha\beta$ after SV infection is replication dependent and mediated by the RNA helicase, retinoic acid-inducible gene-I (RIG-I) and not the related family member, melanoma differentiation-associated gene 5. Furthermore, we characterize a RIG-I-like RNA helicase, Lgp2. In contrast to RIG-I or melanoma differentiation-associated gene 5, Lgp2 lacks signaling caspase recruitment and activation domains. Overexpression of Lgp2 inhibits SV and Newcastle disease virus signaling to IFN-stimulated regulatory element- and NF-κB-dependent pathways. Importantly, Lgp2 does not prevent TLR3 signaling. Like RIG-I, Lgp2 binds double-stranded, but not single-stranded, RNA. Quantitative PCR analysis demonstrates that Lgp2 is present in unstimulated cells at a lower level than RIG-I, although both helicases are induced to similar levels after virus infection. We propose that Lgp2 acts as a negative feedback regulator of antiviral signaling by sequestering dsRNA from RIG-I. *The Journal of Immunology,* **2005, 175: 5260–5268.**

Successful host defense against virus infection relies on the rapid production of IFN-$\alpha\beta$ and the subsequent transcription of hundreds of so-called IFN-stimulated genes (ISGs),[4] the products of which lead to a cellular antiviral state and prevent viral replication. IFN-$\alpha\beta$ also control multiple dendritic cell (DC) functions, thereby activating adaptive immunity and NK cell function, both of which are necessary for viral elimination and protective immunity (reviewed in Ref. 1). Although virtually all cell types can produce type I IFN, plasmacytoid DCs are by far the most potent and are responsible for most of the systemically measurable IFN-α produced early during viral infections (2).

Germline-encoded innate immune receptors, often referred to as pattern recognition receptors (PRRs), control the acute IFN response (3, 4). TLRs are one class of PRRs capable of detecting viral particles or products of viral replication. Viral nucleic acids are the predominant trigger of type I IFNs (reviewed in Ref. 5). TLR3, -7, -8, and -9 serve as receptors for viral nucleic acids. Unmethylated CpG DNA motifs in the genomes of HSV1 and HSV2 induce IFN via TLR9 (6, 7); ssRNA viruses, such as influenza and vesicular stomatitis virus, which enter the cytoplasm through a vesicular pathway activate TLR7 (8); and the dsRNA of reoviruses can induce IFN-$\alpha\beta$ via TLR3 (9). TLRs are transmembrane proteins; their ligand recognition moieties are either extracellular in the case of surface TLRs or in the lumen of endosomes in the case of TLR3, -7, -8, and -9. Many negative-strand RNA viruses replicate in the cytoplasm and are unlikely to expose structures produced during viral replication to TLRs (e.g., dsRNA, a product of viral replication). Accordingly, TLR-mediated sensing of viruses by endosomally localized TLRs occurs without the requirement for viral replication. Recent evidence has suggested that TLR-independent antiviral sensing mechanisms also exist. TLR3-deficient mice fail to show increased susceptibility to many viral infections, and no major changes in morbidity or mortality were observed, when TLR7 or TLR9-deficient mice were infected with influenza or HSV in vivo, although IFN levels were partially reduced (10–12). This TLR-independent induction of IFN is dependent on viral replication and is most likely mediated by cytoplasmic PRRs. The serine threonine kinase protein kinase R, which binds dsRNA, and the nucleotide-binding oligomerization domain (NOD) proteins 1 and 2, which recognize bacterial peptidoglycans, are PRRs located in the cytoplasm. However, there is no evidence supporting a role for these proteins in the type I IFN response (13, 14).

Retinoic acid-inducible gene-I (RIG-I) (15, 16) and melanoma differentiation-associated gene 5 (Mda-5)/Helicard (17, 18), two DExD/H box RNA helicases, have recently been implicated in IFN gene regulation upon cytoplasmic dsRNA treatment (19, 20). Intriguingly, these helicases contain protein interaction caspase recruitment and activation domains (CARD). RIG-I is required for the IFN response to Newcastle disease virus (NDV) (19), and the involvement of Mda-5 in viral recognition has been suggested by the demonstration that paramyxoviruses encode proteins that inhibit Mda-5 (20). Sendai virus (SV), a negative-stranded RNA

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[4] Abbreviations used in this paper: ISG, IFN-stimulated gene; CARD, caspase recruitment and activation domain; DC, dendritic cell; Flt-3L, Flt3 ligand; HAU, hemagglutinating unit; HCV, hepatitis C virus; IRF-3, IFN regulatory factor-3; ISRE, IFN-stimulated regulatory element; Mda-5, melanoma differentiation-associated gene 5; NDV, Newcastle disease virus; NOD, nucleotide-binding oligomerization domain; poly(I-C), polyinosinic-polycytidylic acid; PRR, pattern recognition receptor; RIG-I, retinoic acid-inducible gene I; SV, Sendai virus; TIR, Toll-IL-1 resistance; CFP, cyano-fluorescent protein; IKK, IκB kinase; RIP, receptor-associated protein; shRNA, small hairpin RNA; TRAF, TNFR-associated factor; TRAM, TRIF-related adaptor molecule; TRIF, Toll/IL-1R domain-containing adaptor inducing IFN-β; YFP, yellow-fluorescent protein.

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virus and a member of the paramyxovirus family is a well-established inducer of IFN-$\alpha\beta$ gene expression. SV enters cells by direct fusion with the cell membrane, thereby bypassing endosomal compartments. Data from our own group (and others) have shown that the IκKB kinase (IKK)-related kinases TANK-binding kinase-1 and/or IKKϵ regulate IFN regulatory factor-3 (IRF-3) and IFN gene induction after SV infection (21–24).

In this study we show, using mice with targeted deletions for all Toll-IL-1 resistance (TIR) adapters, that the recognition of SV occurs independently of the TLR system of receptors and adapters. Instead, SV infection is detected by RIG-I. Furthermore, we implicate Lgp2, a protein of previously unknown function (25) in the antiviral response. Lgp2 is a virus-inducible gene, with a DExD/H box RNA helicase domain, highly homologous to that of RIG-I (19). Unlike, RIG-I and Mda-5, Lgp2 lacks the signaling CARD module. Lgp2 negatively regulates SV-induced IRF-3 or NF-κB signaling. Like RIG-I, Lgp2 binds double-stranded, but not single-stranded, RNA. We propose that Lgp2 is a natural inhibitor of antiviral responses, acting as a postinduction repressor of RIG-I signaling.

Materials and Methods

Mice, viruses, and reagents

MyD88$^{-/-}$, TRIF$^{-/-}$, TRAM$^{-/-}$, Mal$^{-/-}$, and TLR3$^{-/-}$ mice were obtained from S. Akira (Osaka University, Osaka, Japan). TRAF6$^{-/-}$ mice were purchased from Tak Mak. Studies with these mice have been reviewed and approved by the University of Massachusetts Medical School institutional animal care and use committee. TLR3-expressing HEK293 cells were previously described (21). SV (300 hemagglutination units (HAU)/ml; Cantrell strain) and NDV (240 HAU/ml; strain VR-699) were purchased from Charles River Laboratories and American Type Culture Collection, respectively. The HSV-KOS strain was obtained from D. Knipe (Harvard Medical School, Boston, MA). Influenza (strain X:31, A/Aichi/68 (H3N2)) was obtained from D. Libraty (University of Massachusetts Medical School, Worcester, MA). CpG-A (oligodeoxynucleotide 2216) was purchased from Coley Pharmaceuticals. Polyinosinic-polycytidylic acid (poly(I-C)) was purchased from Amersham Biosciences. Re-extracted LPS was previously described (26). R-848 was obtained from GL Synthesis.

Plasmid constructs

The pEF-BOS-RIG-I, RIG-I-C, RIG-I-K270A, eIF-4A, IFN-β-luciferase (p125Luc) constructs were obtained from T. Fujita (Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan) (19), pCR-3-flag-murine-Mda5/Helicard was obtained from J. Tschopp (University of Lausanne, Epalinges, Switzerland) (27), and pcDNA3-Nod1 and Nod2 were obtained from G. Nunez (University of Michigan Medical School, Ann Harbor, MI). The NF-κB luciferase reporter was as previously described (28). The ISG54-IFN-stimulated regulatory element (ISRE) was purchased from Stratagene. pCDNA3-RIG-I-CFP (C-terminal fusion protein with cyano-fluorescent protein) was generated by PCR. Lgp2 was amplified by PCR from a human spleen cDNA library and cloned into pEF-BOS or as a C-terminal fusion protein with yellow-fluorescent protein (YFP) into pcDNA3. Lgp2-K30A was generated by site-directed mutagenesis. All PCR-generated constructs were confirmed by sequence analysis.

UV and heat inactivation of the Cantrell strain of SV

SV was inactivated by heating to 55°C for 30 min or through successive cycles of UV cross-linking. To confirm the inability to replicate, fractions of UV-inactivated virus were injected into 12-day-old fertilized eggs. Two days after incubation, the allantoic fluid was harvested and checked for hemagglutination of chicken RBC.

Reporter assays and ELISAs

For reporter assays, HEK293, 293T, or HEK293-TLR3 cells (2×10^4 cells/100 μl/well in 96-well plates) were transfected with 40 ng of the indicated luciferase reporter gene together with 40 ng of thymidine kinase *Renilla*-luciferase reporter gene (Promega) and the indicated amount of expression plasmids using Genejuice (Novagen). Where indicated virus or poly(I-C) (25 μg/ml) was added for 16 h, and luciferase activity was measured as previously described (21). Murine bone marrow-derived DCs were generated in the presence of Flt3 ligand (Flt3-L) as previously described (29). Murine bone marrow-derived macrophages were generated in the presence of recombinant M-CSF (10 ng/ml) as previously described (30). Spleens were isolated from C57BL/6 or TRAF6$^{-/-}$ mice, and splenocytes were harvested by mechanical disruption using 40-μm pore size nylon cell strainers (BD Biosciences), followed by lysis of RBC using Tris-NH_4Cl. DCs, macrophages, or splenocytes (1×10^5/100 μl/well) were stimulated as indicated. Murine IFN-α was measured by ELISA as previously described (29). Using ELISA, we measured murine RANTES and IL-12-p40 according to the manufacturer's recommendations (R&D Systems). Murine IFN-β was measured by ELISA using the following Ab pairs: coating Ab, rabbit polyclonal anti-mouse IFN-β (PBL Biomedical); and detection Ab, rat monoclonal anti-mouse IFN-β (Yamasa). In HEK293 cells, RANTES expression was measured by ELISA using a human RANTES ELISA Duoset (R&D Systems)

RNA interference

RNA interference vectors (piGENE hU6 vector; iGENE Therapeutics) targeting human and mouse RIG-I were obtained from T. Fujita (19). The p53 targeting sequence in pSuper was previously described (21). To determine the efficiency of gene silencing, 5×10^4 293T cells were seeded into 12-well plates and transfected with either 0.5 μg of RIG-I-CFP or 0.5 μg of RIG-I-Flag together with 0.5 μg of the small hairpin RNA constructs. After 48 h, the expression of RIG-I-CFP was measured by flow cytometry. Whole-cell lysates of RIG-I-Flag-transfected cells were also analyzed after silencing by immunoblotting with anti-Flag-M2 (Sigma-Aldrich) and an Ab to caveolin (Transduction Laboratories) as a control. For reporter assays, 2×10^4 HEK 293 or murine L929 cells were transfected with 100 ng/well of the indicated shRNA constructs together with 10 ng of the IFN-β-luciferase reporter/well. After 72 h, the transfected cells were infected with SV, and 9 h later, luciferase activity was measured.

RNA binding analysis of RIG-I and Lgp2

To generate poly(I-C)-coated agarose beads, poly(C)-coated beads (Sigma-Aldrich) were resuspended in 2 vol of 2 mg/ml poly(I) (Sigma-Aldrich) prepared in 50 mM Tris (pH 7.0)-150 mM NaCl. The mixture was then rocked gently overnight at 4°C, collected by centrifugation at $1000 \times g$, washed with 50 mM Tris (pH 7.0)-150 mM NaCl, resuspended in the same buffer as a 10% final slurry, and stored at 4°C for use.

For poly(C) and poly(I-C) pull-down assays, poly(C)- or poly(I-C)-coated beads were equilibrated in binding buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, and 1% Nonidet P-40) as a 10% slurry and combined with an equal volume of whole-cell extract from cells transiently transfected with the indicated YFP/CFP-tagged constructs or from an Lgp2-YFP-expressing stable cell line (lysis buffer: 50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, and 10% glycerol). The cell extracts were supplemented with protease and phosphatase inhibitors and 25 U of RNase inhibitor/ml. The mixtures were incubated with gentle agitation for 2 h at 4°C. For competition experiments, pull-down reactions were supplemented with 10 μg of the competitor RNA/ml. Beads were then centrifuged at $1,000 \times g$, rinsed three times with lysis buffer, and resuspended in 2× SDS-PAGE sample buffer. Samples were boiled at 100°C for 5 min, centrifuged at $13,000 \times g$ for 30 s, loaded immediately onto SDS-PAGE gels, and processed for immunoblot analysis with anti-GFP Abs as indicated.

Quantitative real-time PCR

Raw 264.7 macrophages were infected in six-well plates (1×10^6/well) with SV, and RNA was isolated using RNeasy (Qiagen). cDNA was synthesized, and quantitative RT-PCR analysis was performed on a DNA engine Opticon 2 cycler (MJ Research) using the SuperScript III Two-Step qRT-PCR Kit with SYBR Green (Invitrogen Life Technologies) and the following primers: β-actin: forward, 5′-TTGAACATGGCATTGTTACCAA-3′; reverse, 5′-TGGCATAGAGGTCTTTACGGA-3′; IFN-β: forward, 5′-TGCTCTCCTGTTGTGCTTCTCC-3′; reverse, 5′-CATCTCATAGATGGTCAATGCGG-3′; Lgp2: forward, 5′-TCATCTGTACGGCAGAGTTGT-3′; reverse, 5′-TGTTGTAGACGGTGTCCTTGT-3′; and RIG-I: forward, 5′-AGAGAATTCGGCACCCAGAA-3′; reverse, 5′-AGCTCTCGCTCGGTCTCATC-3′.

The specificity of amplification was assessed for each sample by melting curve analysis, and the size of the amplicon was checked by electrophoresis. Relative quantification was performed using standard curve analysis. All gene expression data were normalized with β-actin and are presented as a ratio of gene copy number per 100 copies of β-actin ± SD.

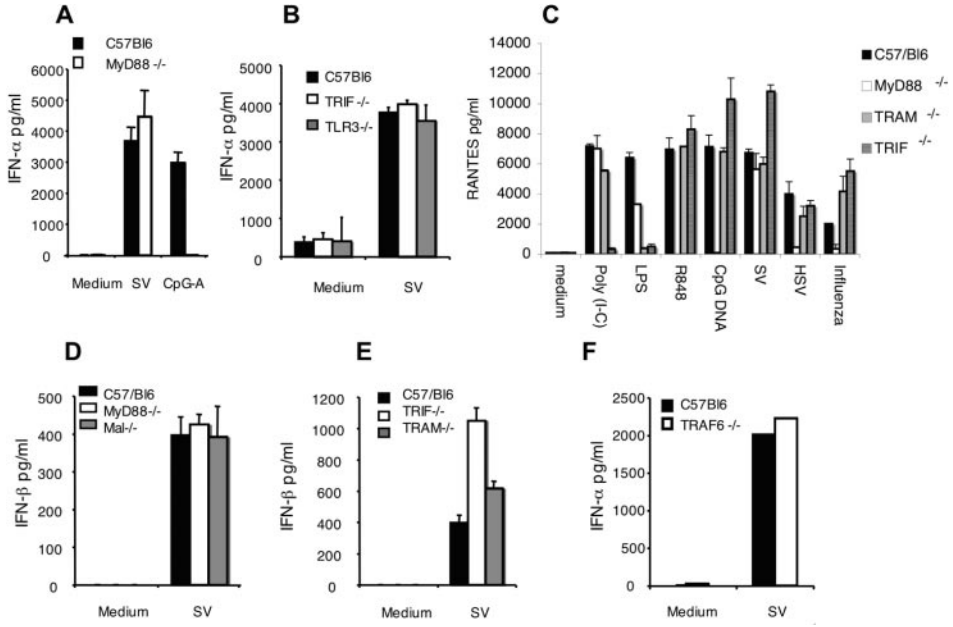

FIGURE 1. SV induces IFN-α via TLR-independent mechanisms. *A* and *B*, Flt-3L-derived DCs from C57BL/6, MyD88$^{-/-}$, TRIF$^{-/-}$, and TLR3$^{-/-}$ mice were infected with SV (60 HAU) or were stimulated with CpG-A DNA (2 μg/ml) for 16 h as indicated, and IFN-α was measured in the supernatants by ELISA. *C*, Flt-3L-derived DCs from C57BL/6, MyD88$^{-/-}$, TRIF$^{-/-}$, and TLR3$^{-/-}$ mice were infected with SV (60 HAU) and stimulated with LPS (20 ng/ml), poly(I-C) (20 μg/ml), R-848 (10 nM), CpG DNA 1668 (2 μg/ml), HSV1-KOS (2×10^5 PFU/ml), or influenza (2×10^4 PFU/ml) for 16 h, and RANTES was measured by ELISA. *D*, Splenocytes from C57BL/6 or the indicated knockout mice were mock infected or infected with SV (60 HAU), and IFN-α or IFN-β was measured by ELISA. Data are expressed as the mean $\pm$ SD. All experiments described were performed a minimum of three times and produced similar results.

Results

SV induces IFN-$\alpha\beta$ independently of TLRs or their early downstream signaling molecules

Our first goal was to determine whether SV-induced IFN-$\alpha\beta$ was dependent on TLR signaling using mice with targeted deletions of TLR adapters. To date, four adapter molecules have been associated with TLR signaling. These include, MyD88, Mal, Toll/IL-1R domain-containing adaptor inducing IFN-β (TRIF), and TRIF-related adaptor molecule (TRAM). TLR7, -8, and -9 induce IFN via MyD88, whereas TLR3 signals via TRIF, and TLR4 via TRIF and the related adapter molecule, TRAM (4). Flt3 ligand-differentiated DCs from either wild-type or MyD88-deficient bone marrow (consisting of 40–50% plasmacytoid DC and 50–60% myeloid DC) were infected with SV or stimulated with CpG-A as a positive control (Fig. 1*A*). Induction of IFN-α after CpG DNA treatment was abrogated in the absence of MyD88, in agreement with published reports (7). In contrast, SV induced normal levels of IFN-α in these cells. Similarly, the SV response was normal in Flt3-L-derived DCs from TRIF or TLR3-deficient mice (Fig. 1*B*). As a control for these measurements, we monitored the production of RANTES, another IRF-3-regulated gene, after SV infection, or stimulation by ligands for the following TLRs: poly(I-C) (TLR3), LPS (TLR4), R-848 (TLR7), and two other viruses, HSV-1 (TLR9) and influenza (TLR7). As shown in Fig. 1*C*, the induction of RANTES was impaired in TRIF-deficient cells after LPS and dsRNA signaling. TRAM-deficient cells were also defective after LPS treatment. MyD88-deficient cells were defective for CpG DNA, R-848, HSV-1, and influenza, consistent with published reports. Macrophages from C57BL/6, MyD88$^{-/-}$, Mal$^{-/-}$, TRIF$^{-/-}$, and TRAM$^{-/-}$ mice produced similar levels of IFN-β

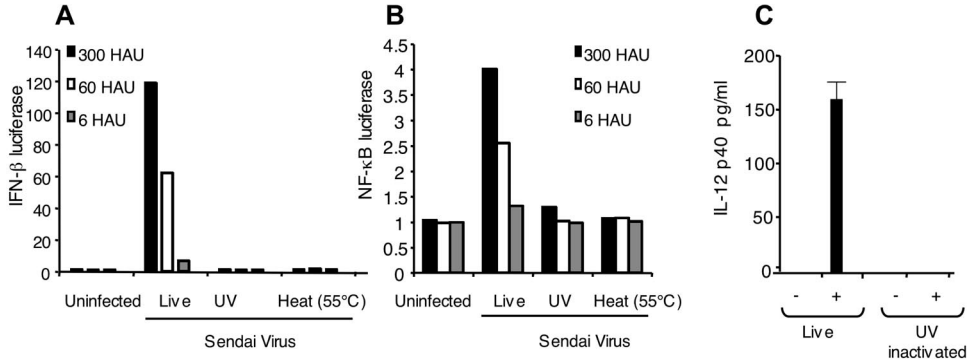

FIGURE 2. SV signaling is dependent on live virus. HEK293 cells expressing an IFN-β-luciferase reporter (*A*) or an NF-κB reporter (*B*) were infected with live, heat-inactivated, or UV-inactivated SV (6, 60, and 600 HAU); luciferase reporter gene activity was measured after 9 h and is depicted as fold induction relative to the reporter-only control. *C*, Purified plasmacytoid DCs (pDCs) from Flt-3L-derived C57BL/6 cultures were infected as indicated with 60 HAU of live or UV-inactivated SV for 16 h, and IL-12-p40 was measured by ELISA. Data are expressed as the mean $\pm$ SD. All experiments described were performed a minimum of three times and produced similar results.

after SV infection (Fig. 1, *D* and *E*). For completeness we also examined TLR2-, TLR4-, and TLR9-deficient cells and in all cases SV induced comparable levels of IFN-α as seen in wild-type mice (data not shown). Recently, the E3 ubiquitin ligase TRAF6 has been implicated in the regulation of IRF-7 and IFN-α responses after CpG DNA treatment (31). We therefore monitored the induction of IFN-α in total splenocytes isolated from TRAF6$^{-/-}$ mice and found normal IFN-α induction after SV infection (Fig. 1*F*). Similar results were obtained in TRAF6, TRAF2, and TRAF2/5 double-knockout embryonic fibroblast cells (data not shown). Collectively, the data from Fig. 1 (and the related data not shown) show that no single TLR or early downstream signaling molecule is required for the measured responses to SV infection.

To determine whether the induction of IFN-β after SV infection required active viral replication, we monitored SV-induced IFN-β reporter gene activation in HEK293 cells and compared live or replication-deficient SV. The Cantrell strain of Sendai virus was inactivated either by heating to 55°C for 30 min or through successive cycles of UV cross-linking, and failure to replicate was controlled as described in *Materials and Methods*. Either method of inactivation abolished the ability of the virus to replicate together with the stimulatory activity and its ability to induce an IFN-β reporter gene (Fig. 2*A*). In addition, we monitored NF-κB reporter gene induction in our HEK cell line. Although SV is a much poorer inducer of this reporter than either the ISRE or the IFN-β reporter, we also found that the UV or heat-inactivated virus failed to induce this response. Furthermore, purified plasmacytoid DCs, which are extremely sensitive to SV, were stimulated with either the live or UV-inactivated virus and IL-12-p40 was measured. There was no IL12-p40 induced with the inactivated virus. Collectively, these observations demonstrate that live virus is required to induce an IFN-β or NF-κB response, suggesting the requirement for active viral replication.

Recognition of SV is mediated by RIG-I

The discovery of RIG-I, as a mediator of NDV signaling and intracellular dsRNA responses (19), prompted us to evaluate its contribution to the SV response. As shown in Fig. 3*A*, infection of HEK293 cells with SV or NDV induces activation of an ISG54-ISRE, NF-κB, or IFN-β reporter gene. Transfection of a cDNA expressing full-length RIG-I synergistically enhanced the response to SV or NDV in each case (Fig. 3*A*). Interestingly, in contrast to RIG-I, overexpression of Mda5 did not enhance SV-induced ISRE activation (Fig. 3*B*), suggesting that Mda-5 is unable to mediate SV recognition. RIG-I contains tandem CARD domains in its N terminus and a COOH-terminal DExD/H box RNA helicase domain. Transfection of cells with RIG-IC (the isolated helicase domain) or RIG-I-K270A (a RIG-I construct that has a point mutation in the Walker-type ATP binding site, which is required for ATP-dependent RNA unwinding) dose-dependently inhibited SV- and NDV-dependent ISRE-reporter gene activation (Fig. 3*C*). Importantly, RIG-IC and RIG-I-K270A had no effect on ISRE activation after TLR3 stimulation (Fig. 3*D*). RIG-IC and RIG-I-K270A also inhibited SV-dependent activation of an NF-κB reporter gene (Fig. 3*E*), suggesting that RIG-I controls both these arms of the innate immune response. Similar results were seen when SV-induced RANTES expression was measured by ELISA. Consistent with the reporter assays, full-length RIG-I weakly induced RANTES expression, an effect that was dramatically enhanced after SV infection. In contrast, both RIG-IC and RIG-I-K270A abolished SV-induced RANTES induction (Fig. 3*F*).

An RNA interference approach using shRNA vectors targeting human and mouse RIG-I also demonstrated a clear role for RIG-I in SV-induced signaling. The efficiency and specificity of silencing RIG-I were examined using a CFP-tagged fusion protein transfected into 293T cells. Efficient and specific silencing of RIG-I by the human shRNA targeting vector was seen by monitoring fluorescence by FACS analysis or immunoblotting for the Flag epitope (Fig. 4*B*). Transfection of the shRNA targeting human RIG-I into HEK293 cells (human cells) greatly reduced SV-dependent IFN-β promoter activation, whereas shRNA targeting murine RIG-I, due to a lack of sufficient complementarity, had no effect (Fig. 4*C*). The shRNA vector targeting murine RIG-I inhibited SV signaling in murine L929 cells, whereas the vector targeting human RIG-I was

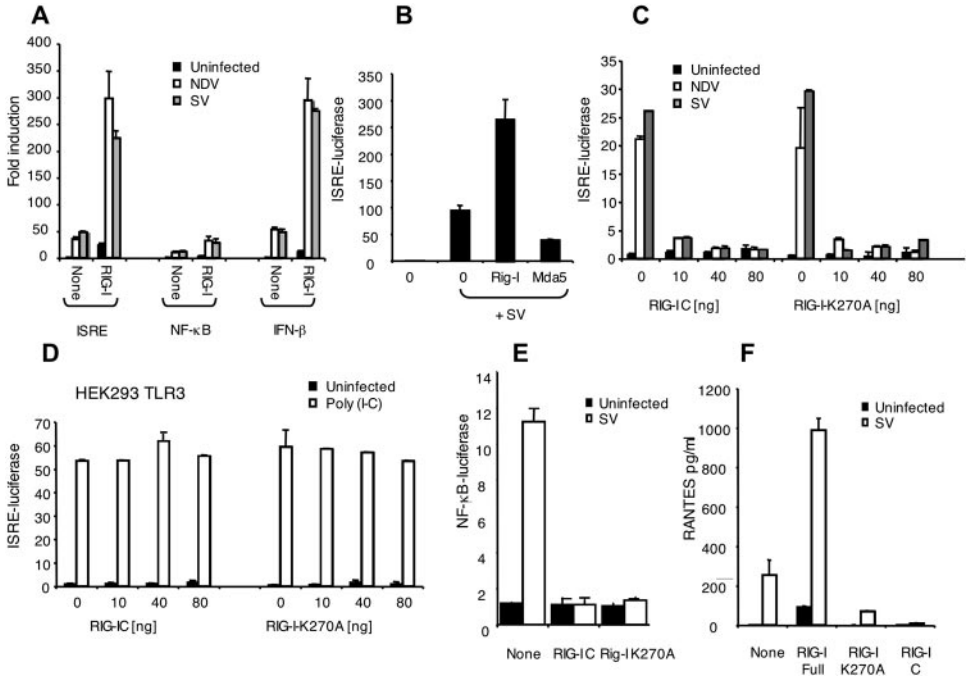

FIGURE 3. SV and NDV induce ISRE and NF-κB reporter activities via RIG-I. *A–E*, 293T cells or HEK293-TLR3 cells were transfected with the indicated reporter genes and cotransfected with full-length RIG-I, RIG-IC, or RIG-I-K270A. Cells were infected with SV (50 HAU) or NDV (50 HAU) or were treated with poly(I-C), where indicated, for 16 h, and luciferase reporter activities were measured. In all cases, data are expressed as fold induction relative to the reporter-only control and are the mean ± SD. *F*, 293T cells were transfected with full-length RIG-I, RIG-IC, or RIG-IK270A. Cells were infected with SV (50 HAU) for 16 h, and RANTES was measured by ELISA. All experiments described were performed a minimum of three times and produced similar results.

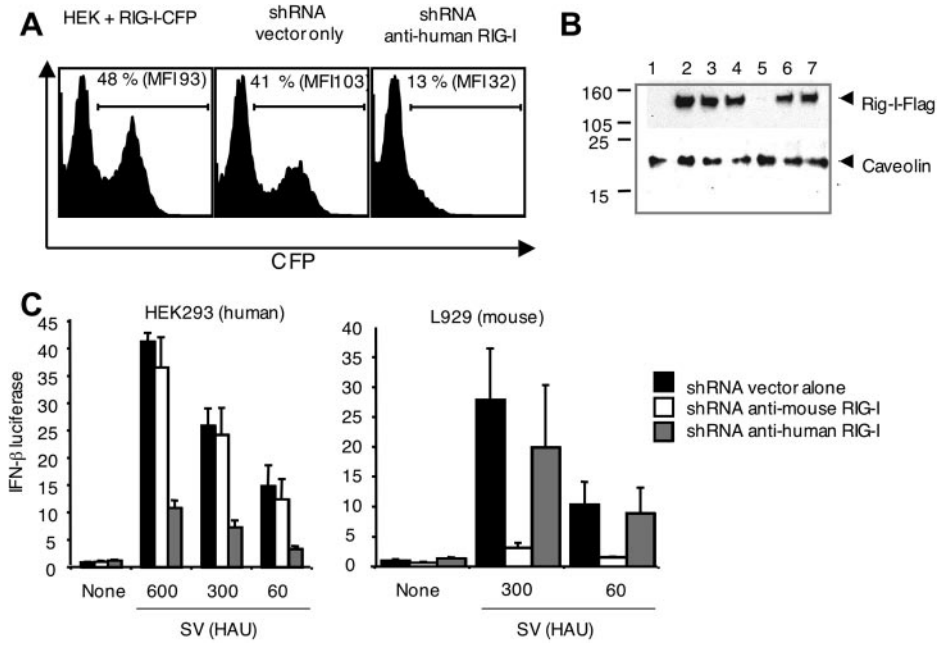

FIGURE 4. RNA interference targeting RIG-I abrogates SV signaling. 293T cells were cotransfected with RIG-I-CFP together with the indicated shRNA vectors for 48 h (*A*). RIG-I-CFP silencing was quantified by FACS analysis. The percentage of CFP^+ cells and the mean fluorescence intensity (MFI) of the CFP^+ cells are indicated. *B*, RIG-I-Flag silencing was quantified by Western blot. *Lane* 1, 293T; *lanes 2–7*, 293T cotransfected with RIG-I-Flag (*lane 2*, control; *lane 3*, piGENE vector; *lane 4*, shRNA anti-mouse RIG-I; *lane 5*, shRNA anti-human RIG-I; *lane 6*, pSuper vector; *lane 7*, shRNA anti p53 HEK293). *C*, HEK 293 or L929 cells were transfected with an IFN-β-luciferase reporter gene and shRNA vectors targeting human or murine RIG-I; 72 h later, cells were infected with SV, and luciferase reporter gene activity was measured. In all cases, data are expressed as fold induction relative to the reporter-only control and are the mean ± SD. All experiments described were performed a minimum of three times and produced similar results.

without effect. This species dependence emphasizes the specificity of the shRNAs. Collectively, the data from Figs. 3 and 4 clearly establish that the SV and NDV-dependent induction of IFN-β, ISRE, and NF-κB is mediated by RIG-I.

Characterization of Lgp2, a RIG-I like molecule which negatively regulates SV and NDV signaling

A genome-wide search for proteins structurally related to RIG-I identified two additional DExD/H box RNA helicases: Mda-5 (melanoma differentiation-associated gene 5) (17) and Lgp2 (25). Interestingly, we had earlier detected both helicases in a genome-wide expression profiling study of LPS treated MyD88-deficient macrophages (32). Mda-5, originally characterized as a CARD containing protein inducible by IFN, contains two N-terminal CARD domains, followed by a DExD/H box-RNA helicase domain (17). Lgp2 contains a DExD/H box RNA helicase domain, highly homologous to that of RIG-I, but lacks CARD domains or indeed any other signaling domain (see schematic representation in Fig. 5*A*). Notably, transfection of HEK293 cells with a cDNA expressing Lgp2 did not induce the NF-κB, ISRE, or the IFN-β reporter gene (Fig. 5*B*). In contrast, transient expression of Mda-5, like RIG-I, activated all three reporters (even in the absence of virus infection). In contrast, NOD1 and NOD2, anti-bacterial CARD-containing proteins induced the NF-κB reporter, consistent with previous reports, but did not activate the ISRE or IFN-β reporters (Fig. 5*B*). The isolated CARDs of RIG-I induced much higher levels of all three reporters than the full-length construct (data not shown) consistent with published reports (19). These data suggest that the CARDs of RIG-I and Mda-5 are functionally distinct from those of the NOD proteins and can initiate signaling to both ISRE- and NF-κB-dependent genes, whereas Lgp2, which lacks CARD domains, has no signaling capability.

We next examined the effect of Lgp2 on SV signaling. Transfection of cells with a cDNA expressing Lgp2 dose-dependently inhibited virus-induced ISRE activation (Fig. 5*C*). Similar results were seen with NDV (not shown). This inhibitory effect was specific for the virus-induced pathway, because Lgp2 did not inhibit TLR3-dependent induction of the ISRE reporter. The distantly related DExD/H box RNA helicase, elF4A (which binds and unwinds RNA duplexes), did not affect either response, demonstrating the specificity for Lgp2. Lgp2 also dose-dependently inhibited virus-induced, but not TLR3-induced, NF-κB or IFN-β reporter activation (Fig. 5*D*). Lgp2 also dose-dependently inhibited SV-induced RANTES production (Fig. 5*E*). A catalytically inactive helicase mutant of Lgp2, in which the Walker-type ATP binding site was mutated to alanine (Lgp2-K30A), which, based on comparisons to other RNA helicases, should still bind, but not unwind, dsRNA, did not reverse the inhibitory effect of Lgp2 on either SV or NDV signaling (data not shown).

RIG-I and Mda5 bind dsRNA (poly(I-C)) via their RNA helicase domains and are activated by dsRNA (19, 20). CARDs, like other structurally related protein interaction modules found in innate immune adaptor molecules, such as death domains, TIR domains, and death effector domains, mediate homotypic interactions to promote signaling events (33). It is therefore possible that a RIG-I dimer/oligomer might be necessary for initiating downstream signaling. One possible explanation for the inhibitory activity of Lgp2 might relate to its ability to bind RIG-I via its helicase domain and prevent the formation or CARD-CARD interactions (because Lgp2 lacks a CARD domain). To test this hypothesis, we first examined the ability of Lgp2 to bind to RIG-I in coimmunoprecipitation experiments in the absence and

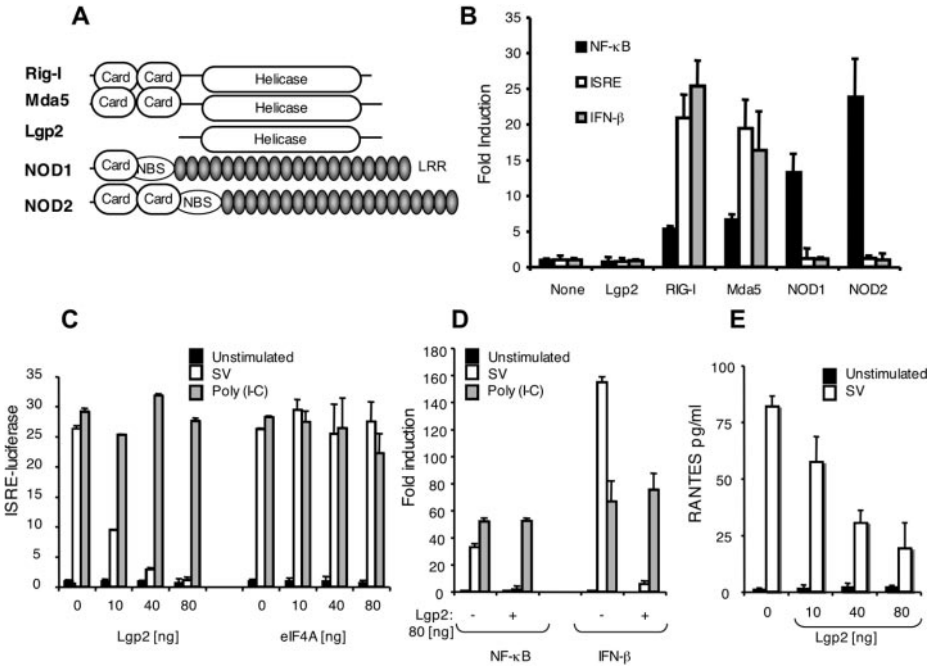

FIGURE 5. Lgp2 acts as a negative regulator of SV-induced signaling. *A*, Schematic representation of Lgp2, RIG-I, Mda-5, NOD1, and NOD2, showing domain architecture. *B*, 293T cells were transfected with the indicated reporter genes and cotransfected with Lgp2, full-length RIG-I, Mda5, NOD1, or NOD2. Luciferase reporter activity was measured after 24 h. *C* and *D*, HEK-293-TLR3 cells were transfected with the indicated reporters and Lgp2 or eIF4A as indicated. Cells were infected with SV (50 HAU) or were treated with poly(I-C) (20 μg/ml) for 16 h, and luciferase activity was measured. *E*, 293T cells were transfected with Lgp2. Cells were infected with SV (50 HAU), and RANTES was measured in the supernatants by ELISA. In all cases, data are expressed as fold induction relative to the reporter-only control and are the mean ± SD. All experiments described were performed a minimum of three times and produced similar results.

the presence of SV infection. Lgp2 did not bind RIG-I or Mda-5 under these conditions (data not shown).

We next took advantage of the fact that full-length RIG-I can elicit a spontaneous induction of downstream signaling pathways (even in the absence of virus infection). We therefore investigated whether Lgp2 could block this constitutive response. Full-length RIG-I induced the ISRE reporter gene normally even in the presence of Lgp2 (Fig. 6*A*). Treating the cells with SV in the presence

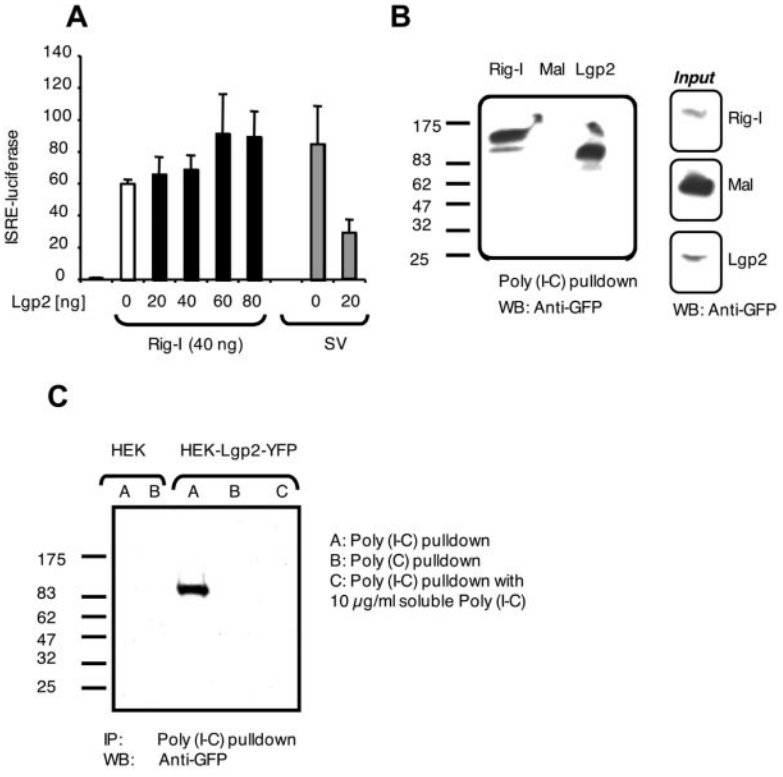

FIGURE 6. Lgp2 binds dsRNA. *A*, HEK293 cells were transfected with an ISRE-luciferase reporter gene together with full-length RIG-I and increasing amounts of Lgp2. Where indicated, cells were infected with SV in the absence of RIG-I as a control. *B*, Cell lysates from HEK293 cells transfected with RIG-I-YFP, Mal-CFP, or Lgp2-YFP were subjected to poly(I-C) pull-down assays as indicated. Poly(I-C)-agarose-associated complexes were subjected to SDS-PAGE and immunoblot analysis with anti-GFP. The expression of each protein before pulldown is shown by immunoblot with anti-GFP in the input lysate. *C*, Lysates from a HEK-Lgp2-YFP stable cell line were subjected to poly(I-C) or poly(C) pull-down. Where indicated, soluble poly(I-C) was added to cell lysates for competition analysis. In each case, immunoprecipitated complexes were subjected to SDS-PAGE and immunoblot analysis with anti-GFP (BD Clontech).

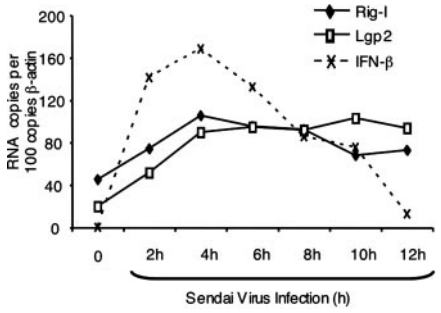

FIGURE 7. Lgp2 and RIG-I are virus-inducible genes. Raw264.7 macrophages were infected with SV (60 HAU), and mRNA expression of RIG-I, Lgp2, IFN-β, and β-actin was measured by quantitative PCR. Data were normalized with β-actin and are presented as a ratio of gene copy number per 100 copies of β-actin.

of Lgp2 inhibited the response, consistent with Fig. 5, *C–E*. These results suggest that Lgp2 blocks at a level upstream of RIG-I in SV signaling, most likely by sequestering dsRNA.

We therefore examined the ability of Lgp2 to bind dsRNA, as has been shown for RIG-I. Consistent with published reports, RIG-I binds dsRNA (Fig. 6*B*). Under similar conditions, Lgp2 also bound dsRNA. As a control for these experiments, the TIR domain adapter, Mal, did not bind dsRNA. We also generated a stable cell line expressing Lgp2-YFP to further examine the ability of Lgp2 to bind dsRNA. As shown in Fig. 6*C*, Lgp2 bound to poly(I-C), but not poly(C), beads. Preincubation of cell lysates with soluble poly(I-C) inhibited Lgp2 binding to dsRNA. Collectively, these data suggest that like RIG-I, Lgp2 is a dsRNA-binding protein.

Because Lgp2 appears to function as a negative regulator of virus-induced signaling, we wanted to compare expression levels of RIG-I and Lgp2 before and after infection with SV. Lgp2 and RIG-I mRNA levels were monitored by quantitative real-time PCR in murine macrophages (RAW264.7) in the first 12 h after infection (Fig. 7). Notably, RIG-I was expressed at a much higher level than Lgp2 in resting cells. The expression of both helicases reached similar levels 4–6 h after infection. IFN-β levels also peaked ~4 h after infection and dropped rapidly thereafter. This expression profile is consistent with a model in which Lgp2 functions as a postinduction repressor that provides a negative feedback signal to RIG-I/Mda5-mediated IFN induction.

Discussion

The data presented in this study demonstrate that the detection of SV and related viruses, such as NDV, requires active viral replication; occurs independently of any single TLR, TIR adaptor, or TRAF family member; and is instead mediated by the cytoplasmic RNA helicase RIG-I. This is in agreement with several recent reports (19, 29, 34, 35). The presence of cytoplasmic RNA helicases, like RIG-I and Mda5, ensures that viruses that avoid recognition by TLRs in endosomal locations will still be detected by the immune system. In this regard, preliminary data from our group suggest that influenza virus-induced IFN-β can also be mediated by RIG-I (N. Goutagny and K. A. Fitzgerald, unpublished observations). It will be intriguing to determine whether these RNA helicases also participate in the detection of other viruses, such as HSV, which is also known to signal by TLR-dependent as well as TLR-independent mechanisms. The relative importance of TLR-dependent and TLR-independent responses in vivo is not well established. Although TLR-mediated recognition of viruses is not essential to overcome local infections of HSV or influenza (6, 11, 12), the recognition of MCMV by TLR9 is essential for survival of a systemic inoculation (36). The ubiquitously expressed cytoplasmic RNA helicases may therefore function to contain local infections, whereas the nucleic acid recognizing TLR3, -7, and -9 with their restricted expression in only specific cells of the immune system might come into play later, when an infection starts to disseminate. The fact that recognition of a virus by TLRs in vivo is not always advantageous for the host was recently demonstrated in the case of West Nile Virus and HSV (37, 38). Inflammatory cytokines induced by these two viruses in a TLR3- and TLR2-dependent fashion, respectively, facilitated the breakdown of the blood-brain barrier, leading to the development of encephalitis with fatal outcome. Mice deficient in the corresponding TLRs, therefore, had an unexpected survival advantage in these models (37, 38).

Our data show that in contrast to RIG-I, overexpression of Mda5 did not enhance SV-induced IRF-3 or NF-κB signaling, suggesting that Mda5 is not involved in the recognition of SV. A recent report demonstrated that the V protein of several paramyxoviruses, including that of SV, binds and inactivates Mda-5 (20). SV has therefore evolved immune evasion strategies to circumvent Mda-5-mediated recognition. The presence of RIG-I enables the host to initiate antiviral signaling despite inactivation of the Mda-5 pathway. Mda5 may play an important role in the recognition of viruses that do not contain such inhibitors. The first evidence that viruses have evolved strategies to evade RIG-I-mediated detection was recently reported (39). RIG-I can mediate hepatitis C virus (HCV) RNA-induced signaling to IFN genes in human hepatoma cells (40). However, HCV expresses a protease NS3/4A, which ablates NF-κB or IRF-3 signaling downstream of RIG-I, thereby attenuating the expression of IFN-$\alpha\beta$ and host antiviral defense genes that otherwise would suppress HCV replication (39).

To date, we do not understand how RIG-I signals to IRF-3 or NF-κB, with the exception that TANK-binding protein 1 is required for the IRF-3 pathway, and the IKK$\alpha\beta\gamma$ complex is required for the NF-κB pathway. RIG-I contains protein interaction caspase recruitment and activation domains (19). CARDs mediate homotypic interactions to promote signaling events (33) and have been linked to signaling from both TLRs and NOD proteins. NODs contain nucleotide-binding sites, leucine-rich repeats, and CARD domains. NODs oligomerize via their nucleotide-binding site domains (33) and recruit a CARD-containing kinase receptor-interacting protein (RIP) (also called CARD3, RICK, and CARDIAK) (41) to elicit NF-κB activation. RIP2 does not elicit IRF-3 reporter gene activation, and RIP2-deficient DCs and macrophages signal normally after SV infection (K. A. Fitzgerald, unpublished observations). Taken together, these studies suggest that RIP2 is not a downstream mediator of the RIG-I pathway, and additional CARD domain-containing proteins instead mediate these events. A recent study has suggested that Fas-associated death domain protein-deficient embryonic fibroblasts have an impaired ability to induce IFN reporters in response to cytoplasmically delivered poly(I-C), suggesting that Fas-associated death domain protein may play a role in this RIG-I pathway (42).

This is the first study to demonstrate that Lgp2 acts as a natural host-derived negative regulator of RIG-I signaling. We hypothesize that Lgp2 functions to prevent overproduction of IFNs, which, if left uncontrolled, could have negative consequences for the host. The presence of tightly controlled negative feedback mechanisms to maintain the balance between host defense and overactivation resulting in chronic inflammation and autoimmune disease has been well recognized. Examples include the IL-1R type II, which

functions as an inducible decoy receptor, sequestering IL-1 (43), MyD88s, an inducible splice variant of MyD88 that inhibits IL-1 and TLR signaling by blocking IRAK-4 recruitment to these receptors (44), and single Ig IL-1R-related molecule, a differentially expressed inhibitory regulator for IL-1 and TLR signaling that sequesters key signaling molecules, such as TRAF6 and IRAKs, away from the signaling receptors (45). It seems most likely that Lgp2, similar to the IL-1R type II, acts as a decoy receptor to sequester the ligand, dsRNA, away from its receptor, RIG-I. A similar mechanism has been suggested for the influenza virus NS1 protein that has the propensity to sequester dsRNA, thereby inhibiting the TLR-independent induction of IFN-α by influenza infection (46, 47). The expression profiles of RIG-I and Lgp-2, with a lower expression of Lgp2 in unstimulated cells that reaches similar levels after 4–6 h, suggest that Lgp2 may function as a postinduction repressor. If sequestering the ligand away from RIG-I is the main mechanism of action of Lgp2, as our data suggest, one might speculate that Lgp2 has a higher affinity for viral dsRNA than RIG-I to explain its powerful inhibition by a competitive mechanism. Additional studies, however, will be required to test this hypothesis directly.

To unveil the cross-talk between these inhibitory and activating receptors sensing the presence of viruses and to uncover strategies by which viruses evade these surveillance mechanisms will open up an exiting new field of virus-host interactions.

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Disclosures

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