THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 38, pp. 25471–25478, September 18, 2009

The Double-stranded RNA Binding Domain of the Vaccinia Virus E3L Protein Inhibits Both RNA- and DNA-induced Activation of Interferon β

Received for publication, May 8, 2009, and in revised form, June 26, 2009 Published, JBC Papers in Press, July 7, 2009, DOI 10.1074/jbc.M109.018895

Jean-Baptiste Marq, Stéphane Hausmann, Jeremy Luban, Daniel Kolakofsky[1], and Dominique Garcin

From the Department of Microbiology and Molecular Medicine, University of Geneva School of Medicine, 9 Avenue de Champel, 1211 Geneva, Switzerland

Vaccinia virus, a large DNA virus that replicates in the cytoplasm, expresses its E3L protein to inhibit the cellular innate immune response and apoptosis. E3L is a bifunctional protein that contains an N-terminal DNA binding domain (BD) and a C-terminal double-stranded RNA (dsRNA)-BD (residues 100–190), both of which contribute to viral pathogenesis by blocking the activation of cellular genes that respond to the viral infection. We report that expression of the dsRNA-BD alone inhibits not only the dsRNA-induced activation of interferon β (IFNβ) but also that of 5′-triphosphate single-stranded RNA and DNA-induced IFNβ activation even though E3L$^{100-190}$ does not bind the latter two pathogen-associated molecular patterns. This inhibition occurs in both human HeLa and A549 cells, where RIG-I appears to be required for dsDNA-induced IFNβ activation. Unexpectedly, the two residues most important for dsRNA binding are also critical for this domain's ability to inhibit all three nucleic acid-induced cellular responses.

The cellular innate immune system is activated by the presence of pathogen-associated molecular patterns (PAMPs),[2] which are recognized by cellular pattern recognition receptors (PRRs). PRRs are present both at the cell surface and in endosomes to survey the extracellular space (*e.g.* toll-like receptors (TLRs)) and in the cytoplasm (*e.g.* NODs, and RIG-I-like receptors) (1, 2). Bacterial PAMPs are generally structures unique to these microorganisms (*e.g.* lipid A or peptidoglycan motifs). Viral PAMPs, in contrast, are based on nucleic acids that are abundantly present in all organisms. The special features of viral nucleic acids that are recognized as PAMPs depend on their intracellular location (cytoplasm and endosome) and, in the case of viral RNAs, on their special structural features; namely, perfectly base-paired dsRNA, presumably the result of convergent transcription (3, 4), and the presence of free 5′-triphosphate ends (ppp-RNAs) (5, 6). All eukaryotic cellular RNAs are initiated with nucleotide 5′-triphosphates, and the possibilities for overlapping convergent transcription from cellular DNA genomes are not uncommon. However, most cellular RNAs are also made in the nucleus, and it is thought that essentially only ssRNAs without free 5′-triphosphate ends are allowed to enter the cytoplasm. Some cellular polymerase III transcripts with 5′-triphosphate ends do enter the cytoplasm, but their 5′-ends are thought to be stably bound to the proteins required for their nuclear export. Thus, perfectly base-paired dsRNAs and ppp-RNAs in the cytoplasm are essentially those which have been made there, *i.e.* they are most likely viral RNAs. Similarly, the presence of DNA in the cytosol elicits an IRF3-dependent innate immune response (7, 8).

RIG-I and mda-5 are DE*X*D/H box helicases with N-terminal CARDs (caspase recruitment domains), which are present in the cytoplasm of most cells and serve as PRRs of viral RNAs (9, 10). RIG-I serves as a PRR in most RNA virus infections and senses both 5′-ppp-ssRNAs and relatively short dsRNAs (<1 kilobase pair), whereas mda-5 is specifically required to mount an innate immune response to picornavirus infections and senses relatively long dsRNAs (>1 kilobase pair) (11). Upon interacting with their RNA PAMPs, RIG-I and mda-5 initiate a signaling cascade that activates the IFNβ promoter, leading to IFN production. IFN binding to its cell surface receptor then leads to the synthesis of multiple IFN-stimulated genes, which together help establish an antiviral state (12, 13). RIG-I and mda-5 bind dsRNAs and signal via the mitochondrial adaptor IPS-1, but these helicases do not bind dsDNA (8). DAI (DNA activator of IRFs), which contains two Z-DNA binding domains and which binds dsDNA but not dsRNA, was the first (and so far only) cytosolic DNA sensor to be identified (14). Binding of dsDNA to DAI enhances its association with IRF3 and the kinase TBK1, and overexpression of DAI in MEFs selectively enhances DNA-mediated induction of type I IFNs and other genes involved in innate immunity. However, the adaptor through which DAI signals to IRF3 has not been identified. Furthermore, RNAi knockdown of DAI and the generation of DAI-deficient mice have indicated that DAI is not the only PRR for cytosolic DNA (15).

Many viruses encode proteins that interfere with the innate immune response. For example, vaccinia virus (VV), a DNA virus that replicates in the cytoplasm, encodes the E3L protein that antagonizes the effects of IFNs intracellularly (16). Part of this antagonism is because of its dsRNA binding domain (residues 100–190), which is thought to sequester the dsRNA produced during virus infection. By binding dsRNAs, E3L inhibits the activation of PKR and other dsRNA-dependent IFN-stimulated genes (17, 18), thus relieving the translational block that

[1] To whom correspondence should be addressed. E-mail: Daniel.kolakofsky@unige.ch.

[2] The abbreviations used are: PAMP, pathogen-associated molecular pattern; PRR, pattern recognition receptor; dsRNA, double-stranded RNA; ssRNA, single-stranded RNA; IFN, interferon; MEF, mouse embryo fibroblast; DAI, DNA activator of IRF; VV, vaccinia virus; GFP, green fluorescent protein; BD, binding domain; TK, thymidine kinase; WT, wild type; KD, knock down; PKR, protein kinase activated by RNA; IRF, interferon regulatory factor.

is in part responsible for VV-induced apoptosis (19). VV deleted of E3L has a narrower host range because of the failure to inhibit apoptosis (20), and the deleterious effects of VV-Δ E3L infection can be compensated in large part by reducing cellular PKR levels (21). Besides antagonizing the antiviral effects of the IFN-stimulated genes, E3L is also known to inhibit the dsRNA-induced activation of IFNβ (22), presumably because of its ability to interfere with the recognition of viral RNAs by the cytoplasmic helicases. This paper reports that the VV E3L dsRNA-BD alone ($E3L^{100-190}$) also inhibits the 5′-ppp-ssRNA- and dsDNA-induced activation of IFNβ in both murine and human cells even though it does not bind to these nucleic acids. Unexpectedly, the two residues most important for dsRNA binding are also critical for this domain's ability to inhibit all three nucleic acid induced cellular responses.

EXPERIMENTAL PROCEDURES

Cells, Viruses, and Antibodies—HeLa, A549, and MEF cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Some A549 cells were "knocked down" for RIG-I or IRF3 by infection with lentiviruses expressing micro RNAs targeting either RIG-I (5′-tgcaggtattctggactttat) or IRF3 mRNAs (5′-ggcccttcattgtagatctgat) (Rig-I-KD and IRF3-KD). 24 h post-inoculum, cells were selected with puromycin (10 μg/ml) for 72 h.

rSeV-GFP(+), which expresses green fluorescent protein (GFP) from a transgene between the M and F genes, and rSeV-$E3L^{100-190}$, which expresses $E3L^{100-190}$ from a similarly located transgene, were prepared as previously described (23). DI-H4 stocks were described previously (24). Primary antibodies used included mouse anti-Rig-I (Alexis), mouse anti-actin (Chemicon), mouse anti-HA (Berkeley Antibody Co., Inc.), and rabbit anti-Sendai P,V,C (homemade).

Plasmids, Transient Transfections, Infections, Inductions, and Luciferase Assay—FLAG-tagged RIG-I was obtained from Jurg Tshopp (Lausanne) and inserted into our expression vector pEBS. pβ-IFN-fl-lucter, which contains the firefly luciferase gene under the control of the human IFN-β promoter, was described previously (25). pTK-rl-lucter, used as a transfection standard, contains the herpes simplex virus TK promoter region upstream of the *Renilla* luciferase gene (Promega). pEBS-$E3L^{100-190}$ and pEBS-$E3L^{100-190}$ (K167A/R168A) were described previously (26).

Transfections—100,000 cells were plated into 6-well plates 20 h before transfection with 1.5 μg of pβ-IFN-fl-lucter, 0.5 μg of pTK-rl-lucter, 1 μg of plasmids expressing RIG-I, E3L (according to the various figure legends), and TransIT-LT1 transfection reagent (Mirus). At 24 h post-transfection, the cells were (or were not) infected with various SeV stocks or transfected with 5 μg of poly(I-C) (sigma) or 10 μg of poly-(dAdT) using TransIT-LT1 transfection reagent. Twenty hours later, cells were harvested and assayed for firefly and *Renilla* luciferase activity (dual-luciferase reporter assay system; Promega). Relative expression levels were calculated by dividing the firefly luciferase values by those of *Renilla* luciferase.

Immunoblotting—Cytoplasmic extracts were prepared using 0.5% Nonidet P-40 buffer. Equal amounts of total proteins were separated by SDS-PAGE and transferred onto Immobilon-P membranes by semidry transfer. The secondary antibodies used were alkaline phosphatase-conjugated goat anti-rabbit (or mouse) immunoglobulin G (Bio-Rad). The immobilized proteins were detected by light-enhanced chemiluminescence (Pierce) and analyzed in a Bio-Rad light detector using Quantity One software.

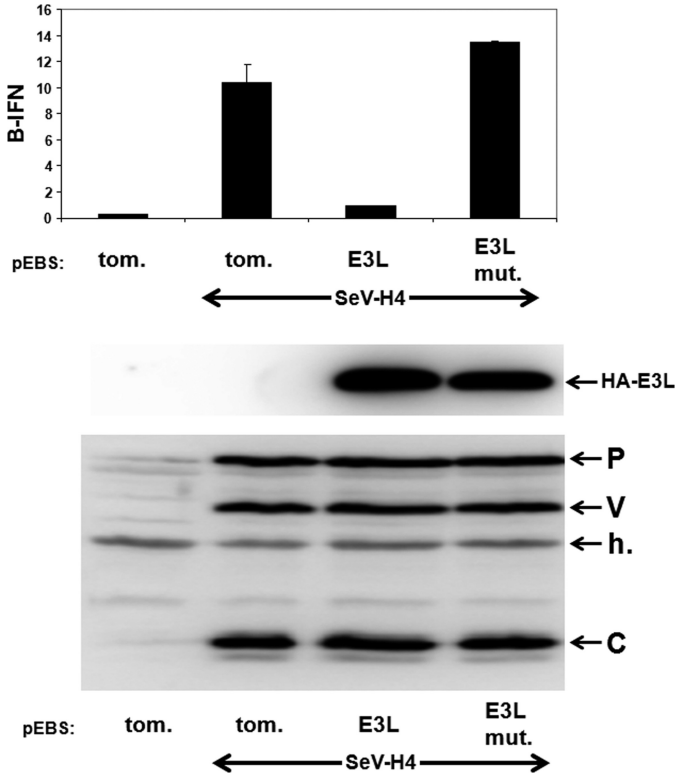

FIGURE 1. **The effect of $E3L^{100-190}$ expression on SeV DI-H4 induced IFNβ activation.** Parallel cultures of MEFs were transfected with pIFNβ-(ff)luciferase and pTK-(ren)luciferase plus either pEBS-(HA)$E3L^{100-190}$ or mutant (HA)$E3L^{100-190}$/K167A/R168A or pEBS-tomato as a negative control. The cultures were then mock-infected or infected with SeV DI-H4 (*H4*) at 24 h post-transfection. Cell extracts were prepared at 20 hours post-inoculum, and their luciferase activities were determined. Equal amounts of the extracts (total proteins) were also Western-blotted with anti-HA (*middle panel*) and anti-SeV P/V/C (3 SeV proteins expressed from the viral P gene; *lower panel*). A cross-reacting host band (*h*) serves as a loading control.

In Vitro Synthesis of RNA, Purification, Transfection, and Treatment—DNA for T7 RNA polymerase synthesis of model RNA1 was prepared by PCR using the partially complementary primers 5′-TAATACGACTCACTATAgggACACACCACA-ACCAACCCACAAC-3′ (forward) (start sites are in lowercase type) and 5′-GAAAGAAAGGTGTGGTGTTGGTGTGGTT-GTTGTGGGTTGGTTGTGG-3′ (reverse). Transcription was performed with 100 pmol of purified PCR product using T7 MEGAshortscript (Ambion) according to the manufacturer's instructions. Biotinylated RNA1 was synthesized using equal amounts of 5′-biotin-UTP and UTP. The total T7 transcripts (unpurified ppp-ssRNA1) were digested with DNase I and then chromatographed on NucAway Spin columns (Ambion) to remove unincorporated nucleotides and DNA fragments. Slightly radiolabeled ([α-^{32}P]CTP) T7 transcripts were further electrophoresed on 10% preparative denaturing gels. The major 55-nucleotide band was excised from the gel, and the RNA was eluted followed by ethanol precipitation.

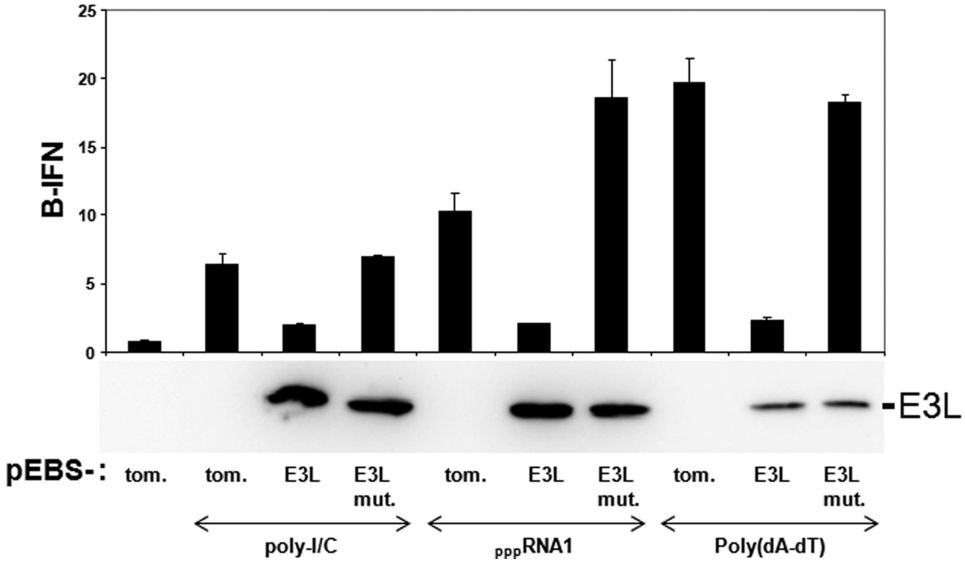

FIGURE 2. **The effect of WT and mutant E3L$^{100-190}$ expression on nucleic acid-induced IFNβ activation.** Parallel cultures of MEFs were transfected with pIFNβ-(ff)luciferase and pTK-(ren)luciferase and either pEBS-HA-E3L$^{100-190}$, pEBS-mutant HA-E3L$^{100-190}$/K167A/R168A, or pEBS-tomato (as a negative control). The cultures were then further transfected 24 h later with either poly-I/C (5 μg), ppp-RNA1 (2.5 μg), or poly(dA-dT) (10 μg) as indicated. Cell extracts were prepared 20 h later, and their luciferase activities were determined. Equal amounts of the extracts (total proteins) were also Western-blotted with anti-HA.

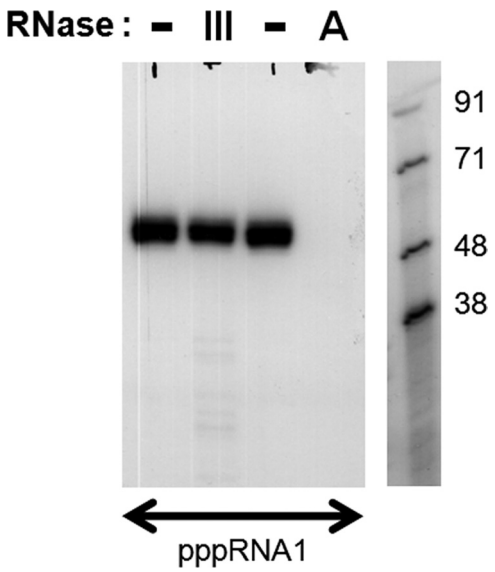

FIGURE 3. **Sensitivity of *in vitro* transcripts to RNase.** The products of the ppp-RNA1 transcription reaction, which had been DNase-treated, phenol-extracted, and recovered from a preparative denaturing gel by ethanol precipitation were either left untreated (−) or digested with RNase III (III) or RNase A (*A*) under conditions that are specific for dsRNA and ssRNA, respectively ("Experimental Procedures") as indicated. The RNAs were then further phenol-extracted and separated on a 10% sequencing gel. ssRNA size markers were run alongside (*right lane*).

RNase Treatment—1 μg of ppp-ssRNA1 was digested with 1 unit of RNase III (Ambion) for 60 min at 37 °C in 40 μl of 50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol (pH 7.9), or 50 ng of RNase A in 40 μl of 10 mM Tris-HCl, 10 mM EDTA, and 0.4 M NaCl (pH 7.9). The digestion products were then phenol/chloroform=extracted, ethanol-precipitated with 10 μg of glycogen, and electrophoresed on a denaturing 10% polyacrylamide gel.

IFN Assay—Supernatants (400 μl (1×) or 1 600 μl (4×)) of MEF cells transfected with poly-I/C or poly(dA-dT) for 20 h were added to fresh MEF cells for 12 h, which were then infected with 20 plaque-forming units/cell of rVSV-GFP (Jacques Perrault, San Diego State University) for 14 h. Then cells were trypsinized and analyzed by fluorescence-activated cell sorter for GFP expression.

Poly(dA-dT)—Poly(dA-dT) was purchased from Sigma (catalog no. P0883).

RESULTS

Wild-type SeV infection or infection with SeV-GFP activates the IFNβ promoter very poorly under normal conditions because of the robust expression of the viral C and V proteins, which counteract the host innate immune response. However, co-infection of cells with rSeV-GFP- and rSeV-expressing mRNA containing the complement of the GFP open reading frame does lead to IFNβ activation (26). This activation presumably results from the formation of GFP dsRNA, as this IFNβ activation does not occur when a SeV-expressing RFP replaces either of the SeV-expressing GFP RNAs. Moreover, the simultaneous expression of the dsRNA binding domain of the vaccinia virus E3L protein (residues 100–190) prevented the IFNβ activation, whereas expression of a mutant form of this domain that no longer bound dsRNA (K167A/R168A) (27) had no effect. More commonly, the SeV infection used to activate IFNβ is carried out with stocks containing relatively short, copyback DI genomes (Fig. 1, *upper panel*). In this case IFNβ activation is thought to be because of the combined action of unassembled DI genome ppp-RNA (whose ends can also form dsRNA panhandles intramolecularly) and to the overexpression of trailer ppp-ssRNA (26). When the ability of E3L$^{100-190}$ to prevent IFNβ activation induced by SeV DI-H4 infection was examined, we found that this dsRNA binding domain was also highly effective in inhibiting this activation. Moreover, this inhibitory activity was also lost in the mutant protein (K167A/R168A) unable to bind dsRNA (Fig. 1).

We next examined whether the ability of E3L$^{100-190}$ to efficiently inhibit DI-H4-induced IFNβ activation was due solely to its ability to bind dsRNA (and thereby prevent its interaction with PRRs such as RIG-I and mda-5) by comparing the ability of E3L$^{100-190}$ to inhibit poly-I/C and 5′-ppp-ssRNA-induced IFNβ activation. At the same time we also examined the ability of E3L$^{100-190}$ to inhibit dsDNA (poly(dA-dT):poly(dA-dT) or poly(dA-dT))-induced IFNβ activation, as E3L is known not to bind either ssRNA or dsDNA (28). We have confirmed that E3L$^{100-190}$ does not bind our ppp-ssRNA1 or poly(dA-dT) (data not shown). Other than cytoplasmic 5′-ppp-ssRNA and dsRNA that are recognized by RIG-I/mda-5 (and which signal

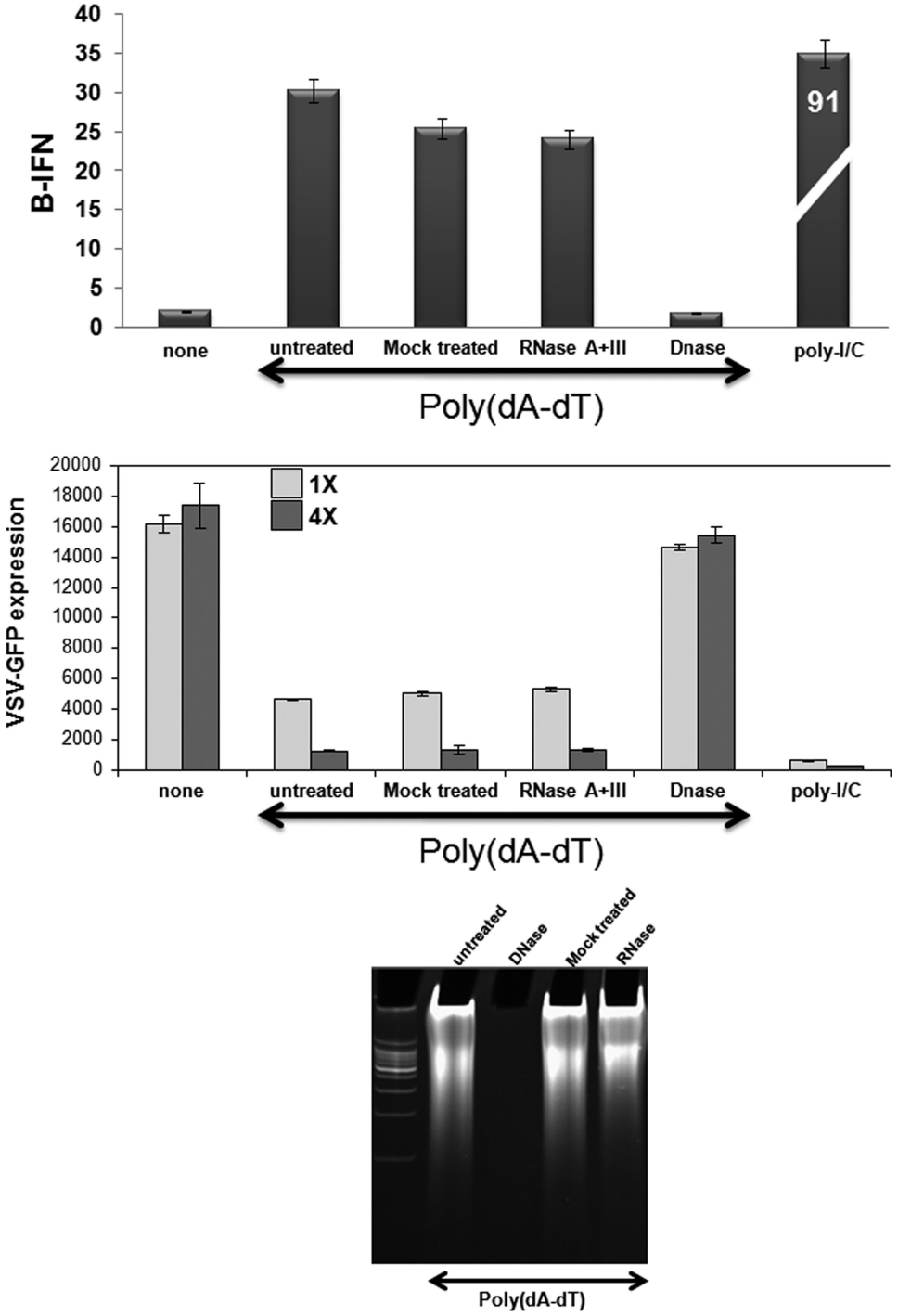

FIGURE 4. **Characterization of DNA-induced IFNβ activation.** Poly(dA-dT) (10 μg) that was either untreated, mock (DNase)-treated, digested with RNase III plus RNase A or DNase I and transfected into MEFs that had previously been transfected with pIFNβ-(ff)luciferase and pTK-(ren)luciferase. A sample of these DNAs was examined by non-denaturing PAGE (*bottom panel*). Poly-I/C was also transfected as a positive control. Cell extracts were prepared 20 h later, and their luciferase activities were determined (*top panel*). 400 (1×) and 1600 μl (4×) samples of the culture media of these transfections were added to naïve MEFs for 12 h, which were then infected with vesicular stomatitis virus (*VSV*)-GFP. After 121 h of incubation, the infected cells were harvested and analyzed by fluorescence-activated cell sorter (*middle panel*) for GFP expression.

via the mitochondrial membrane adapter IPS-1), cytoplasmic dsDNA is also recognized as a PAMP that activates IFNβ. Different synthetic dsDNAs are known to vary widely in their ability to stimulate an IFN response (8, 29). Poly(dA-dT) has by far the strongest effect on type I IFN expression, possibly because of its propensity to adopt different DNA conformations, including B-DNA and Z-DNA (30, 31). dsDNA is recognized in part by DAI in murine cells, which binds dsDNA, but the adapter through which the signaling occurs is unknown.

As shown in Fig. 2, we unexpectedly found that coexpression of $E3L^{100-190}$ strongly inhibited all three nucleic acid PAMP-induced IFNβ activations, whereas coexpression of mutant $E3L^{100-190}$ was again unable to prevent any of the nucleic acid PAMP-induced IFNβ activations. To examine whether our 5′-ppp-ssRNA unexpectedly contained significant amounts of dsRNA character, we treated this RNA with RNase III or RNase A under conditions that are specific for the digestion of dsRNA or ssRNA, respectively, followed by PAGE. We found that our 5′-ppp-ssRNA was essentially resistant to RNase III digestion but entirely sensitive to RNase A digestion (Fig. 3). To ensure that dsDNA was responsible for the IFNβ activation, our poly(dA-dT) was treated with either RNase A plus RNase III or DNase I before transfection, and the effects of these treatments were controlled by PAGE (Fig. 4, *bottom panel*)). Poly-I/C was also transfected as a positive control. Both the level of IFNβ reporter gene (luciferase) activation and the presence of endogenous IFN released into the medium were then examined (Fig. 4). Our poly(dA-dT)-induced IFNβ reporter gene activation was sensitive to DNase treatment but resistant to RNase digestion, and the extent of IFNβ reporter gene activation in all cases correlated well with the antivesicular stomatitis virus activity (IFN) released into the medium.

The expression of E3L by plasmid transfection has the added complication that additional dsDNA is simultaneously being introduced into the cytosol (at least during its transit to the nucleus). Moreover, the effects of its expression are examined in the absence of a viral infection, during which it normally functions. We, therefore, prepared rSeV that express WT $E3L^{100-190}$ as a transgene and tested the ability of this protein expressed via SeV infection

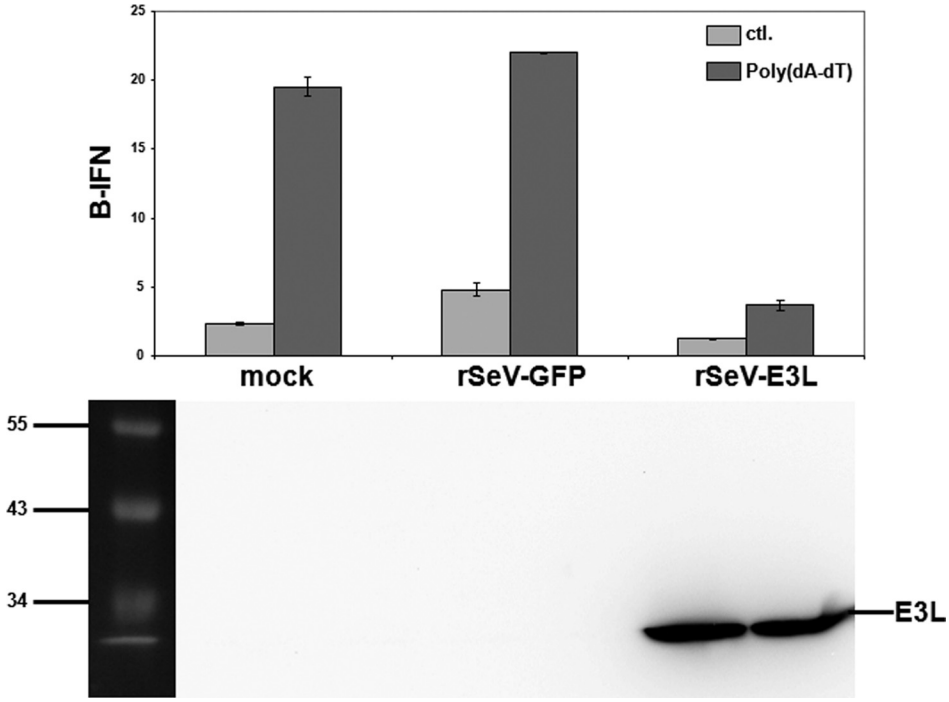

FIGURE 5. **The effect of E3L$^{100-190}$ expressed from rSeV on poly(dA-dT)-induced IFNβ activation.** Parallel cultures of MEFs were transfected with pIFNβ-(ff)luciferase and pTK-(ren)luciferase and then either mock-infected or infected with either SeV expressing HA-E3L$^{100-190}$ or GFP (as a negative control). The cultures were then further transfected 24 h later with poly(dA-dT) as indicated. Cell extracts were prepared 20 h later, and their luciferase activities were determined. Equal amounts of the extracts (total proteins) were also Western-blotted with anti-HA. *ctl.*, control.

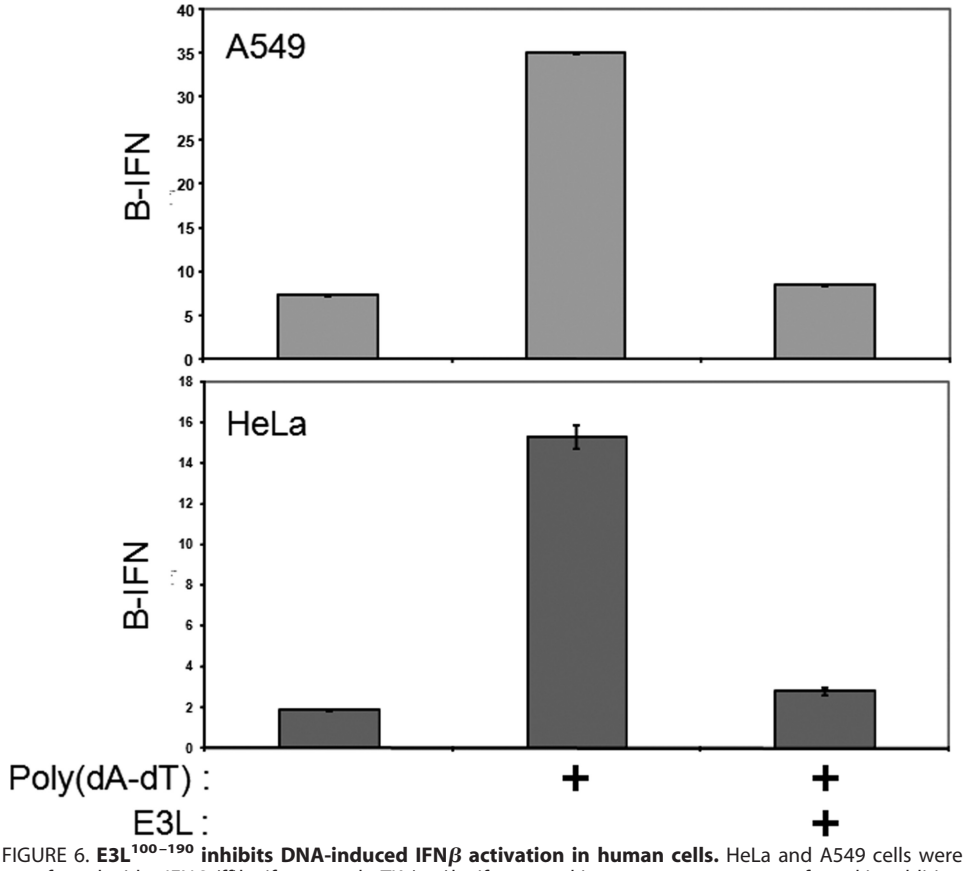

FIGURE 6. **E3L$^{100-190}$ inhibits DNA-induced IFNβ activation in human cells.** HeLa and A549 cells were transfected with pIFNβ-(ff)luciferase and pTK-(ren)luciferase and in some cases were transfected in addition with pEBS-(HA)E3L$^{100-190}$. Some cultures were then further transfected 24 h later with poly(dA-dT) as indicated. Cell extracts were prepared 20 h later, and their luciferase activities were determined.

to inhibit poly(dA-dT)-induced IFNβ activation. As a neutral control, some cultures were infected with SeV-GFP. As shown in Fig. 5, the transgenic expression of E3L$^{100-190}$ during SeV infection largely eliminated the poly(dA-dT)-induced IFNβ activation relative to that of the GFP control. Thus, the co-expression of E3L$^{100-190}$ strongly inhibits 5′-ppp-ssRNA-, dsRNA-, and dsDNA-induced IFNβ activation independent of whether this protein domain can bind these nucleic acids. Nevertheless, the two residues most important for dsRNA binding are also critical for the ability of this domain to inhibit all three nucleic acid-induced cellular responses.

DNA Induced IFNβ Activation in Human Cells—Poly(dA-dT)-induced IFNβ activation occurs normally in RIG-I deficient MEFs (8). In contrast, RIG-I appears to be required for this IFNβ activation in human Huh-7 hepatoma cells (29). As E3L$^{100-190}$ expression is equally effective in inhibiting poly(dA-dT)-induced IFNβ activation in human HeLa and A549 cells (fig. 6), we examined whether RIG-I was required for this primary innate immune response in A549 cells. An A549 cell line in which RIG-I levels were strongly reduced was established by infecting these cells with a lentivirus expressing a micro RNA targeting the human RIG-I mRNA (RIG-I-KD) (Fig. 7). RIG-I levels were also restored in RIG-I-KD cells by plasmid transfection (pEBS-RIG-I; RIG-I-KI). When poly-I/C or poly(dA-dT) were transfected into RIG-I-KD *versus* control A549 cells, both double-stranded nucleic acids were considerably less effective in inducing IFNβ activation (Fig. 7*B*). In addition, the restoration of RIG-I to WT levels in the RIG-I-KD cells clearly restored the ability of both poly-I/C and poly(dA-dT) to induce IFNβ activation (RIG-I-KI; Fig. 7*B*). Thus, RIG-I is equally important for dsDNA- and dsRNA-induced IFNβ activation in human A549 cells, similar to Huh-7 cells.

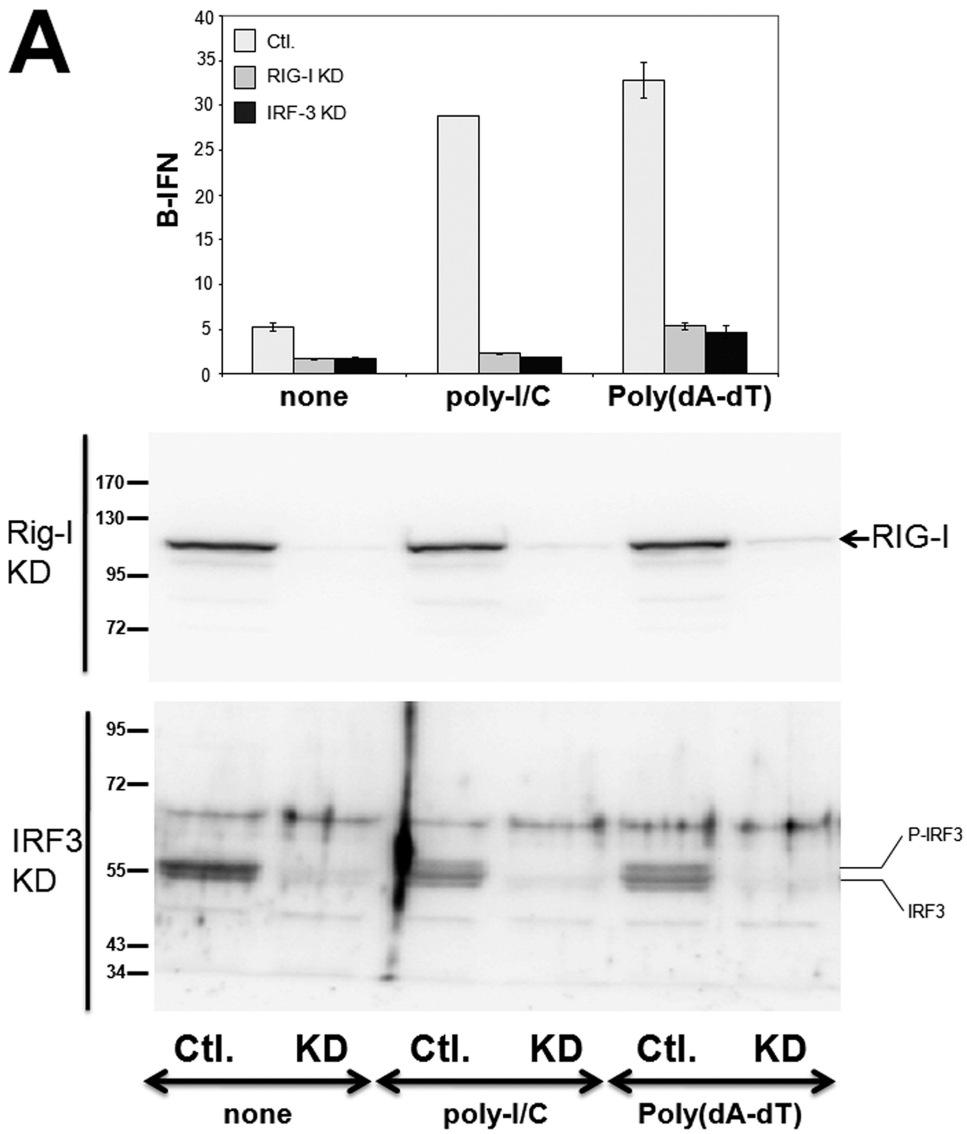

FIGURE 7. **RIG-I and IRF3 are required for DNA induced IFNβ activation in human A549 cells.** *A*, A549 cell lines in which either IRF3 or RIG-I levels were strongly reduced (by infection with lentiviruses expressing micro RNAs targeting their respective mRNAs) (IRF3-KD and RIG-I-KD) and control A549 cells were transfected with pIFNβ-(ff)luciferase and pTK-(ren)luciferase. Some cultures were then further transfected 24 h later with either poly-I/C (5 μg) or poly(dA-dT) (10 μg) as indicated. Cell extracts were prepared 20 h later, and their luciferase activities were determined. Equal amounts of the extracts (total proteins) were also Western-blotted with either anti-IRF3 or anti-RIG-I. The phosphorylated form of IRF3 is also indicated. *Ctl.*, control. *B*, RIG-I-KD cells were transfected with pIFNβ-(ff)luciferase and pTK-(ren)luciferase and in some cases were transfected in addition with pEBS-RIG-I (RIG-I-KI cells). Some cultures were then further transfected 24 h later with either poly-I/C or poly(dA-dT), as indicated. Cell extracts were prepared 20 h later, and their luciferase activities were determined. Equal amounts of the extracts (total proteins) were also Western-blotted with anti-RIG-I and anti-actin. The *right lane* shows the level of RIG-I in control (WT) A549 cells.

innate immune response by all three of these nucleic acid PAMPs. What is surprising is that all these activities are present in the dsRNA-BD of the E3L protein. The bifunctional VV E3L protein contains both a Z-DNA-BD at its N terminus (which to date has been found only on DAI, ADAR1, and PKZ (a pseudo-log of PKR)) (15)) and a dsRNA-BD at its C terminus. Both these E3L nucleic acid binding domains contribute to viral pathogenesis in animal models and in cell culture at least in part by blocking the activation of cellular genes that respond to the viral infection (22). It had been assumed that the ability to E3L to block IFNβ activation induced by cytosolic DNA was because of its Z-DNA-BD, which was thought to sequester cytoplasmic DNA and prevent its interaction with DAI and other DNA sensors (14), similar to the action of the E3L dsRNA-BD and dsRNA. Although this may be so, this report provides evidence that the dsRNA-BD alone, expressed either via plasmid transfection or transgenic SeV infection, can also prevent DNA-induced IFNβ activation. Specifically, we found that the co-expression of the dsRNA-BD of the vaccinia virus E3L protein ($E3L^{100-190}$) strongly inhibits 5′-ppp-ssRNA-, dsRNA (poly-I/C)-, and dsDNA (poly(dA-dT))-induced IFNβ activation independent of whether this protein domain can bind these nucleic acids. Moreover, $E3L^{100-190}$ is equally effective in cells of human and murine origin, where RIG-I is and is not, respectively, required for this DNA-induced activation. Equally surprising, the ability of $E3L^{100-190}$ to inhibit these nucleic acid-induced IFNβ activations appears to depend in all cases on its ability to bind dsRNA.

A similar approach showed that IRF3 was also required for poly(dA-dT)-induced IFNβ activation in A549 cells as expected (Fig. 7*A*). Notably, within the limits of this approach, RIG-I appeared to be as important as IRF3 in dsDNA-induced IFNβ activation.

DISCUSSION

VV is a DNA virus that replicates in the cytoplasm, and infected cells presumably contain some uncapped transcripts (5′-ppp-ssRNA), overlapping convergent transcripts (dsRNA) and viral dsDNA. It is, therefore, not surprising that VV encodes a function(s) that interferes with the induction of the

As neither RIG-I nor IPS-1 are apparently required for dsDNA-induced IFNβ activation in murine cells, whereas both these proteins appear to be required for this activation in human hepatoma cells, we have examined this requirement in another human cell line, A549, along with that of dsRNA (poly-I/C) as a positive control. We found that RNAi-induced reduction of RIG-I strongly reduced IFNβ activation by both double-stranded nucleic acids and that restoration of the RIG-I to WT levels clearly restored the activation

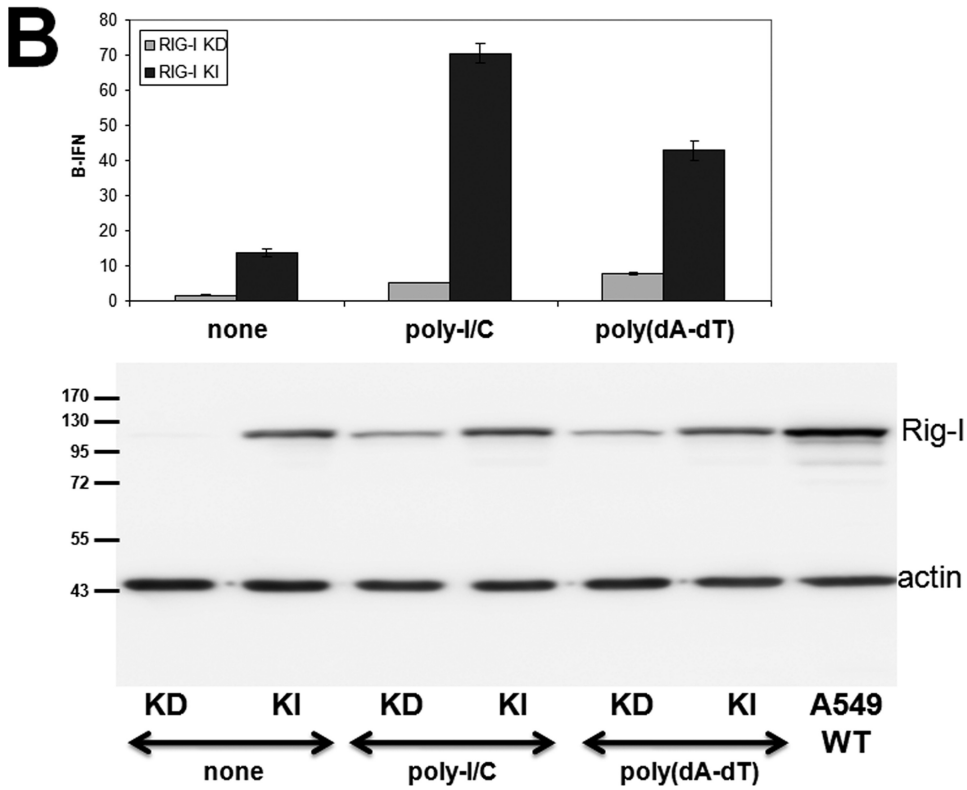

FIGURE 7—*continued*

by both dsRNA and dsDNA (Fig. 7). However, as RIG-I does not bind dsDNA and poly(dA-dT) is totally inactive in stimulating the ATPase activity of RIG-I *in vitro* (a good indicator of RIG-I signaling[3]), the apparent requirement of RIG-I in dsDNA induced IFNβ activation remains unclear and may well be indirect.

Perhaps the most baffling of our findings concerning $E3L^{100-190}$ inhibition of both RNA- and DNA-induced innate immune responses is that in all cases two point mutations designed to specifically abrogate dsRNA binding (27) also eliminated the activity of this domain in preventing 5′-ppp-ssRNA- and DNA-induced IFNβ activation (Fig. 4). Two possible explanations come to mind. The most obvious is that $E3L^{100-190}$ interaction with its downstream signaling target requires its association with dsRNA. There may be an interesting precedent for this (32). Expression of PKR in yeast is lethal as it inhibits protein synthesis by phosphorylating eIF2α. Remarkably, co-expression of E3L in yeast overcomes the lethal effect of PKR in a manner requiring the same two key residues (Lys-167 and Arg-168) needed for dsRNA binding by E3L. In yeast two-hybrid assays and *in vitro* protein binding experiments, segments of E3L and PKR containing their respective dsRNA-BDs interacted in a manner requiring E3L residues Lys-167 and Arg-168. Romano *et al.* (32) proposed that effective inhibition of PKR in yeast requires formation of an E3L-PKR-dsRNA complex. However, the source of the dsRNA here, as in the case of 5′-ppp-ssRNA- and DNA-induced IFNβ activation described above, remains unclear.

dsRNA provides a platform on which various dsRNA binding proteins can interact with each other, and the binding of E3L to dsRNA greatly contributes to the stability of the E3L-PKR complex (32). However, it is also possible that E3L can form a complex with its downstream signaling target independent of the presence of dsRNA but, nevertheless, in a Lys-167/Arg-168-dependent manner. In this second case, E3L binding to dsRNA and the signaling target would presumably be mutually exclusive. The dsRNA binding motif is known to be a versatile macromolecular interaction platform that can in some cases also interact with DNA and proteins (for review, see Ref. 33). dsRNA-BDs are known to heterodimerize with other dsRNA-BDs and to also recognize structurally different protein targets. However, the nature of these dsRNA-BD protein-protein interactions is not well characterized, and it is not known whether Lys-167 and Arg-168 of E3L are essential here as well. Evidence has recently been provided that PKR facilitates the host innate immune response to viral infection by mediating IRF3 activation through the IPS-1 signal transduction pathway (34). $E3L^{100-190}$ can, thus, also act indirectly to prevent the host innate immune response by preventing PKR facilitation of IRF3 phosphorylation. It will be of interest to determine whether Lys-167 and Arg-168 of E3L are essential for any of its protein-protein interactions independent of dsRNA.

Note Added in Proof—Ablasser *et al.* have just reported that AT-rich dsDNA serves as a template for RNA polymerase III and is transcribed into 5′ ppp-dsRNA, which is then sensed by RIG-I to induce IFN. The existence of this dsDNA-sensing pathway provides a cogent explanation for how E3L(100–190) inhibits poly-(dA-dT)-induced IFNβ activation and why this inhibitory activity is lost when this E3L domain can no longer bind dsRNA.

[3] J.-B. Marq, S. Hausmann, J. Luban, D. Kolakofsky, and D. Garcin, unpublished data.

REFERENCES

1. Medzhitov, R., and Janeway, C. A., Jr. (1997) *Cell* **91,** 295–298
2. Meylan, E., Tschopp, J., and Karin, M. (2006) *Nature* **442,** 39–44
3. Colby, C., and Chamberlin, M. J. (1969) *Proc. Natl. Acad. Sci. U.S.A.* **63,** 160–167
4. Jacobs, B. L., and Langland, J. O. (1996) *Virology* **219,** 339–349
5. Hornung, V., Ellegast, J., Kim, S., Brzózka, K., Jung, A., Kato, H., Poeck, H., Akira, S., Conzelmann, K. K., Schlee, M., Endres, S., and Hartmann, G. (2006) *Science* **314,** 994–997
6. Pichlmair, A., Schulz, O., Tan, C. P., Näslund, T. I., Liljeström, P., Weber, F., and Reis e Sousa, C. (2006) *Science* **314,** 997–1001
7. Stetson, D. B., and Medzhitov, R. (2006) *Immunity* **24,** 93–103
8. Ishii, K. J., Coban, C., Kato, H., Takahashi, K., Torii, Y., Takeshita, F., Ludwig, H., Sutter, G., Suzuki, K., Hemmi, H., Sato, S., Yamamoto, M., Uematsu, S., Kawai, T., Takeuchi, O., and Akira, S. (2006) *Nat. Immunol.*

7, 40–48
9. Kawai, T., and Akira, S. (2007) *J. Biochem.* **141,** 137–145
10. Yoneyama, M., and Fujita, T. (2007) *J. Biol. Chem.* **282,** 15315–15318
11. Kato, H., Takeuchi, O., Mikamo-Satoh, E., Hirai, R., Kawai, T., Matsushita, K., Hiiragi, A., Dermody, T. S., Fujita, T., and Akira, S. (2008) *J. Exp. Med.* **205,** 1601–1610
12. Stark, G. R., Kerr, I. M., Williams, B. R., Silverman, R. H., and Schreiber, R. D. (1998) *Annu. Rev. Biochem.* **67,** 227–264
13. Samuel, C. E. (2001) *Clin. Microbiol. Rev.* **14,** 778–809
14. Wang, Z., Choi, M. K., Ban, T., Yanai, H., Negishi, H., Lu, Y., Tamura, T., Takaoka, A., Nishikura, K., and Taniguchi, T. (2008) *Proc. Natl. Acad. Sci. U.S.A.* **105,** 5477–5482
15. Takaoka, A., and Taniguchi, T. (2008) *Adv. Drug Deliv. Rev.* **60,** 847–857
16. Chang, H. W., Watson, J. C., and Jacobs, B. L. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89,** 4825–4829
17. Chang, H. W., and Jacobs, B. L. (1993) *Virology* **194,** 537–547
18. Rivas, C., Gil, J., Mělková, Z., Esteban, M., and Díaz-Guerra, M. (1998) *Virology* **243,** 406–414
19. Lee, S. B., and Esteban, M. (1994) *Virology* **199,** 491–496
20. Langland, J. O., and Jacobs, B. L. (2002) *Virology* **299,** 133–141
21. Zhang, P., Jacobs, B. L., and Samuel, C. E. (2008) *J. Virol.* **82,** 840–848
22. Langland, J. O., Kash, J. C., Carter, V., Thomas, M. J., Katze, M. G., and Jacobs, B. L. (2006) *J. Virol.* **80,** 10083–10095
23. Strähle, L., Garcin, D., Le Mercier, P., Schlaak, J. F., and Kolakofsky, D. (2003) *J. Virol.* **77,** 7903–7913
24. Strahle, L., Garcin, D., and Kolakofsky, D. (2006) *Virology* **351,** 101–111
25. King, P., and Goodbourn, S. (1994) *J. Biol. Chem.* **269,** 30609–30615
26. Strähle, L., Marq, J. B., Brini, A., Hausmann, S., Kolakofsky, D., and Garcin, D. (2007) *J. Virol.* **81,** 12227–12237
27. Ho, C. K., and Shuman, S. (1996) *J. Virol.* **70,** 2611–2614
28. Ho, C. K., and Shuman, S. (1996) *Virology* **217,** 272–284
29. Cheng, G., Zhong, J., Chung, J., and Chisari, F. V. (2007) *Proc. Natl. Acad. Sci. U.S.A.* **104,** 9035–9040
30. Vorlícková, M., and Kypr, J. (1985) *J. Biomol. Struct. Dyn.* **3,** 67–83
31. Rich, A., Nordheim, A., and Wang, A. H. (1984) *Annu. Rev. Biochem.* **53,** 791–846
32. Romano, P. R., Zhang, F., Tan, S. L., Garcia-Barrio, M. T., Katze, M. G., Dever, T. E., and Hinnebusch, A. G. (1998) *Mol. Cell. Biol.* **18,** 7304–7316
33. Chang, K. Y., and Ramos, A. (2005) *FEBS J.* **272,** 2109–2117
34. Zhang, P., and Samuel, C. E. (2008) *J. Biol. Chem.* **283,** 34580–34587
35. Ablasser, A., Bauernfeind, F., Hartmann, G., Latz, E., Fitzgerald, K. A., and Hornung, V. (July 16, 2009) *Nat. Immunol.* 10.1038/ni.1779