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RIG-I Detects Viral Genomic RNA during Negative-Strand RNA Virus Infection

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SUMMARY

RIG-I is a key mediator of antiviral immunity, able to couple detection of infection by RNA viruses to the induction of interferons. Natural RIG-I stimulatory RNAs have variously been proposed to correspond to virus genomes, virus replication intermediates, viral transcripts, or self-RNA cleaved by RNase L. However, the relative contribution of each of these RNA species to RIG-I activation and interferon induction in virus-infected cells is not known. Here, we use three approaches to identify physiological RIG-I agonists in cells infected with influenza A virus or Sendai virus. We show that RIG-I agonists are exclusively generated by the process of virus replication and correspond to full-length virus genomes. Therefore, nongenomic viral transcripts, short replication intermediates, and cleaved self-RNA do not contribute substantially to interferon induction in cells infected with these negative strand RNA viruses. Rather, single-stranded RNA viral genomes bearing 5′-triphosphates constitute the natural RIG-I agonists that trigger cell-intrinsic innate immune responses during infection.

INTRODUCTION

Vertebrates possess a variety of defense mechanisms to detect, contain, and clear viral infections. Chief among these is the interferon (IFN) system, which plays a key role in inducing an antiviral state and contributes to the subsequent antigen specific adaptive immune response (Samuel, 2001). Type I IFNs (IFN-α and -β, hereafter simply referred to as IFN) and type III IFNs are induced very rapidly in all cell types by receptors that monitor the cytosol for the presence of nucleic acids indicative of virus presence. Such receptors include RIG-I-like receptors (RLRs) that recognize RNA and are themselves IFN inducible (Yoneyama and Fujita, 2009). The three members of this family—RIG-I (retinoic acid-inducible gene I), MDA5 (melanoma differentiation factor-5) and LGP-2 (laboratory of genetics and physiology-2)—all contain a DExD/H-box RNA helicase domain. RIG-I and MDA5 additionally posses two N-terminal caspase activation and recruitment domains that allow for interaction with the mitochondrial adaptor protein MAVS (Yoneyama and Fujita, 2009). MAVS triggers the activation of NF-κB, IRF-3, and IRF-7, which in turn induce transcription of IFNs and other innate response genes. Notably, mice deficient in RIG-I, MDA5, or MAVS readily succumb to infection with RNA viruses, highlighting the importance of RLRs in antiviral defense (Gitlin et al., 2006; Kato et al., 2006; Kumar et al., 2006).

Total RNA extracted from virally infected cells can stimulate specific RLRs (Hornung et al., 2006; Kato et al., 2008; Pichlmair et al., 2009). For example, RNA from cells infected with influenza A virus (flu) potently induces IFN-β when transfected into wild-type or MDA5-deficient, but not RIG-I-deficient mouse embryonic fibroblasts (Kato et al., 2008). However, the actual stimulatory RNA molecules within these pools remain largely unidentified. Instead, RLR agonists have been defined with chemically or enzymatically synthesized nucleic acids (reviewed in Schlee et al., 2009a). We and others identified RNAs transcribed in vitro by phage polymerases as potent RIG-I agonists (Hornung et al., 2006; Pichlmair et al., 2006). These RNAs carry a 5′-triphosphate (5′-PPP) moiety that is absolutely required for their activity (Hornung et al., 2006; Pichlmair et al., 2006). Other synthetic RIG-I agonists lack 5′-PPPs. These include poly I:C, which is prepared by annealing inosine and cytosine polymers that have monophosphate or diphosphate 5′ ends (Grunberg-Manago et al., 1955). Although long poly I:C activates MDA5 (Gitlin et al., 2006; Kato et al., 2006), short poly I:C (200–1000 nt) is reported to trigger RIG-I (Kato et al., 2008). Chemically synthesized RNA oligonucleotides 70 or 25 nt long and lacking 5′-PPPs also trigger RIG-I when annealed to a complementary strand (Kato et al., 2008; Takahasi et al., 2008). Thus, data obtained with synthetic RNAs suggest the possibility that there may be distinct types of RIG-I triggers in virally infected cells, including RNAs bearing 5′-PPPs or not and composed of either a single strand or two short complementary RNA strands.

In addition to synthetic RNAs, some natural RNAs also serve as RIG-I agonists. For example, RIG-I-dependent IFN production can be observed in response to transfection with genomic RNA from viruses such as flu or rabies virus that bear 5′-PPPs but not genomes of viruses that have no 5′-phosphates, such as encephalomyocarditis virus, or that have a 5′-monophosphate, such as Hantaan virus, Crimean-Congo hemorrhagic fever virus, and Borna disease virus (Habjan et al., 2008; Hornung et al., 2006; Pichlmair et al., 2006). This has led to the hypothesis that, in infected cells, RIG-I may be activated by viral genomes bearing 5′-PPPs. However, transfection of naked viral RNA does not mimic infection, and viral genomes in infected cells are in the form of viral ribonucleoprotein particles (vRNPs) in which viral proteins may prevent access of RIG-I to the RNA. For example, the flu polymerase binds to the 5′ end of the viral genome and is predicted to obscure the 5′-PPP necessary for RIG-I activation (Fodor et al., 1994; Tiley et al., 1994). Further doubt on the notion that RIG-I is primarily activated by viral genomes has come from reports that measles virus and Epstein-Barr virus transcripts (Plumet et al., 2007; Samanta et al., 2006), as well as products of host RNA cleavage by RNase L bearing 3′-monophosphates (Malathi et al., 2007), serve as the triggers for RIG-I in virally infected cells. Thus, the identification of natural RNA molecules with the potential to activate RIG-I has not clarified the identity of the actual RIG-I stimulus responsible for initiating antiviral immunity. As such, there is a pressing need to study relevant RIG-I agonists isolated from virally infected cells as opposed to characterizing the types of synthetic or natural RNA that can activate RIG-I in experimental models.

RIG-I is indispensable for IFN responses to negative-strand RNA viruses, including Sendai virus (SeV) and flu (Kato et al., 2006). SeV has a nonsegmented genome consisting of a single RNA molecule and belongs to the paramyxoviridae family. This virus family includes important human pathogens such as measles, mumps, and respiratory syncytial virus. Flu is a segmented RNA virus, and annual flu epidemics result in an estimated 250,000–500,000 deaths worldwide (Kilbourne, 2006). In addition, the ability of flu to infect different mammalian and avian species and generate reassortants constantly poses the threat that a new highly pathogenic virus will emerge, leading to a pandemic outbreak. Notably, the virulence of some flu strains is due, at least in part, to a deregulation of the innate immune response (Maines et al., 2008). Therefore, understanding how RIG-I becomes activated during infection with flu and other RNA viruses not only is of basic research interest but may also allow the development of new ways of containing viral spread and preventing disease.

Here, we characterize the RNA species responsible for activating RIG-I in cells infected with flu or SeV. Reconstitution of flu vRNPs in cell culture showed that only 5′-PPP-bearing viral genomic RNA triggered RIG-I. Furthermore, isolation of RIG-I complexes from infected cells revealed the presence of full-length viral genomes that accounted for stimulatory activity. Taken together, our data show that 5′-PPP-bearing viral genomes rather than short double-stranded RNAs, viral transcripts, or cleaved self-RNA constitute the physiological source of RIG-I stimulation and IFN induction during infection with negative-strand RNA viruses.

RESULTS

Reconstitution of Influenza A Virus vRNPs Induces IFN-α/β

To simplify the search for RIG-I agonists in flu-infected cells, we started with a mock infection system involving reconstitution of vRNPs (Fodor et al., 2002; Pleschka et al., 1996) (Figure 1A). We confirmed expression of viral RNA (vRNA), complementary RNA (cRNA), and messenger RNA (mRNA) in vRNP reconstitution experiments with each of the eight PR8 genome segments (Figure S1A available online). We then tested the IFN-inducing activity of total RNA from vRNP reconstitution experiments in an IFN-β promoter luciferase reporter assay (Figure 1B). RNA isolated from nontransfected cells (no TF) or from cells expressing the viral polymerase but no genome segment (no template) did not induce reporter activity (Figure 1B). However, RNA extracted from cells expressing the wild-type viral polymerase and any of the eight genome segments was stimulatory (Figure 1B), as reported for RNA isolated from flu-infected cells (Kato et al., 2008). Cells expressing a polymerase mutated in its active site (PB1a) did not accumulate stimulatory RNA (Figure 1C). In addition, the stimulatory activity of total cellular RNA from vRNP reconstitutions, like that of in vitro-transcribed (IVT) RNA, was RIG-I dependent (Figure 1D). The accumulation of stimulatory RNA in transfected cells was accompanied by RIG-I-dependent secretion of IFN-α/β into the culture medium, although this was not always detectable unless the cells were pretreated with IFN to upregulate RIG-I and downstream mediators prior to transfection (Figures 1E and 1F; see below for NS segment). In sum, like live infection, flu vRNP reconstitution induces RIG-I-dependent production of IFN and promotes the accumulation of RIG-I-stimulatory RNA in cells.

Transcription of vRNPs Is Dispensable for IFN Induction

Stimulatory RNA accumulated in reconstitutions with a modified template (Pleschka et al., 1996) in which the bacterial chloramphenicol acetyltransferase gene was flanked by a viral polymerase promoter composed of the 5′ and 3′ noncoding regions of the NS segment (vCAT, Figure 1C). Therefore, apart from this short promoter, specific viral sequence elements are not required for the generation of stimulatory RNA. We next asked whether transcription and/or replication are necessary. We used point mutations in the PA subunit of the viral polymerase that selectively impair one or the other process (Hara et al., 2006). As shown in Figure 2A, the PA-E410A polymerase (replication mutant) generated normal amounts of viral mRNA from an NA template but vRNA and cRNA levels were reduced about 3-fold. This was accompanied by a 3-fold reduction in IFN secretion by the transfected cells and by accumulation of lower amounts of stimulatory RNA (Figures 2B and 2C). In contrast, when transcription was selectively abrogated with the PA-D108A transcription mutant, mRNA production was blocked, yet we did not see a loss but rather observed an increase in stimulatory RNA accumulation, as well as elevated IFN secretion (Figure 2). Similar results were obtained when the PB2 genome segment was used as the template (Figure S2). Therefore, in vRNP reconstitutions, transcription is dispensable for IFN induction and for the accumulation of stimulatory RNA, which

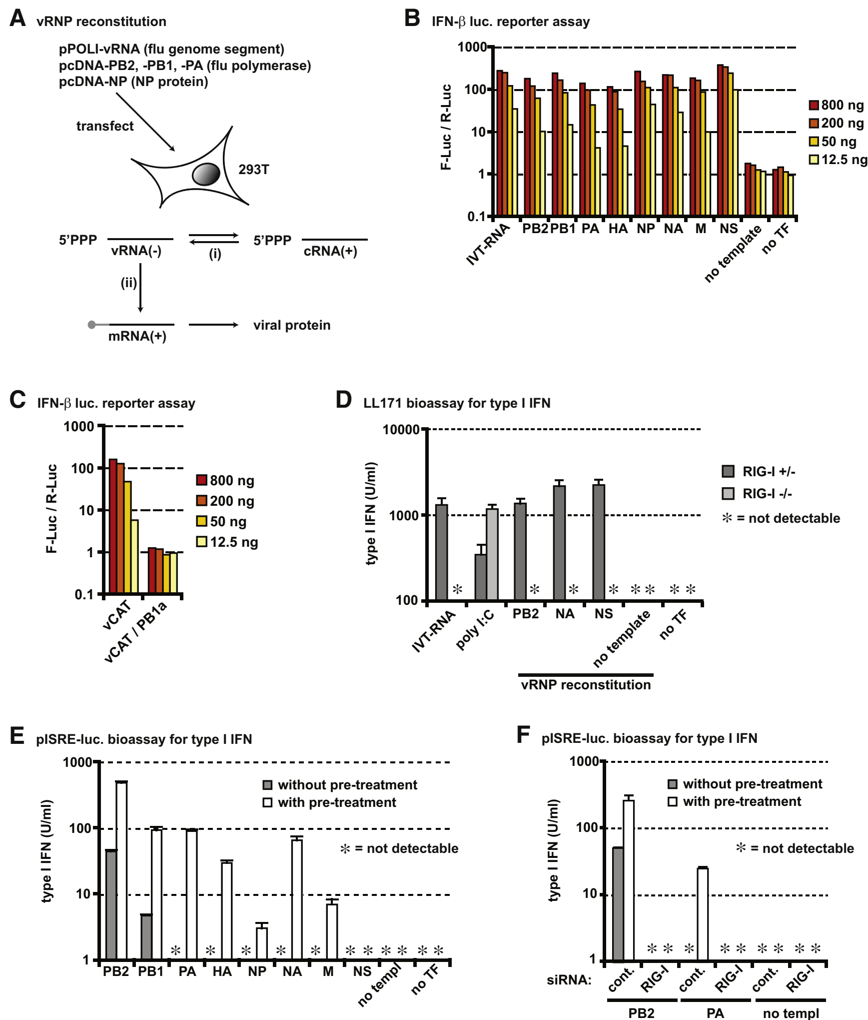


Figure 1. vRNP Reconstitution Induces IFN

(A) Scheme of the vRNP reconstitution system. Individual segments of the flu genome (vRNAs) are expressed off a promoter for RNA polymerase I, which generates uncapped RNA transcripts. These act as templates for the viral polymerase, which is expressed, together with the NP protein, from a different set of plasmids. The viral polymerase (i) replicates the negative sense genome segment via a positive sense cRNA intermediate (antigenome) and (ii) snatches short stretches of capped RNA (depicted in gray) from cellular mRNAs to serve as primers for transcription of viral mRNA, which is then translated into viral protein.

(B) Each genome segment (PB2, PB1, PA, HA, NP, NA, M, or NS) was used for the vRNP reconstitution or the genome segment was omitted (no template). Two days after transfection, total RNA was extracted and tested in an IFN-β promoter luciferase reporter assay. Results were normalized to a Renilla luciferase control and are shown as fold increase relative to cells treated with transfection reagent only. RNA extracted from non-transfected cells (no TF) and IVT-RNA (Neo$^{1-99}$) were included as controls.

(C) The bacterial CAT gene was flanked by viral 5′ and 3′ noncoding sequences (vCAT) and expressed instead of an authentic viral genome segment. In one group, pcDNA-PB1 was replaced by pcDNA-PB1a that encodes a mutant abrogating polymerase activity (vCAT/PB1a). Extracted RNA was tested as in (B).

(D) Mouse embryonic fibroblasts of the indicated genotype were transfected with 100 ng RNA extracted from vRNP reconstitutions with the PB2, NA, or NS genome segment. RNA from reconstitutions without a genome segment (no template), RNA from nontransfected cells (no TF), IVT-RNA (Neo$^{1-99}$) and poly I:C were included as controls. After overnight culture, cell culture supernatants were tested for mouse IFN content.

(E) Supernatants from vRNP reconstitutions were harvested 2 days after transfection. A bioassay was used to test for the presence of human IFN. Cells were pretreated or not with IFN-A/D (100 units/ml overnight) prior to transfection.

(F) siRNA targeting RIG-I or a control siRNA (cont.) were cotransfected with the plasmids for vRNP reconstitution. Supernatants were tested as in (E).

Representative examples of three (A–C and E) or two (D and F) independent experiments are shown. (D)–(F) show average values and standard deviation of triplicate measurements. See also Figures S1 and S7.

correlate instead with the amount of vRNA and cRNA produced by viral replication.

Full-Length Viral Genomes Bearing 5′-PPPs Trigger IFN Induction in vRNP Reconstitutions

Resistance to DNase and susceptibility to RNase V1+A treatments confirmed that extracted RNA accounted for stimulatory activity in vRNP reconstitutions (Figure 3A and Figure S3A). Digestion with Terminator (TER), a 5′-to-3′ exonuclease that degrades RNA bearing 5′-monophosphate, led to disappearance of ribosomal RNAs but did not alter the potency of the preparations (Figure 3A and Figure S3A). In contrast, treatment with calf intestinal phosphatase (CIP), which removes 5′-phosphates, completely abolished the stimulatory activity (Figure 3A and Figure S3A).

We then determined the size of the stimulatory RNA. RNA from vRNP reconstitutions using the PB2, NA, or NS segments was separated into eight fractions of decreasing size by agarose gel electrophoresis. Nucleic acid was extracted from each fraction and tested in the IFN-β reporter assay. As a control, we fractionated a 99 nt long IVT-RNA and showed that its stimulatory activity was recovered exclusively in fraction 7, as expected (Figure 3B and Figure S3B). Notably, stimulatory RNA from vRNP reconstitutions with PB2, NA, and NS segments was recovered in fractions 2, 3, and 5/6, respectively, correlating with the respective size of these segments (2341, 1413, and 890 nt). This observation excludes a dominant role for short replication intermediates (or small stimulatory RNAs of self origin), which would elute in fractions 7 or 8 (Figure 3B and Figure S3B). In sum, RNAs corresponding in size to the viral genome or antigenome and bearing more than one 5′-phosphate account for the majority of the stimulatory RNA generated during vRNP reconstitution.

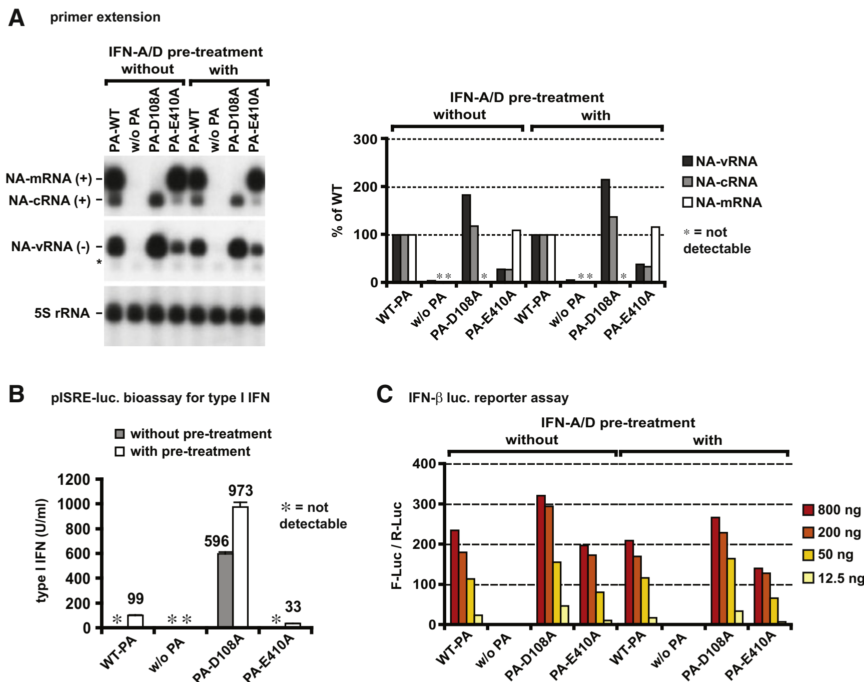


Figure 2. vRNP Replication but Not Transcription Induces IFN

(A) Cells were pretreated or not with IFN-A/D as in Figure 1E and used for vRNP reconstitution with the NA genome segment in conjunction with an intact viral polymerase (WT), a polymerase lacking the PA subunit (w/o PA), or with two point mutants, PA-D108A and PA-E410A. Extracted RNA was tested by primer extension for NA-cRNA, -mRNA, and -vRNA. A primer specific for 5S rRNA was used as a control. Signals were quantified by phosphoimager, normalized to the 5S rRNA control, and are expressed relative to WT polymerase. A nonspecific band is marked with an asterisk.

(B) Supernatants from (A) were tested in the human IFN bioassay.

(C) Total RNA extracted from (A) was tested in the IFN-β reporter assay.

(A) is representative of two independent experiments, and (B) and (C) of four experiments. (B) shows average values and standard deviation of triplicate measurements. See Figure S2 for equivalent experiments using the PB2 genome segment.

NS1 Inhibits IFN Induction in vRNP Reconstitutions and Associates with RIG-I and Stimulatory RNA

The NS segment encodes the viral NS1 and NS2 proteins, the former of which binds RNA and, in the PR8 strain, acts as an inhibitor of IFN induction (Gack et al., 2009; Guo et al., 2007; Mibayashi et al., 2007; Opitz et al., 2007; Pichlmair et al., 2006). Accordingly, we did not detect secretion of IFN in vRNP reconstitutions using the NS genome segment (Figure 1E) even though stimulatory RNA accumulated in these cells (Figure 1B and Figure S3A). However, when we modified the PR8 NS genome segment by introducing two point mutations (R38A and K41A) in the sequence encoding the RNA binding domain (Donelan et al., 2003), vRNP reconstitution resulted in secretion of significant amounts of IFN (Figures S1B–S1E). These results show that, as expected, NS1 inhibits IFN induction during vRNP reconstitution.

To explore the possibility that IFN inhibition involves simultaneous binding of NS1 to stimulatory RNAs and to RIG-I, we used a two-step immunoprecipitation approach (Figure S4A). Lysates from cells expressing NS1 and FLAG-tagged RIG-I were combined with IVT-RNA, and RIG-I complexes were precipitated with α-FLAG antibodies. As described (Pichlmair et al., 2006), wild-type NS1 but not the NS1 R38A/K41A mutant associated with RIG-I (Figure S4B). Next, native complexes were eluted using FLAG peptide and were reprecipitated with α-NS1 antibody (Figure S4C). The majority of the stimulatory RNA was

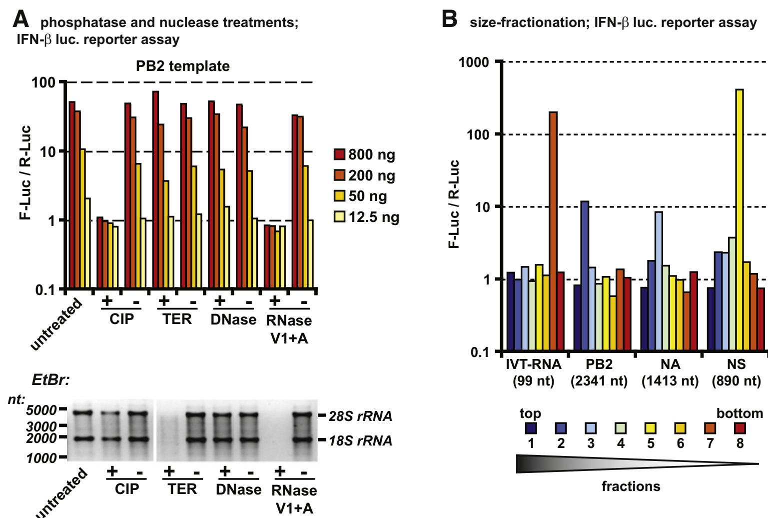


Figure 3. Full-Length Viral Genomes Trigger IFN Induction in vRNP Reconstitutions

(A) RNA extracted from reconstitutions using the PB2 genome segment was subjected to CIP, TER, DNase, or RNase A+V1 digestion. Parallel reactions with (+) and without (−) enzyme were performed and RNAs were analyzed by gel electrophoresis and ethidium bromide staining (bottom) and in the IFN-β reporter assay (top). Extracted RNA without any further treatment was also included (untreated).

(B) RNA extracted from reconstitutions using the PB2, NA, or NS genome segments was size fractionated on agarose gels (fractions 1 to 8 from the pockets to the bottom). RNA was reisolated and tested as in (A). IVT-RNA (Neo^{1-99}) was included in the fractionation. The length of this RNA and that of the viral genome segments is given in brackets.

Data are representative of three independent experiments. See also Figure S3.

retained in the second immunoprecipitation with wild-type NS1 (Figure S4D), indicating that the viral protein traps stimulatory RNA and RIG-I in a trimolecular complex. However, RIG-I was not necessary for NS1-dependent sequestration of stimulatory RNA as the latter still occurred in RIG-I-deficient cells (Figure S4E).

Association with NS1 Marks the Natural RIG-I Agonist during Flu Infection

The above experiments suggested that NS1 immunoprecipitation might allow the isolation of RIG-I agonists from flu-infected cells. Indeed, nucleic acids extracted from NS1 pulldowns from flu-infected cells potently induced the IFN-β reporter (Figure 4A). This approach did not require artificial overexpression of any protein and allowed quantitative recovery of all relevant RIG-I agonists as evidenced by the fact that it depleted cell lysates from stimulatory activity (Figure 4B). The stimulatory activity of NS1-associated RNAs was sensitive to RNase A treatment but not DNase digestion and was RIG-I dependent as it was greatly diminished by siRNA depletion of mouse but not human RIG-I in NIH 3T3 cells and vice versa in HEK293T cells (Figure S4F and Figure 4C). We conclude that RIG-I agonistic RNAs generated during flu infection are associated with NS1 and can be purified from infected cells by NS1 immunoprecipitation.

Flu Genomes Constitute the Physiological RIG-I Agonist

NS1-associated stimulatory activity was sensitive to CIP but not to TER treatment and encompassed RNA species ranging from 0.5 to 6 kb (Figures 5A and 5B). The phosphatase sensitivity and size characteristics suggested that stimulatory activity might be attributable to 5′-PPP-containing genomic or antigenomic RNA segments. Indeed, primer extension analysis revealed that all eight negative sense genome segments were highly enriched in the NS1 but not a control immunoprecipitate (Figures 5C and 5D). For example, PB2 vRNA was 21-fold enriched among NS1 associated RNAs compared to RNA extracted from the lysate (Figures 5C and 5D). We also detected some viral cRNA and mRNA in the NS1 precipitate, albeit not for all segments (Figures 5C and 5D). In northern blots with a full-length probe for M segment vRNA, the NS1-associated RNA migrated at around 1000 nt, corresponding to the size of the genome segment (1027 nt, Figure 5E). Thus, full-length flu genomes are highly enriched in the NS1-associated RIG-I stimulatory fraction.

To validate these findings by an independent approach, we generated a cell line expressing FLAG-tagged RIG-I and infected these cells with PR8 flu or a mutant that does not express the NS1 protein (ΔNS1). RIG-I was precipitated with α-FLAG antibody, and associated nucleic acids were tested in the IFN-β promoter reporter assay (Figure 6A). We recovered stimulatory RNA from the FLAG immunoprecipitation but not from a control reaction (Figure 6A and Figure S5A). Consistent with the observations from vRNP reconstitution and NS1 precipitation experiments, RIG-I-associated RNA lost its stimulatory activity after CIP but not TER treatment (Figure 6B). We characterized RNA from RIG-I precipitates by three approaches. First, we used oligonucleotides complementary to the PB2 and NA segments in primer extension experiments. We found that both vRNAs and cRNAs were retained specifically in the RIG-I purification,

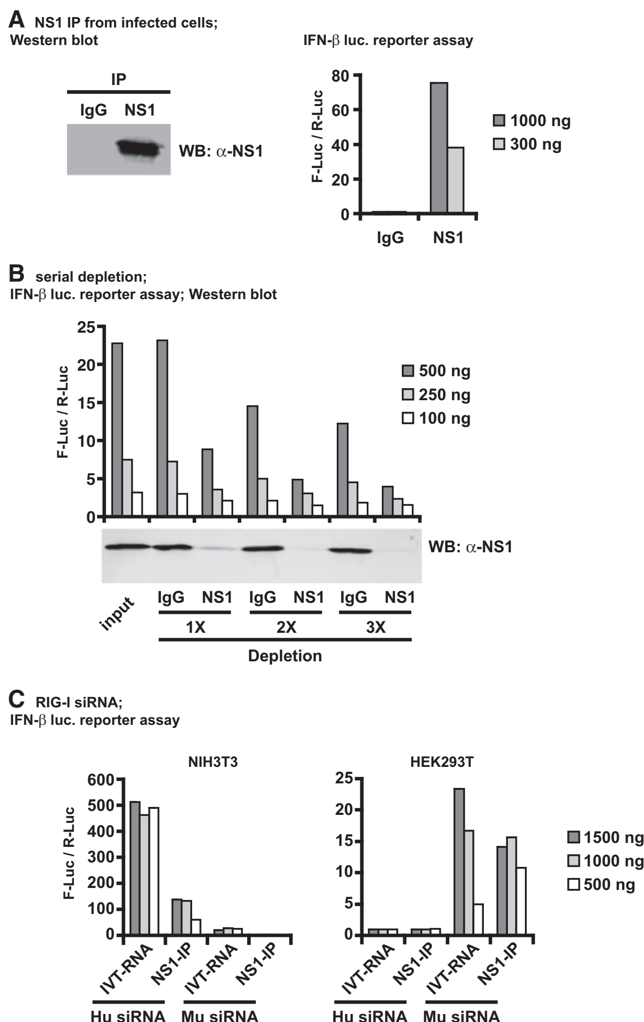


Figure 4. NS1 Associates with the Actual RIG-I Agonist during Flu Infection

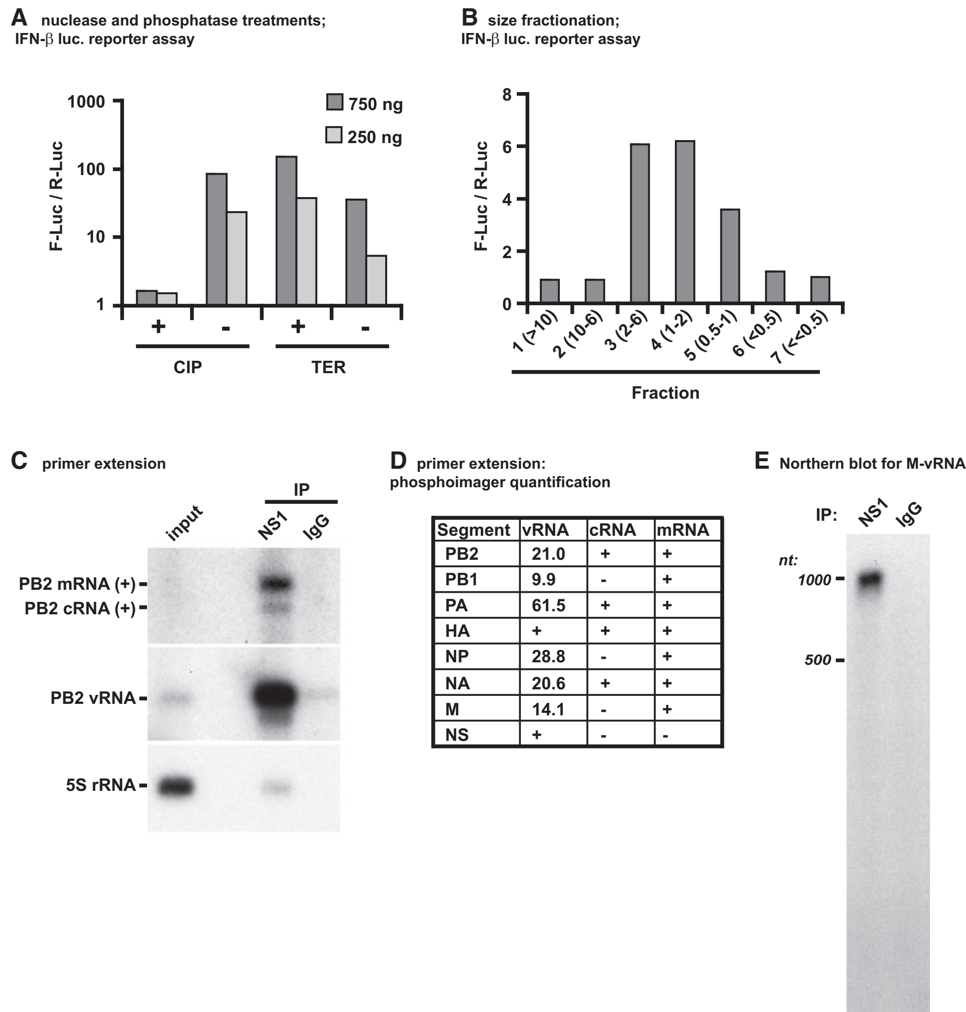
(A) NS1 was immunoprecipitated from total lysate of MDCK cells infected with PR8 WT flu for 48 hr at a multiplicity of infection (MOI) of 0.01. Nucleic acids associated with control IgG or NS1 immunoprecipitates (IP) were extracted and tested in the IFN-β reporter assay. Western blot (WB) shows the presence of NS1.

(B) Total lysate of MDCK cells infected with flu was subjected to three rounds of mock or NS1 immunoprecipitation. Nucleic acids were extracted from the lysate and the depleted fractions and tested as in (A). The depletion of NS1 was monitored by WB.

(C) Mouse NIH 3T3 or human HEK293T cells were cotransfected with the IFN-β promoter reporter plasmid and siRNAs specific for human or mouse RIG-I. After 48 hr, cells were transfected with the indicated amount of IVT-RNA or NS1-associated RNA (NS1-IP), and luciferase activity was measured 12 hr later.

(A), (B), and (C) are representative of five, three, and two independent experiments, respectively. See also Figure S4.

while contaminating 5S rRNA was detectable in both RIG-I and control precipitates (Figure 6C). Second, in northern blots for vRNA of the M segment, the RIG-I-associated RNA migrated at the size expected for the full-length genome segment (1027 nt), and we did not detect faster-migrating RNA species (Figure 6D). Third, the size profile of RIG-I-associated stimulatory



Segment	vRNA	cRNA	mRNA
PB2	21.0	+	+
PB1	9.9	-	+
PA	61.5	+	+
HA	+	+	+
NP	28.8	-	+
NA	20.6	+	+
M	14.1	-	+
NS	+	-	-

Figure 5. NS1 Binds 5′-PPP Influenza A Virus Genomes and Antigenomes

(A) NS1 associated stimulatory nucleic acid (see Figure 4A) was subjected to CIP or TER treatment and tested in the IFN-β reporter assay.

(B) Size profile of the NS1-associated stimulatory RNA. NS1-associated nucleic acid (see Figure 4A) was resolved by agarose gel electrophoresis. The gel was cut into seven fractions, and RNA was re-extracted and analyzed as in (A). The relative molecular weight of each fraction in nucleotides (×1000) is based on comparison with an RNA marker.

(C) NS1-associated RNA was immunoprecipitated from infected cells and PB2 mRNA, cRNA, and vRNA as well as 5S rRNA were detected by primer extension. For all fractions, the same amount of RNA (400 ng) was used.

(D) The primer extension shown in (C) was repeated with oligonucleotides complementary to all other segments and quantified by phosphoimager analysis. Results are expressed as fold enrichment in the NS1 precipitate compared to input RNA. HA and NS vRNAs and some mRNAs and cRNAs were present in the NS1 precipitate (denoted by the "plus" sign), but not detectable in the input material, hence fold enrichments could not be calculated.

(E) Northern blot analysis of RNA associated with α-NS1 or control antibody using a full-length, internally labeled probe specific to M-vRNA.

(A)–(E) show representative examples of two independent experiments.

RNA matched the size range of flu genome segments (890–2341 nt) and was not found in smaller fractions (Figure 6E). Thus, direct RIG-I precipitation reveals only the presence of flu viral genomes and not other stimulatory nucleic acids in flu-infected cells.

RIG-I Is Triggered by Viral Genomic RNA during Sendai Virus Infection

To extend our findings to other viruses sensed by RIG-I, we chose SeV. The SeV genome consists of a single negative sense 5′-PPP-bearing RNA molecule 15,384 nt long that serves as a template for the synthesis of capped mRNAs and 5′-PPP antigenomes. Unlike flu, SeV makes short (∼50 nt long) leader and trailer 5′-PPP RNAs during infection. Thus, in SeV-infected cells, these RNAs could serve as RIG-I agonists, as proposed for the related measles virus (Plumet et al., 2007).

Total RNA from SeV-infected cells but not from uninfected cells potently induced the IFN-β promoter upon transfection into reporter cells (Figure 7A). As for flu, stimulatory activity was RIG-I dependent and sensitive to RNase, but not DNase treatment (Figures S6A and S6B) and was additionally sensitive

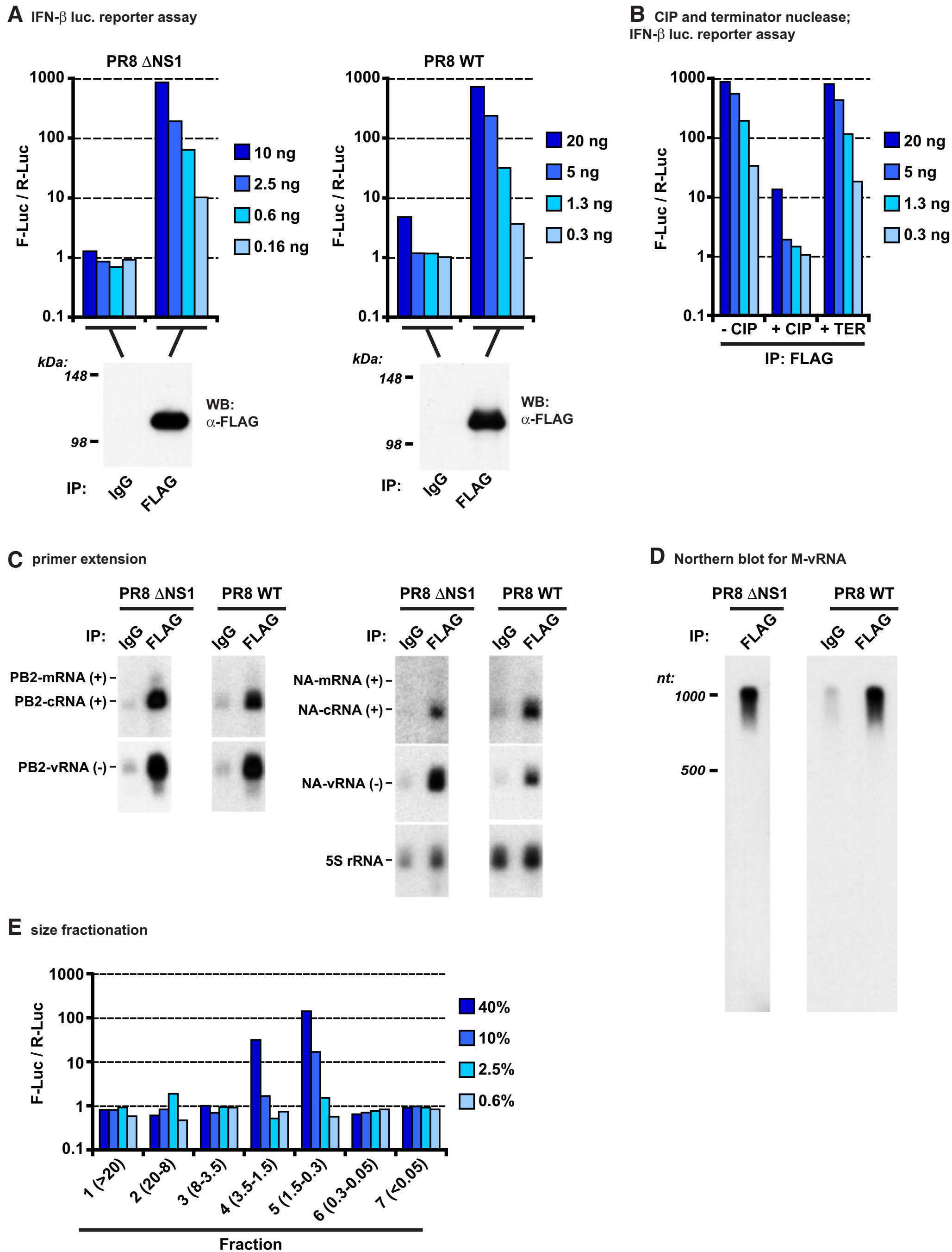


Figure 6. Influenza A Virus Genomes and Antigenomes Trigger IFN Induction during Infection

(A) HEK293 cells expressing FLAG-RIG-I were infected with flu PR8 WT or PR8 ΔNS1 at an MOI of 1. After 16 hr, RIG-I was precipitated with α-FLAG antibody. An isotope matched antibody (IgG) was used as a control. RNA extracted from the precipitates was tested in the IFN-β reporter assay (top). The precipitates were also tested by WB using α-FLAG antibodies (bottom).

(B) RNA associated with FLAG-RIG-I (from PR8 ΔNS1 infection) was analyzed by CIP and TER treatment and tested as in (A).

(C) Stimulatory RNA in the control and FLAG-RIG-I precipitates was analyzed by primer extension for the presence of PB2 and NA mRNA, cRNA, and vRNA, as well as 5S rRNA.

(D) Northern blot analysis of RNA bound to FLAG-RIG-I using a full-length probe specific for M-vRNA.

(E) Size profile of the FLAG-RIG-I associated stimulatory RNA (from PR8 ΔNS1 infection) determined as in Figure 5B.

(A) and (B)–(E) are representative examples of four and two independent experiments, respectively. See also Figures S5 and S7.

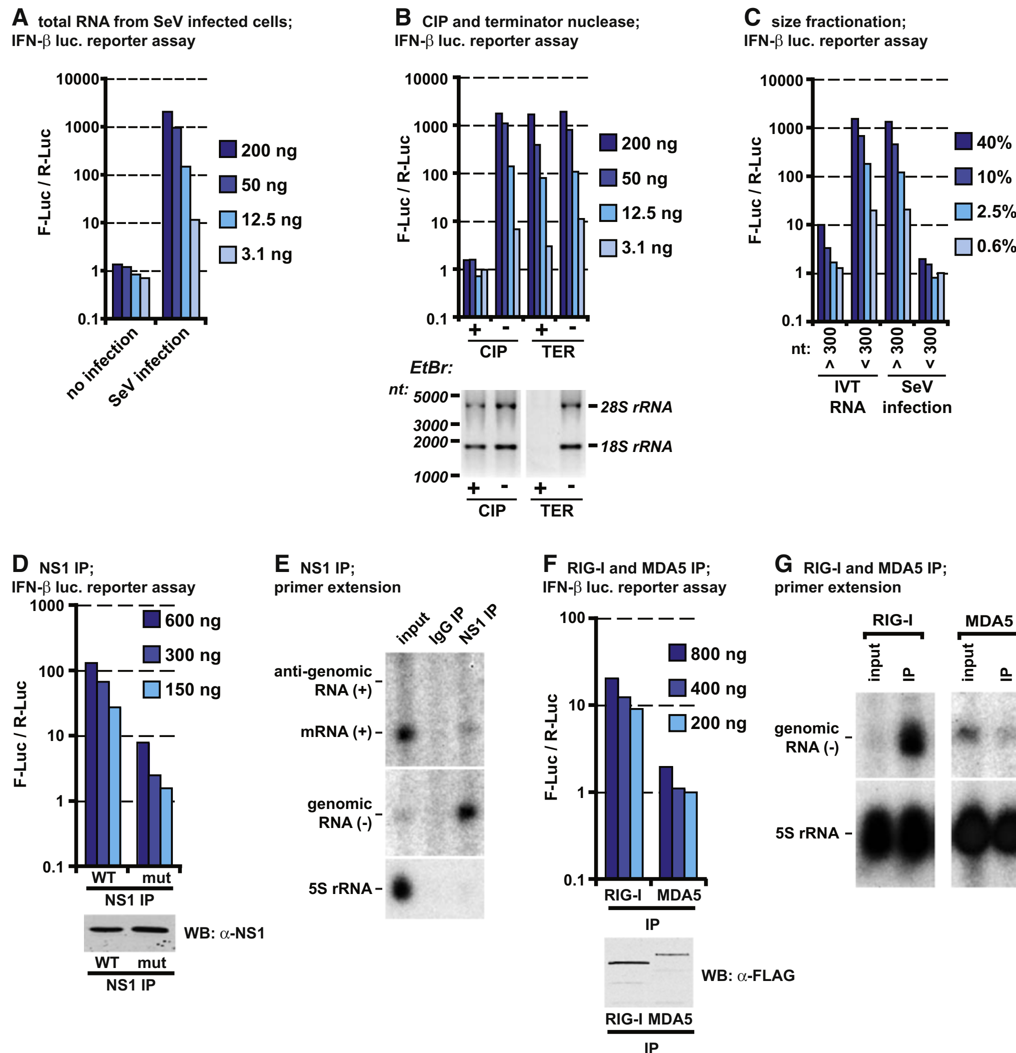


Figure 7. Viral Genomes Constitute the Primary RIG-I Agonist during Sendai Virus Infection

(A) HEK293 cells were infected or not with SeV at an MOI of 5. After 16 hr, RNA was extracted with TRIZOL and tested in the IFN-β reporter assay as in Figure 1B.

(B) Stimulatory RNA extracted from SeV-infected cells was treated with CIP or TER. The minus sign denotes a control reaction without enzyme. Aliquots were tested as in (A) (top) and by agarose gel electrophoresis and ethidium bromide staining (bottom).

(C) Size profile of stimulatory RNA extracted from SeV-infected cells. RNA from (A) was resolved by agarose gel electrophoresis. The gel was cut into two fractions, corresponding to RNAs migrating above or below 300 nt by comparison with an RNA marker. RNA was re-extracted and analyzed as in (A). IVT-RNA (Neo$^{1–99}$, 99 nt) was included as a control.

(D) HEK293T cells were transfected with plasmids expressing NS1 or NS1-R38A/K41A. After 24 hr, cells were infected with SeV at an MOI of 5. Cell lysates were prepared 20 hr later, and NS1 was precipitated. The precipitates were analyzed by WB (bottom) and nucleic acids were extracted and tested as in (A) (top).

(E) Transiently transfected HEK293T expressing NS1 were infected with SeV and NS1 was precipitated as in (D). Nucleic acid extracted from the cell lysate (input) or associated with α-NS1 or IgG control antibodies (IP) was analyzed for the presence of SeV genomic, antigenomic, and messenger RNA and 5S rRNA by primer extension.

(F) FLAG-RIG-I and FLAG-MDA5 were expressed in HEK293T cells by transient transfection, followed by infection with SeV after 24 hr. Cell lysates were prepared after 20 hr and RIG-I and MDA5 were precipitated using α-FLAG antibodies. Nucleic acids were extracted from precipitates (IP) and analyzed as in (A) (top panel). The bottom panel shows a WB using an aliquot of the IP.

(G) Nucleic acids extracted from cell lysates (input) and precipitates (IP) as in Figure 7F were tested by primer extension for SeV genomic RNA and 5S rRNA.

Panels (A), (C), (F), and (G) and panels (B), (D), and (E) are a representative examples of three and two independent experiments, respectively. See also Figures S6 and S7.

to CIP but not TER (Figure 7B). Size fractionation into "large" (>300 nt) and "small" (<300 nt) RNAs showed that stimulatory RNA from SeV infected cells was "large," whereas that of a control 99 nt IVT RNA was "small" (Figure 7C). This excludes a major role for leader and trailer RNAs in triggering RIG-I and together with the CIP sensitivity suggests that genomic and/or antigenomic RNA is the primary RIG-I agonist during SeV infection.

To capture the physiologically relevant RIG-I agonist during SeV infection, we again made use of flu NS1, which is able to inhibit IFN responses to SeV (Wang et al., 2000). As predicted, WT NS1 but not the NS1 mutant was able to precipitate stimulatory RNAs from extracts of transiently transfected cells infected with SeV (Figure 7D). We next tested by primer extension if NS1-associated stimulatory RNA contains SeV genomic, antigenomic, and/or messenger RNA (Figure S6D). Compared to RNA extracted from the cell lysate, SeV genomic RNA was enriched between 6.6- and 11.5-fold in the NS1 precipitate, while the antigenome and N-mRNA were not detectable (Figure 7E and Figure S6C). We validated these results by precipitating FLAG-RIG-I (or FLAG-MDA5 as a control) from transiently transfected cells infected with SeV. Stimulatory RNA was recovered only in the FLAG-RIG-I immunoprecipitation and SeV genomic RNA was enriched between 7.4- and 17-fold in the RIG-I precipitate compared to cell lysate (Figures 7F and 7G). Thus, flu NS1 or RIG-I precipitation selectively enriches for SeV viral genomes. Taken together with the size characteristics of the stimulatory RNA and the CIP sensitivity, these observations show that 5′-PPP bearing genomic RNA is the main trigger for RIG-I during SeV infection.

DISCUSSION

Sensing of virus presence and cytokine induction via the RIG-I pathway are crucial for successful host defense against infections with RNA viruses (Pichlmair and Reis e Sousa, 2007; Yoneyama and Fujita, 2009). Although the signaling cascade from RIG-I to IFN induction is well defined, the identity and properties of RIG-I agonists and the mechanisms that allow the helicase to be activated specifically in infected cells are controversial. Viral genomes, shorter viral transcripts, double-stranded RNA, or cellular RNA cleaved by RNase L have all been suggested to trigger RIG-I (Habjan et al., 2008; Hausmann et al., 2008; Hornung et al., 2006; Kato et al., 2008; Malathi et al., 2007; Pichlmair et al., 2006; Plumet et al., 2007; Ranjith-Kumar et al., 2009; Samanta et al., 2006; Takahasi et al., 2008). Such RNAs have been variably defined as containing no phosphates, 5′-monophosphates, 5′-triphosphates, or 3′-monophosphates (Hornung et al., 2006; Kato et al., 2008; Malathi et al., 2007; Pichlmair et al., 2006; Takahasi et al., 2008) and, in some cases, to require specific structural determinants or sequence motifs (Marques et al., 2006; Saito et al., 2008; Schlee et al., 2009b; Schmidt et al., 2009; Uzri and Gehrke, 2009). Most of these studies, however, have been limited to the analysis of RIG-I activation by defined RNAs, including synthetic RNAs made by chemical or enzymatic synthesis or vRNAs isolated from virus particles. Although such studies have been instrumental in defining the range of RNAs that can activate RIG-I, they have fallen short of identifying physiological RIG-I agonists that are actually responsible for activating RIG-I and triggering IFN production in virus-infected cells. Here, we analyze the properties of relevant RIG-I agonists in cells infected with flu or SeV. Using three complementary approaches, we find that genomic RNA generated by viral replication constitutes the major trigger for RIG-I and conclude that viral transcripts, RNase L cleavage products, and/or other RNA species make only a minor contribution to cell-intrinsic antiviral innate immunity.

We started with a mock infection system that allows the reconstitution of flu vRNP complexes and leads to IFN induction and accumulation of stimulatory RNA (Figure 1). Using this system, we found that an artificial genome segment that retains the viral promoter but otherwise lacks viral sequences behaved similarly to bona fide flu vRNA segments (Figure 1C). Therefore, RIG-I activation in this setting is largely sequence independent. This is in contrast to recent reports suggesting that a polyuridine motif in the hepatitis C virus 3′ untranslated region is required for triggering RIG-I (Saito et al., 2008; Uzri and Gehrke, 2009). Importantly, those conclusions were based on cellular responses to transfected IVT-RNAs, whereas the vRNP reconstitution system used here allowed us to look at RNAs made endogenously by the mock-infected cell. Nevertheless, it remains possible that sequence motifs may facilitate RIG-I activation in some instances. Indeed, such motifs could contribute to the observed quantitative differences in accumulation of stimulatory RNA and IFN secretion depending on which of eight flu genome segments was used for reconstitution (Figures 1B and 1E). Alternatively, those differences may be due to the expression of viral proteins associated with the viral genome (such as the PB2, PB1, PA, NP, M1, NS1, and NS2 proteins), which may inhibit or facilitate the access and/or function of RIG-I.

In a particularly striking example of the latter point, the viral NS1 protein completely blocked IFN induction during vRNP reconstitution (Figures S1B–S1E). NS1 can interact with RIG-I (Mibayashi et al., 2007), especially in the presence of stimulatory RNA through formation of a trimeric complex (Figures S4A–S4D) (Pichlmair et al., 2006). This activity of NS1 is dependent on the integrity of the RNA binding domain, which is reported to bind double-stranded RNA (Hatada and Fukuda, 1992). Interestingly, recent studies demonstrate that, in addition to the 5′-PPP, synthetic RNAs require base pairing at the 5′ end in order to trigger RIG-I (Schlee et al., 2009b; Schmidt et al., 2009). Such 5′ base-paired regions can be found within the genomes of flu and SeV (Knipe and Howley, 2007). We therefore envisage that one mechanism of NS1 action may be to bind to the base-paired region at the 5′ end of viral genomes. This does not prevent RIG-I binding to the 5′-PPP via its C-terminal domain (Cui et al., 2008; Takahasi et al., 2008) but may block translocation along the base paired stretch, which has been proposed to be necessary for signaling (Myong et al., 2009). This model (Figure S4G) therefore suggests that the ability of NS1 to associate with stimulatory RNAs is due to its propensity to recognize RNA secondary structure determinants important for RIG-I activation (Schlee et al., 2009b; Schmidt et al., 2009) and is consistent with the finding that NS1 binds agonistic RNA in the absence of functional RIG-I (Figure S4E). This model does not exclude additional modes of NS1 action, such as inhibition of TRIM25-mediated RIG-I ubiquitination (Gack et al., 2009).

Given the finding that NS1 associates with stimulatory RNA, we used it as one of our strategies to purify RIG-I agonists from infected cells. As a complementary approach, we immunoprecipitated epitope-tagged RIG-I from infected cells. Both precipitations enriched for flu and SeV genomic RNAs. Furthermore, NS1- or RIG-I-associated stimulatory RNA matched the

size of vRNA and required 5′-phosphates for stimulatory activity. Thus, viral genomic RNAs represent the major RIG-I agonist in flu- and SeV-infected cells. Antigenomes, which have an identical size to the genome and also bear 5′-PPP, may also contribute to IFN induction. Indeed, flu cRNAs were present in the NS1 and RIG-I immunoprecipitates (Figures 5C, 5D, and 6C). Their contribution, however, is likely to be minor, as cRNA accumulates to much lower levels compared to vRNA (Robb et al., 2009) (Figure S1A and Figure 5C).

In contrast to vRNA and cRNA, flu or SeV transcripts do not appear to trigger RIG-I, based on the size distribution of the stimulatory RNA, the fact that the transcription-defective PA-D108A mutant flu polymerase was fully capable of inducing IFN in vRNP reconstitution experiments, and the fact that viral mRNAs, like cellular mRNAs, are capped. These findings do not exclude a role for viral transcripts in activating RIG-I in other virus infections such as measles virus and Epstein-Barr virus (Plumet et al., 2007; Samanta et al., 2006) as those viruses use mechanisms for transcription that can result in transcripts bearing 5′-triphosphates. However, it is worth noting that measles-related SeV also generates uncapped short 5′-triphosphate-bearing leader and trailer RNA transcripts, yet our size fractionation experiments exclude a role for these short RNAs in RIG-I stimulation in SeV-infected cells (Figure 7C). We speculate that leader and trailer RNAs lack a sufficient degree of secondary structure to potently trigger RIG-I and/or are sequestered by association with cellular proteins. Similarly, during infection with another paramyxovirus, respiratory syncytial virus, leader RNAs do not play an important role in IFN induction (Bitko et al., 2008). Thus, the ability of a virus to generate uncapped transcripts during its life cycle does not necessarily mean that these will act as RIG-I agonists.

Our results also appear to exclude RNase L cleavage products as major RIG-I agonists during infection with negative-strand RNA viruses. Such cleavage products have 5′-hydroxyl and 3′-monophosphate ends and are expected to be shorter than 200 nt (Malathi et al., 2007; Wreschner et al., 1981). Yet we found that the stimulatory activity of RNA isolated from both vRNP reconstitutions and infected cells strictly required 5′-phosphates and was longer than 200 nt. It may therefore be the case that RNase L-cleaved self or viral RNAs are not obligate RIG-I agonists but primarily serve to amplify RIG-I activation driven by vRNA. Consistent with such a model, RNase L-deficient mice show only a 6-fold reduction in serum IFN-β after infection with SeV (Malathi et al., 2007).

Virus entry into cells can induce innate immune responses in the absence of replication (Collins et al., 2004) and, in fact, 56°C-inactivated influenza virus was originally used to discover IFNs (Isaacs and Lindenmann, 1957). In retrospect, the latter observations may be explained by RIG-I-mediated recognition of incoming viral genomes delivered by high doses of fusogenic virus. However, the fact that NS1, a nonstructural protein only produced after infection, can effectively prevent IFN induction by flu indicates that the incoming genomes of virus particles are not the major triggers of RIG-I activation during live infection. Consistent with that notion, infection in the presence of drugs that block translation (and, consequently, inhibit the virus life cycle) prevents accumulation of stimulatory RNA in the cytoplasm of flu-infected cells (Figure S7A). Therefore, we believe that progeny genomes are the likely source of RIG-I stimulatory activity. However, flu replication is confined to the nucleus (Jackson et al., 1982; Krug et al., 1987), raising the question of how progeny viral genomic RNA is sensed in the cytoplasmic compartment monitored by RIG-I. It is clear that progeny genomes traverse the cytoplasm for assembly of new virions, but these genomes are bound at the ends by the flu polymerase and along their length by the NP protein. For RIG-I to interact with the genome and the critical 5′-PPP moiety, these viral proteins need either to be displaced or to dissociate from the vRNA. The former process could be facilitated by the ATP-driven helicase activity of RIG-I (Takahasi et al., 2008), whereas the latter may occur naturally as part of an equilibrium reaction. Indeed, viral RNA within vRNPs is accessible to nucleases (Duesberg, 1969) and might therefore also permit RIG-I docking, at least for a fraction of the estimated 100,000 viral genome segments present within an infected cell.

Here, we report that viral genomes are the major trigger for RIG-I in cells infected with negative-sense single-stranded RNA viruses. Our findings confirm earlier suggestions that single stranded RNAs bearing 5′-PPPs constitute effective agonists for RIG-I (Hornung et al., 2006; Pichlmair et al., 2006). It is worth noting that single-strandedness does not mean absence of base pairing. Flu genome segments and SeV genomic RNA adopt a "panhandle" conformation by pairing of complementary 5′ and 3′ ends (Knipe and Howley, 2007). Interestingly, Myong et al. showed that RIG-I translocates on synthetic double-stranded RNA molecules and that this movement is enhanced the presence of 5′-PPP (Myong et al., 2009). Notably, treatment of the stimulatory RNAs studied here with the double-stranded RNA specific nuclease RNase III abolishes RIG-I stimulatory activity (Figure S7B), which indicates that these RNAs contain base-paired regions. Therefore, we envisage that base-pairing within the "panhandle" structure of single-stranded flu and SeV genomic RNAs acts in cooperation with the presence of 5′-PPP to allow for potent RIG-I activation (Pichlmair et al., 2006). This model is likely to apply to other viruses sensed by RIG-I as panhandle structures are found in many single stranded RNA virus genomes (Schlee et al., 2009b). Thus, RIG-I integrates RNA secondary structure determinants and the presence of a 5′-PPP to effectively discriminate viral genomes from self-RNA.

EXPERIMENTAL PROCEDURES

Reconstitution of Flu vRNPs

One million HEK293T cells were transiently transfected using lipofectamine 2000 (Invitrogen) with 1 μg each of pcDNA-PB2, -PB1, -PA, and -NP (all from the flu WSN strain) and a pPOLI construct expressing a flu genome segment (derived from the flu A/PR/8/34 [PR8] strain). Two days after transfection, cell culture supernatants were collected and total RNA was extracted with TRIZOL (Invitrogen).

RNA Analysis

CIP (New England Biolabs), TER (Epicenter Biotechnologies), RQ1 DNase (Promega), and RNase V1 (Ambion) combined with RNase A (Sigma) or RNase III (Ambion) were used according to manufacturer recommendations. A control reaction omitting the enzyme was carried out in parallel. RNA was recovered by extraction with phenol:chloroform:isoamylalcohol (25:24:1), followed by chloroform extraction and precipitation with ethanol and sodium acetate in

the presence of glycogen. For size fractionation, RNAs were separated on 0.75% TBE-agarose gels at 70 V for 3 hr. Gels were cut into slices (including the well and bottom of the gel) and RNA was recovered from gel pieces with Quantum Prep Freeze N Squeeze Spin Columns (Bio-Rad) and precipitation (as above). Primer extension and northern blot assays are described in the Extended Experimental Procedures. Oligonucleotide sequences are given in Table S1.

Immunoprecipitation from Virally Infected Cells

Lysates from cells infected with flu PR8, flu PR8 ΔNS1, or SeV were used for immunoprecipitation as detailed in the Extended Experimental Procedures. Aliquots of the beads were boiled in SDS sample buffer for western blot analysis or RNA was recovered from the beads by extraction and precipitation as described above.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, seven figures, and one table and can be found with this article online at doi:10.1016/j.cell.2010.01.020.

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