5′-Triphosphate RNA Is the Ligand for RIG-I

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The structural basis for the distinction of viral RNA from abundant self RNA in the cytoplasm of virally infected cells is largely unknown. We demonstrated that the 5′-triphosphate end of RNA generated by viral polymerases is responsible for retinoic acid–inducible protein I (RIG-I)–mediated detection of RNA molecules. Detection of 5′-triphosphate RNA is abrogated by capping of the 5′-triphosphate end or by nucleoside modification of RNA, both occurring during posttranscriptional RNA processing in eukaryotes. Genomic RNA prepared from a negative-strand RNA virus and RNA prepared from virus-infected cells (but not from noninfected cells) triggered a potent interferon-α response in a phosphatase-sensitive manner. 5′-triphosphate RNA directly binds to RIG-I. Thus, uncapped 5′-triphosphate RNA (now termed 3pRNA) present in viruses known to be recognized by RIG-I, but absent in viruses known to be detected by MDA-5 such as the picornaviruses, serves as the molecular signature for the detection of viral infection by RIG-I.

Receptor-mediated detection of pathogen-derived nucleic acids assists in protecting the host genome from invading foreign genetic material. Retinoic acid–inducible protein I (RIG-I) recognizes a specific set of RNA viruses (Flaviviridae, Paramyxoviridae, Orthomyxoviridae, and Rhabdoviridae) (*1*–*3*), whereas a second member of this protein family, melanoma differentiation–associated gene 5 (MDA-5), is responsible for the antiviral defense against a reciprocal set of RNA viruses (Picornaviridae) (*3*). The four members of the Toll-like receptor (TLR) family (TLR3, TLR7, TLR8, and TLR9) involved in viral nucleic acid recognition are located in the endosomal membrane. TLRs are largely dispensable for effective antiviral defense, whereas the two cytosolic helicases MDA-5 and RIG-I (*1*) are essential for controlling viral infection.

The molecular characteristic of "double-strandedness" seems to allow for the distinction of self and nonself RNA. In the endosome, long double-stranded RNA (dsRNA) and its mimic polyinosinic-polycytidylic acid [poly(I:C)] but not single-stranded RNA (ssRNA) are recognized by TLR3 (*4*). In the cytosol, abundant self RNA complicates our understanding of recognition of nonself RNA. It is generally accepted that long dsRNA in the cytoplasm is detected as nonself and is thought to be recognized by MDA-5 and RIG-I (*1*, *5*). Recently, it was demonstrated that poly(I:C) is a ligand for MDA-5 but not RIG-I (*3*, *6*), whereas long dsRNA was found to activate RIG-I but not MDA-5 (*3*). One study suggests that synthetic short dsRNA with blunt ends is recognized by RIG-I and that two-nucleotide overhangs at the 3′ end block this recognition (*7*). Another group reported that short dsRNA (such as small interfering RNA), when generated by in vitro transcription, induced type I interferon (IFN) in cell lines (*8*). Together, these results suggest that there is more to cytoplasmic RNA recognition than long dsRNA.

We hypothesized that motif patterns or sequences in RNA may exist that are preferentially recognized. Long in vitro–transcribed RNA was transfected in monocytes and plasmacytoid dendritic cells (PDCs), and IFN-α production was assessed. We found that a 2500-nucleotide-long RNA molecule, but not the TLR9 ligand CpG-A (*9*) or the TLR7/8 ligand R848, stimulated a strong IFN-α response in primary human monocytes (fig. S1A). In vitro–transcribed RNA required a minimal length of 19 bases to efficiently induce IFN-α in monocytes (fig. S1B). Our results suggest that a molecular characteristic shared by all in vitro–transcribed RNA molecules, such as the 5′-triphosphate, rather than a specific sequence motif is responsible for IFN-α induction in monocytes.

To study the sequence-independent contribution of the 5′-triphosphate, we compared IFN-α induction of synthetic (5′-hydroxyl) and in vitro–transcribed (5′-triphosphate) versions of the immunostimulatory ssRNA oligonucleotide 9.2s (isRNA9.2s), which is composed of 19 nucleotides (*10*). We found that only the in vitro–transcribed version of isRNA9.2s, not the synthetic isRNA9.2s,

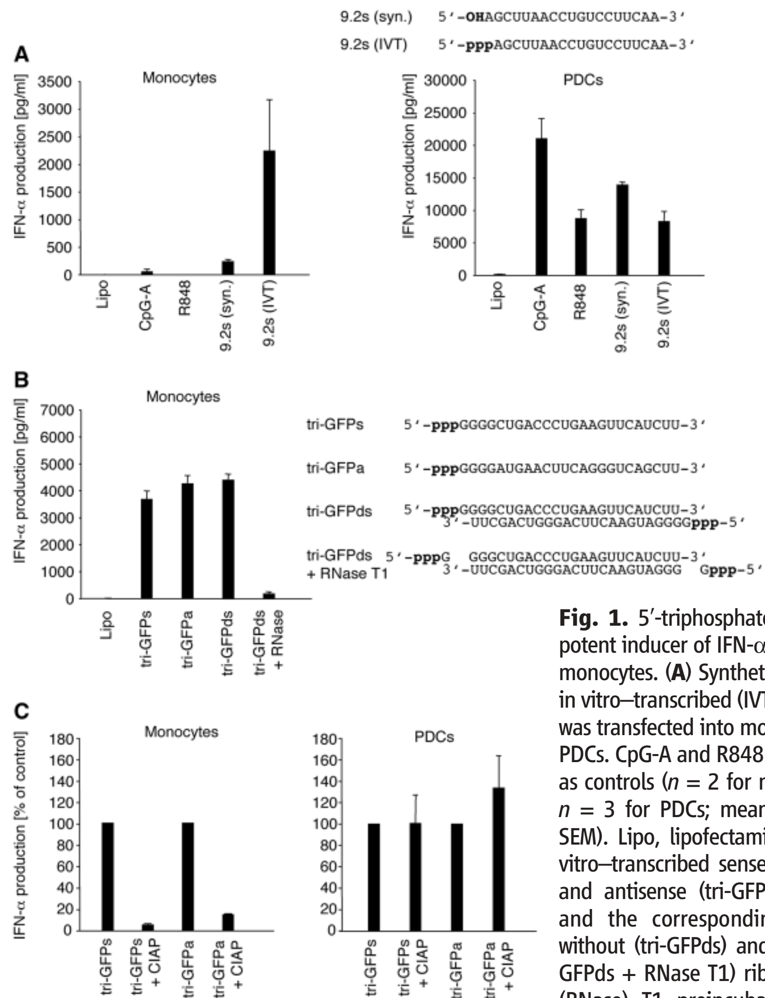

Fig. 1. 5′-triphosphate RNA is a potent inducer of IFN-α in human monocytes. (**A**) Synthetic (syn.) or in vitro–transcribed (IVT) RNA9.2s was transfected into monocytes or PDCs. CpG-A and R848 were used as controls (n = 2 for monocytes, n = 3 for PDCs; mean values ± SEM). Lipo, lipofectamine. (**B**) In vitro–transcribed sense (tri-GFPs) and antisense (tri-GFPa) strands and the corresponding dsRNA without (tri-GFPds) and with (tri-GFPds + RNase T1) ribonuclease (RNase) T1 preincubation were transfected into monocytes (n = 2; mean values ± SEM). (**C**) Tri-GFPs and tri-GFPa were dephosphorylated with calf intestine alkaline phosphatase (CIAP) and subsequently transfected into monocytes and PDCs (n = 2; mean values of normalized data ± SEM).

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strongly induced IFN-α production in monocytes (Fig. 1A). PDCs, known to detect ssRNA oligonucleotides via TLR7, produced IFN-α in response to both in vitro–transcribed and synthetic isRNA9.2s (Fig. 1A). Next, we used in vitro transcription to generate a dsRNA oligonucleotide with an overhang of one nucleotide at the 5′ position. The two single-stranded oligonucleotides (tri-GFPs and tri-GFPa) and the double-stranded oligonucleotide (tri-GFPds) induced comparable levels of IFN-α in monocytes (Fig. 1B). Cleavage of the 5′ overhang (including the 5′-triphosphate) of the dsRNA (tri-GFPds) (Fig. 1B) or dephosphorylation of the 5′ end completely abrogated the IFN response (Fig. 1C). PDCs, however, showed no decrease in IFN production when oligonucleotides were dephosphorylated (Fig. 1C). Together, these data indicate that the 5′-triphosphate is at least one well-defined structural feature responsible for IFN-α–inducing activity of in vitro–transcribed RNA in monocytes and that a 5′-triphosphate confers IFN-α–inducing activity to both ssRNA and dsRNA.

We examined the influence of a 7-methyl-guanosine cap on the IFN-α–inducing activity of 5′-triphosphate RNA. We found that RNA transcribed in the presence of a synthetic cap analog (containing ~80% capped RNA) was much less active in inducing IFN-α in monocytes as compared to uncapped in vitro–transcribed RNA (Fig. 2A). A strong decrease in IFN-α production in both monocytes and PDCs could be seen when either pseudouridine or 2-thiouridine was substituted for uridine (Fig. 2B). Analogous results were obtained when 2′-O-methylated uridine 5′-triphosphate (UTP) was incorporated into the triphosphate RNA oligonucleotides instead of UTP (Fig. 2C). These results indicate that common eukaryotic posttranscriptional modifications suppress the immunostimulatory activity of triphosphate RNA.

Triphosphate RNA–mediated IFN-α induction required neither endosomal maturation nor TLR7 (fig. S4). Human embryonic kidney (HEK) 293 cells, overexpressing full-length RIG-I, strongly responded to 5′-triphosphate RNA9.2s, whereas synthetic isRNA9.2s was inactive (fig. S5A). Consistent with these results, wild-type and MDA-5 $^{/}$ mouse embryo fibroblasts (MEFs) produced large amounts of IFN-β in response to 5′-triphosphate RNA, whereas no response was detected in RIG-I $^{/}$ MEFs (Fig. 3A). Endogenous transcription of triphosphate RNA, by means of a cytosolically expressed T7 RNA polymerase (Pol), also induced a strong IFN response in a RIG-I–dependent fashion (fig. S5, B and C). Together, these data provide evidence that RIG-I but not MDA-5 is required for the recognition of 5′-triphosphate RNA and that recognition of 5′-triphosphate RNA is not confined to immune cells.

To assess the importance of 5′-triphosphate RNA in the recognition of virus infection by RIG-I, we used rabies virus (RV), which is a prototypical rhabdovirus. Wild-type RV (SAD L16) encodes a potent antagonist of IFN induction, the phosphoprotein P. In contrast, RV genetically engineered to express little P (SAD ΔPLP) efficiently induces IFN (*11*). SAD ΔPLP infection per se triggered a potent IFN response in African green monkey kidney (Vero) cells that could be further increased through overexpression of RIG-I and strongly suppressed by the dominant-negative mutant RIG-IC (Fig. 3B). These results indicate that RIG-I is required for the initiation of an IFN response upon RV infection, as has been observed for other negative-strand viruses (NSVs), such as vesicular stomatitis virus and influenza (*3*). In addition, RNA isolated from RV-infected BSR cells, but not from noninfected cells, induced a potent IFN response in HEK 293T cells. This IFN production was abrogated when the RNA isolates were dephosphorylated (Fig. 3C), indicating that the 5′-phosphorylation status was critical for recognition. RNA from NSVs and NSV-infected cells is not considered infectious and does not allow the initiation of a replicative cycle. Indeed, the fact that RNA from RV SAD L16–infected cells was equally potent in terms of IFN-β induction as RNA from RV SAD ΔPLP–infected cells indicated that little or no productive translation and replication was initiated through

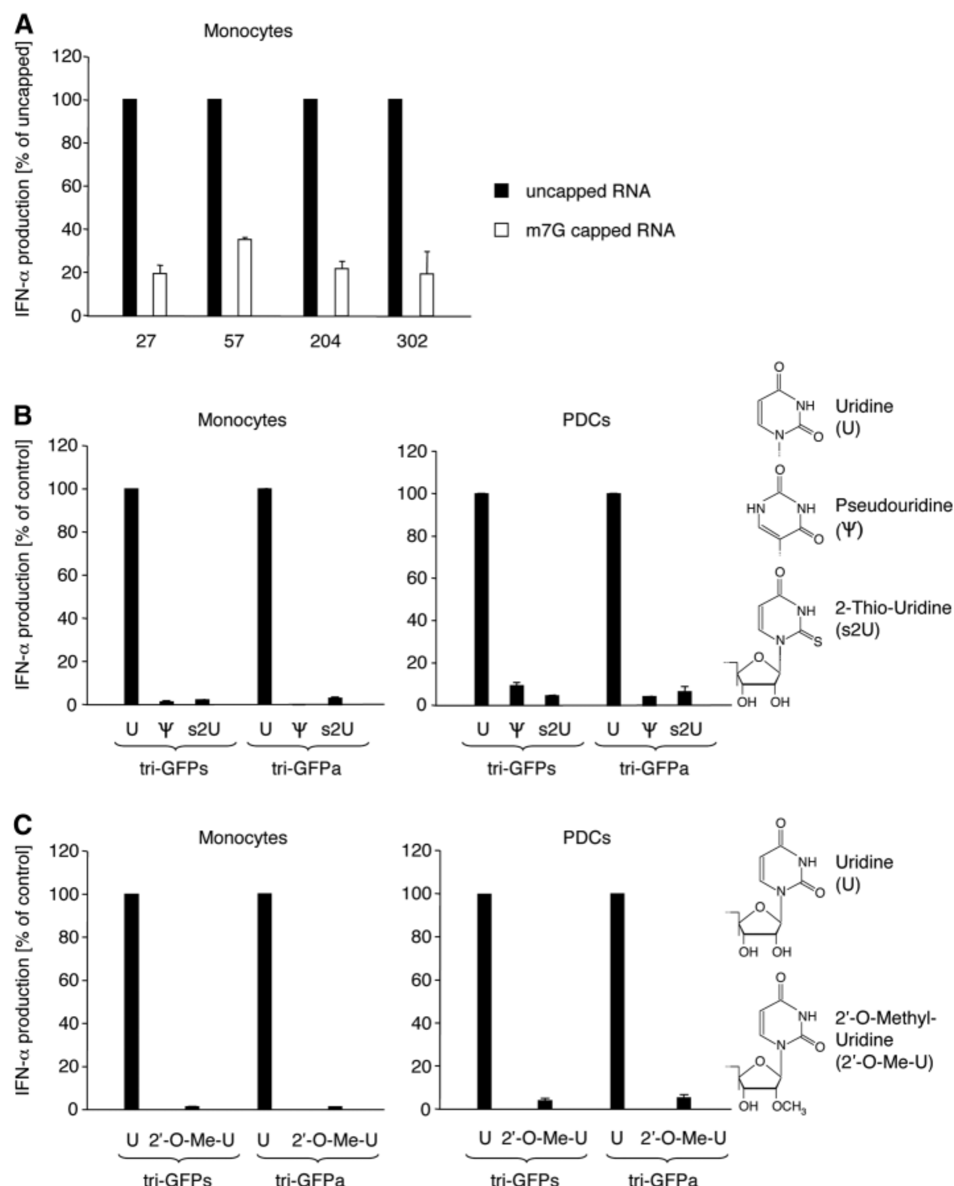

Fig. 2. 7-methyl-guanosine capping and base or sugar modifications abolish IFN-α induction by 5′-triphosphate RNA. (**A**) RNA molecules of various lengths (27, 57, 204, and 302 nucleotides) transcribed in the presence of the cap analog *N*-7-methyl-GpppG (m7G-capped RNA) or standard nucleoside triphosphates (uncapped RNA) were transfected into monocytes (n = 2; mean values of normalized data ± SEM). The absolute values for the respective RNA transcripts were 1401, 2351, 797, and 2590 pg/ml. (**B** and **C**) Tri-GFPs and tri-GFPa were transcribed in vitro in the presence of either UTP, pseudouridine 5′-triphosphate, or 2-thiouridine-5′-triphosphate (B), or 2′-*O*-methyluridine-5′-triphosphate (C) and subsequently transfected into monocytes or PDCs [n = 2 for (B), n = 3 for (C); mean values of normalized data ± SEM].

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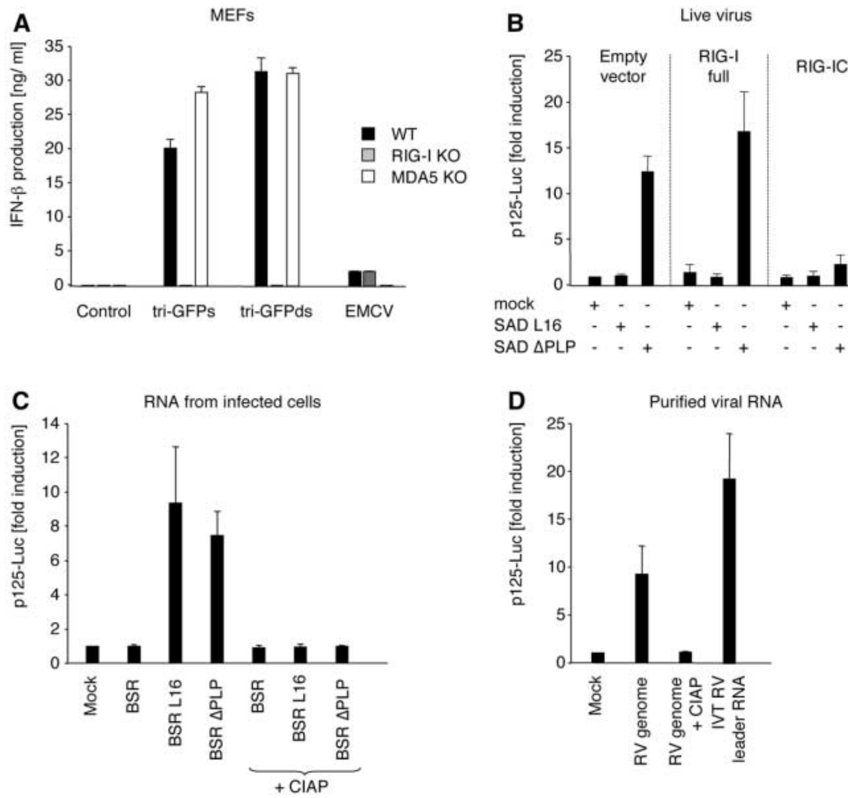

Fig. 3. 5′-triphosphate RNA and viral RNA induce a type I IFN response with RIG-I. (**A**) IFN-β production of MEFs from RIG-I– or MDA-5–deficient (KO) mice or wild-type (WT) mice transfected with tri-GFPs or tri-GFPds or infected with encephalomyocarditis virus (EMCV) at a multiplicity of infection (MOI) of 1 (mean values ± SEM of one representative experiment out of three). (**B**) Vero cells were transfected with the empty vector, RIG-I full, or RIG-IC and subsequently were either mock infected or infected with RV SAD L16 or RV SAD ΔPLP at an MOI of 3. p125-Luc, reporter plasmid encoding firefly luciferase downstream of the IFN-β gene promoter. (**C**) HEK 293T cells were transfected with 1 μg of total RNA isolated from noninfected BSR cells or from BSR cells infected with RV L16 (BSR L16) or RV ΔPLP (BSR ΔPLP). RNA was pretreated with CIAP as indicated [(B) and (C), mean values of normalized data (mock = 1) ± SEM of one representative experiment out of two]. (**D**) RNA from gradient-purified virions (RV L16) or CIAP-treated RNA from purified virions or in vitro–transcribed RNA corresponding to the 5′-terminal leader sequence of the RV SAD L16 complementary RNA were used to stimulate HEK 293T cells. Data from the experiment are shown as mean fold values (mock = 1) of triplicates ± SEM.

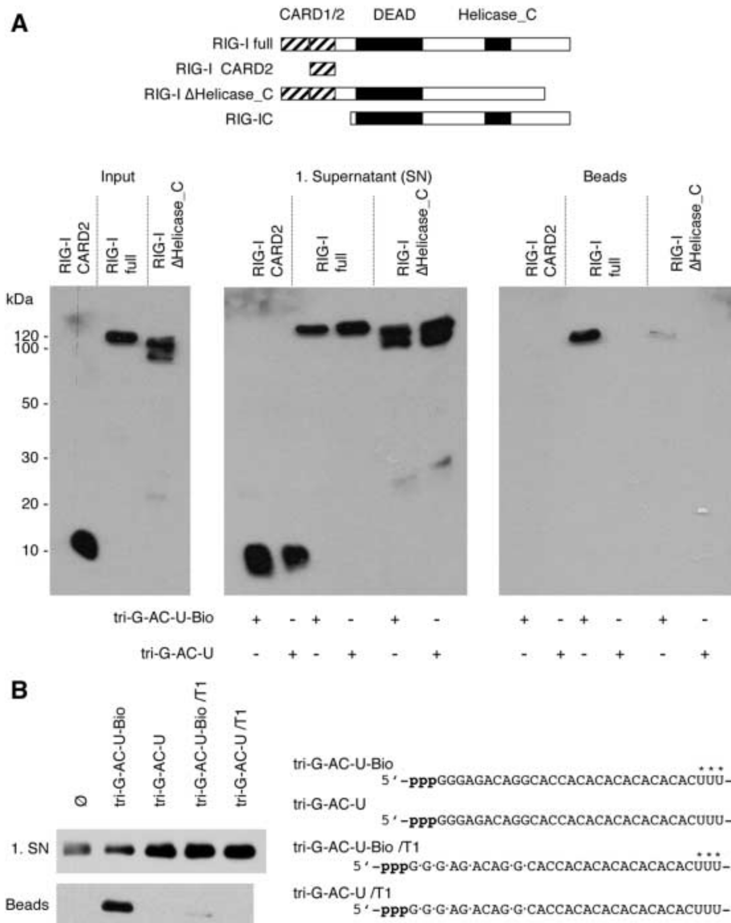

Fig. 4. 5′-triphosphate RNA directly binds to RIG-I. (**A**) HEK 293 cells were transfected with full-length RIG-I, RIG-I CARD2, or RIG-I ΔHelicase_C. Cell lysates were co-incubated with the indicated RNA oligonucleotides. For one representative experiment out of two, the input, the supernatants of the first wash (1. SN), and the bead-bound fraction (Beads) are depicted. (**B**) RNA with and without RNase T1 pretreatment (removal of the 5′ portion of the oligonucleotide containing the triphosphate group) was co-incubated with purified RIG-IC. The first supernatant and the bead-bound fraction of one representative pulldown experiment out of three are shown. Circled slash, control without oligonucleotide.

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the transfection of the respective RNA isolates. To completely rule out the possibility that replication of RV was required to trigger an IFN response, we isolated full-length RNA from virions and assessed it for the induction of IFN expression. Transfection of purified RV RNA effectively stimulated IFN induction in HEK 293T cells, whereas dephosphorylation of the genomic RV RNA isolates completely abrogated the observed IFN response (Fig. 3D). Together, these results demonstrate that RIG-I directly recognizes genomic RNA from RV independently of replication.

We next performed in vitro binding assays, testing the ability of 5′-triphosphate RNA to pull down RIG-I. RNA oligonucleotides with 3′-terminal biotin tags were generated and coincubated with whole-cell lysate from HEK 293 cells overexpressing full-length RIG-I and truncated versions of RIG-I. Although the 5′-triphosphate biotin oligonucleotide (tri-G-AC-U-Bio) was able to immunoprecipitate full-length RIG-I, no or little pulldown was seen when truncated versions of RIG-I (RIG-I CARD2 and RIG-I ΔHelicase_C) were tested for binding of the triphosphate RNA (Fig. 4A, right panel). Purified RIG-IC was also efficiently pulled down by triphosphate RNA oligonucleotides (Fig. 4B, second lane), but not if the initial 5′-triphosphate group was enzymatically removed before coincubation (Fig. 4B, fourth lane). These results indicate that 5′-triphosphate RNA directly binds to full-length RIG-I, and therefore RIG-I is the direct receptor responsible for the recognition of 5′-triphosphate RNA.

Our results provide evidence that uncapped unmodified 5′-triphosphate RNA (now termed 3pRNA) is a well-defined molecular structure of viral nucleic acids that is detected by RIG-I in the cytosol of eukaryotic cells. Many of the RNA species in the cytosol of eukaryotes are known to lack a free 5′-triphosphate group, although all RNA transcripts generated in the nucleus of a eukaryotic cell initially contain a 5′-triphosphate. Matured Pol I–transcribed ribosomal RNAs (rRNAs) in the cytosol have a monophosphate group at the 5′ end (*12*). mRNAs and small nuclear RNAs transcribed by Pol II are capped with a 7-methyl-guanosine group that is attached to the 5′-triphosphate (*13*). All mature tRNAs (Pol III) have a 5′-monophosphate (*14*), as is likely to apply to 5S rRNA. U6 RNA receives a γ-monomethylphosphate cap structure after transcription (*15*). 7SL RNA (Pol III), however, has a triphosphate at the 5′ end and is present at high copy numbers in the cytosol. Therefore, the presence or absence of a 5′-triphosphate might not be the only structural feature of RNA responsible for the distinction of self and viral RNA. Eukaryotic RNA undergoes substantial posttranscriptional modifications. The host machinery that guides nucleoside modifications and 2′-O-methylation of the ribose backbone is located in the nucleus (*16*). Because most RNA viruses do not replicate in the nucleus, extensive modification of viral RNA seems unlikely.

The mRNAs of viruses infecting eukaryotic cells commonly contain 7-methyl-guanosine cap structures at their 5′ ends and polyadenylate tails at their 3′ ends. Nonetheless, in many viruses, RNA synthesis leads to transient cytosolic viral RNA intermediates with an uncapped 5′-triphosphate end. RNA transcripts of all positive-strand RNA viruses of the family Flaviviridae start with an uncapped 5′-triphosphate, and members of all of these virus genera were reported as being recognized by RIG-I (*2*, *3*, *17*). Segmented NSVs initiate genomic and the complementary antigenomic RNA replication by a primer-independent de novo mechanism resulting in a 5′-triphosphate–initiated transcript (*18*). NSVs with a nonsegmented genome, including the paramyxoviruses and rhabdoviruses, initiate both replication and transcription de novo leading to 5′-triphosphate RNA in the cytosol. Consequently, genomic RNA from NSVs per se is expected to trigger an IFN response without the need for replication and presumed dsRNA formation. All viruses in the picornavirus-like supergroup use an RNA-dependent RNA Pol that exclusively uses a protein as a primer for both positive- and negative-strand RNA production; as a consequence, during the life cycle of picornaviruses, uncapped triphosphorylated 5′ ends are absent (*19*). Thus, although RIG-I is expected to be involved in the detection of Flaviviridae and NSVs, it cannot detect picornaviruses.

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RIG-I–Mediated Antiviral Responses to Single-Stranded RNA Bearing 5′-Phosphates

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Double-stranded RNA (dsRNA) produced during viral replication is believed to be the critical trigger for activation of antiviral immunity mediated by the RNA helicase enzymes retinoic acid–inducible gene I (RIG-I) and melanoma differentiation–associated gene 5 (MDA5). We showed that influenza A virus infection does not generate dsRNA and that RIG-I is activated by viral genomic single-stranded RNA (ssRNA) bearing 5′-phosphates. This is blocked by the influenza protein nonstructured protein 1 (NS1), which is found in a complex with RIG-I in infected cells. These results identify RIG-I as a ssRNA sensor and potential target of viral immune evasion and suggest that its ability to sense 5′-phosphorylated RNA evolved in the innate immune system as a means of discriminating between self and nonself.

The innate immune response to viral infection is characterized by the rapid production of a range of cytokines, most prominently type I interferons (IFN-α/β) (*1*). Specialized plasmacytoid dendritic cells (pDC) produce IFN-α/β when RNA or DNA viral

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