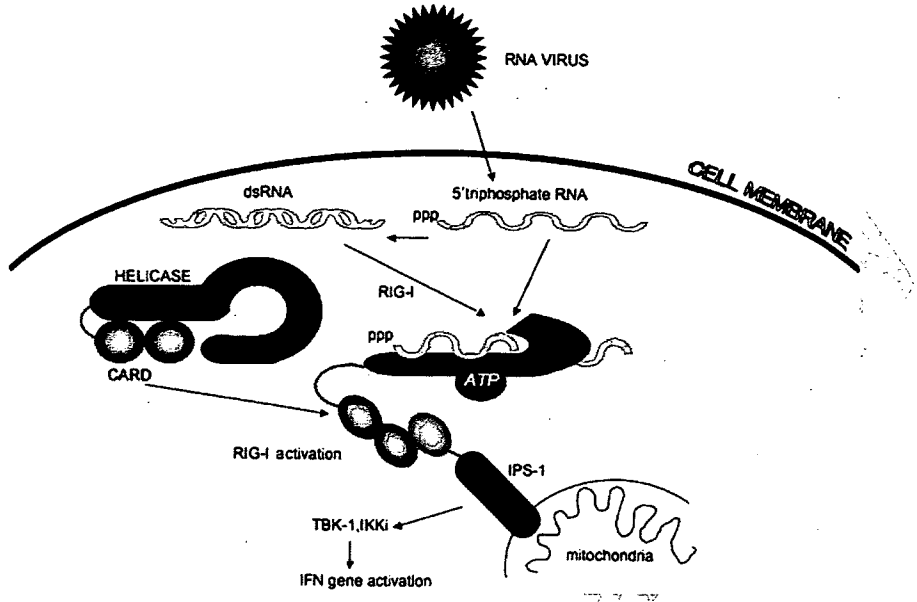

Fig. 2. Activation of RIG-I by viral RNA in cytoplasm. Normally, RIG-I conforms a "closed" structure, in which CARD is masked. On binding with viral RNA (dsRNA or 5′-pppRNA, see below) and ATP, RIG-I drastically alters its conformation and exposes CARD. RIG-I CARD specifically interacts with another CARD-containing adaptor, IPS-1, which is localized on the mitochondrial membrane. IPS-1 activates the regulatory kinases TBK-1 and IKK-i to induce IFN gene expression.

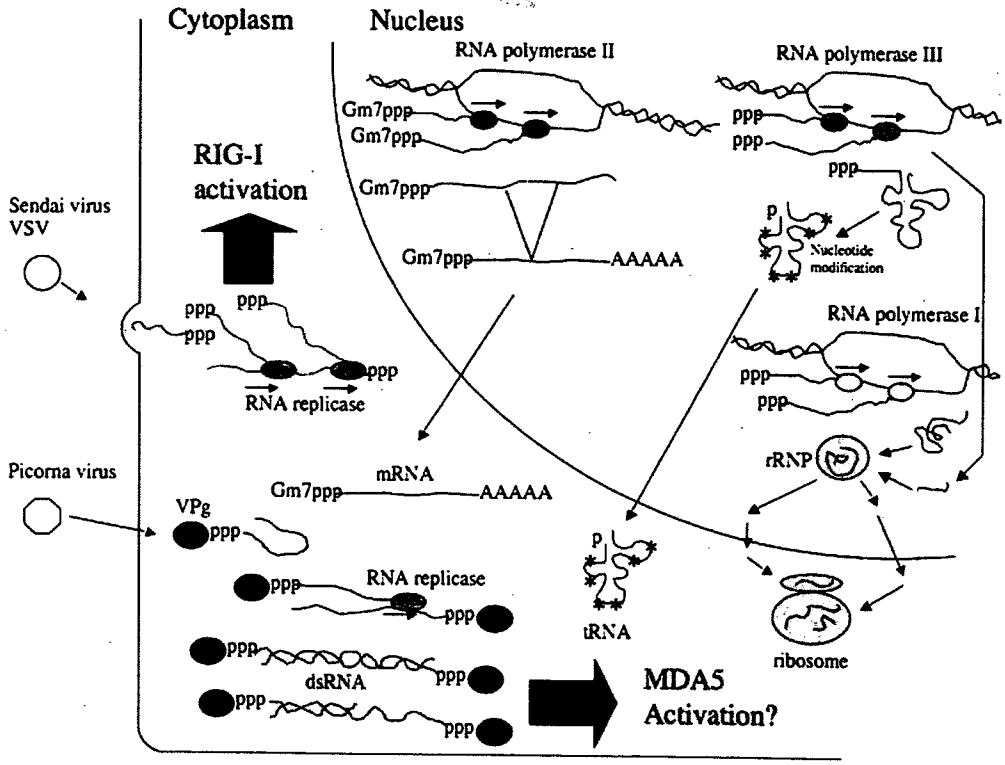

Fig. 3. Self and non-self RNA discrimination by RIG-I. In the cytoplasm of virus-infected cells, viral RNA containing 5′ tri-phosphate (ppp) is specifically recognized by RIG-I, and IFN production is induced. Cellular RNA syntheses take place in the nucleus by 3 different RNA polymerases. The 5′ ppp of these RNA are eventually removed or masked, thus self RNA species do not activate RIG-I. Picorna viruses encode VPg protein, which covalently attaches at 5′ of viral RNA. VPg may mask 5′ ppp to avoid detection by RIG-I. dsRNA produced as by-products of picorna viral replication may be detected by MDA5.

domain, but lacks CARD. When over-expressed in cultured cells, LGP2 dominantly inhibited IFN promoter activation induced by viral infection [46,49,50]. Knockdown by siRNA resulted in augmented IFN promoter activation by the virus. Similar to RIG-I and MDA5, the LGP2 expression level is highly inducible by IFN. These results strongly suggest that LGP acts as a feedback negative regulator of the virus-induced signal. Analysis of LGP2 knockout mice will define its role in vivo.

5. Self and non-self discrimination by RIG-I

During the screening of various RNA species for the activation of IFN genes, Hornung et al. observed that RNA produced by in vitro transcription but not by chemical synthesis is capable of inducing the signal [51]. They elucidated that the 5′ triphosphate structure of single-stranded (ss)RNA is critical for detection by RIG-I (Fig. 2). Pichlmair et al. observed that influenza virus does not accumulate dsRNA in infected cells while being capable of activating IFN genes [52]. They found that ssRNA extracted from purified influenza virion is capable of inducing a signal when transfected; however, the dephosphorylation of RNA abolished this activity. Taking these observations together the authors hypothesized that the primary product of viral replication containing 5′ ppp structure can serve as a signature of non-self RNA. Cellular self RNA escapes detection by RIG-I because they are known to undergo several modifications before being transported to the cytoplasm: messenger RNA acquires a 7-methyl-guanosine moiety at its 5′ end; transfer RNA undergoes 5′ cleavage and a series of nucleotide modifications; and ribosomal RNA readily associates with ribosomal proteins (Fig. 3). Although the details remain to be proven, the 5′ ppp structure hypothesis well explains the experimental observations.

6. Viral inhibitors of RIG-I/MDA5 signaling

If innate immune responses are engaged appropriately, viral replication will never take place. Viruses apparently acquire the means to avoid immune responses. Picornaviruses are not detected by RIG-I presumably because viral Vpg protein is covalently attached at the 5′ end of viral RNA to hinder the ppp structure [53]. V protein of paramyxoviruses specifically associates and blocks the function of MDA5 [46,54]. NS3/4A of hepatitis C virus cleaves IPS-1 adaptor protein, thus terminating the signals activated by RIG-I and MDA5 [41,43–45]. Several reports demonstrate that NS1 of influenza A virus inhibits RIG-I by physical association; however, the action mechanism of NS1 is controversial [52,55–58]. Although the precise mechanism is not clear, VP35 of Ebola virus and the G1 cytoplasmic tail of Hantavirus inhibit RIG-I signaling [59,60]. Mouse hepatitis virus (MHV) neither induces IFN production nor blocks poly (rI): poly (rC)-induced activation of the IFN promoter [61]. These observations led to the speculation that MHV sequesters its own dsRNA to avoid cellular detections, including those by RIG-I/MDA5. A growing list of viral inhibitors for RIG-I/MDA5 signaling indicates the crucial function of this cascade in host defense. As shown in Fig. 4, cellular antiviral response and viral inhibitors oppose each other. When innate immunity dominates viral replication, both viral replication and the production of viral inhibitor are blocked. If viral replication dominates, viruses manage to accumulate sufficient inhibitors to totally circumvent immune responses; thus, a subtle initial balance may determine outcome of infection.

7. Cell type specificity of signaling

It has been known that pDC produces a large amount of IFN-α upon viral infection [62]. pDC recruits a distinct set of signaling components to activate IFN genes including TLR7/8, TLR9, MyD88, IRAK1 and IRF-7 (Fig. 5). In natural infections, several distinct pathways, summarized in Fig. 5, are potentially activated. Each pathway depends on a specific signaling molecule: MyD88, TLR3, RIG-I and MDA5 are exclusively required for the pathways mediated by TLR7/8/9, TLR3, RIG-I and MDA5 sensors, respectively. Thus, to explore the role of these pathways in vivo, viral challenge experiments using respective knockout mice were performed [37,47]. When challenged with Japanese encephalitis virus (JEV), RIG-I-deficient mice exhibited increased mortality

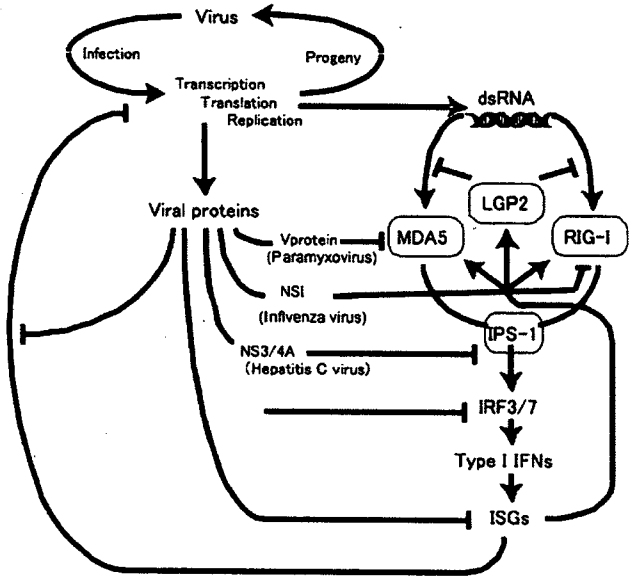

Fig. 4. RIG-I-mediated antiviral responses and viral inhibitors. When a virus infects cells and initiate replication, viral RNA accumulates in cytoplasm. Viral RNA triggers activation of RIG-I and MDA5, resulting in the activation of the IFN system. Since RIG-I and MDA5 are IFN-inducible at the level of transcription, the initial signal by viral RNA is amplified through autocrine IFN. Another helicase, LGP2, may act as a negative regulator of this signaling. Viruses encode various inhibitory proteins targeting various steps of the cascade, including RIG-I, MDA5 and IPS-1. These inhibitors accumulate in cells as viruses replicate successfully; therefore, initial conditions, such as the multiplicity of infection and cellular levels of RIG-I and MDA5, will determine the result of infection.

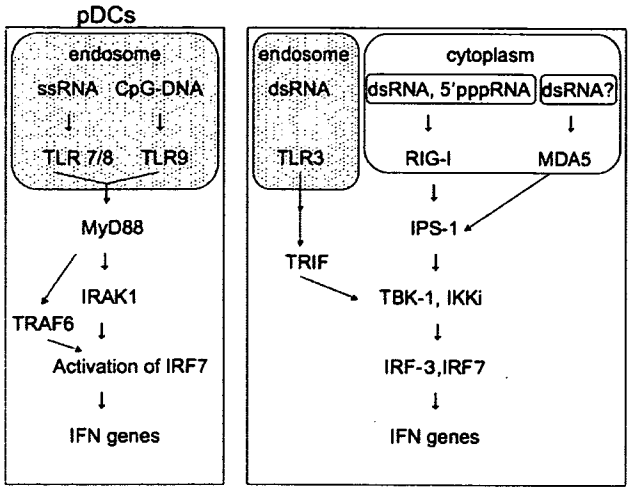

Fig. 5. Cell-type specificity of the signaling. TLR7/8, and -9 function in a pDC-specific manner, activating MyD88-dependent signaling. In other cells, RIG-I and MDA5 play a major role in antiviral responses. TLR3 is activated in some cell types and triggers TRIF-dependent signaling.

whereas mice deficient in MDA5 or MyD88 did not show a significant change in mortality. Upon challenge by EMCV, MDA5-deficient mice exhibited high mortality; however, mice deficient for RIG-I, TLR3 and MyD88 were not significantly susceptible. These results are consistent with studies using knockout cells and re-emphasize the significance of RIG-I and MDA5 in vivo. On the other hand, these results do not exclude the role of MyD88- and TLR3-dependent signaling in antiviral responses. These pathways may cooperate to maximize the defense for a variety of viruses with different tissue tropism and the route of infection.

8. Perspective

After 50 years of IFN discovery, we have an outline of how IFN production is induced. Several sensors, including TLRs, RIG-I and MDA5, recognize viral RNA to initiate the reaction; however the precise mechanism of self or non-self discrimination is not well understood. It is possible that some cellular transcripts are actually recognized in cytoplasm by RIG-I or MDA5 to participate in certain physiological regulation. *C. elegans* encodes a RIG-I-like helicase (Dicer Related Helicase-1) which is essential for RNA interference in nematodes [63]; however, no evidence has been reported on the role of RIG-I or MDA5 in mammalian RNA interference, suggesting that structural similarity may be a consequence of evolution.

The identification of numerous virus-encoded inhibitors against RIG-I and MDA5 function suggests the development of new antiviral drugs, which indirectly target these inhibitors. Also, RNA medicines, which mimic viral RNA to selectively activate antiviral responses, may be developed to enforce cellular innate immunity for the treatment of viral infections.

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MINIREVIEW

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Function of RIG-I-like Receptors in Antiviral Innate Immunity*

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Mitsutoshi Yoneyama and Takashi Fujita[1]

From the Laboratory of Molecular Genetics, Institute for Virus Research and Graduate School of Biostudies, Kyoto University, Kyoto 606-8507, Japan

Various cells in the body are capable of sensing infectious viruses and initiating reactions collectively known as antiviral innate responses. These responses include the production of antiviral cytokines such as type I interferon (IFN)[2] and subsequent synthesis of antiviral enzymes, which are responsible for the impairment of viral replication and promoting adaptive immune responses (1). In this minireview, we focus on a subset of molecules known as RIG-I-like receptors, which sense viral RNA molecules that trigger a danger signal.

RIG-I-like Receptors

Three genes encode RIG-I-like receptors (RLR) in human and mouse genomes (2). Three DExD/H box helicases, termed retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated antigen 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2), exhibit marked primary structure conservation in their helicase domain (Fig. 1). As the analysis of RIG-I precedes the other two, the biochemical characteristics of RIG-I will be described in this section.

As shown in Fig. 1, RIG-I contains two repeats of the caspase recruitment domain (CARD)-like motif at its N terminus. The cDNA clone was initially obtained by functional screening based on reporter gene activation, essentially consisting of tandem CARD (3). Although less efficient than the signal by full-length RIG-I activated by viral infection, overexpression of the tandem CARD alone is sufficient to generate signaling and subsequent type I IFN production (2). CARD acts as a signaling domain, which interacts with a downstream molecule (IPS-1, see below) to relay the signal. Single amino acid substitution within the first CARD (T55I) is sufficient to inactivate CARD function, and tandem CARD is necessary for its function (4). So far there is no report showing the signal-dependent proteolytic release of CARD from full-length RIG-I, suggesting that processing is unlikely in the mechanism of RIG-I activation. Full-length RIG-I exhibits undetectable or very low constitutive activity in the cell transfection assay, suggesting that the C-terminal region contains a domain for autorepression. Indeed, functional analysis revealed that the C-terminal domain (Fig. 1, *Repression Domain*) is responsible for autorepression by interacting with both CARD and helicase domains (5). Interestingly, RIG-I with loss of function of CARD, either by deletion (RIG-IC) or point mutation (T55I), is incapable of transmitting a signal upon viral infection and dominantly inhibits virus-induced signaling (3, 4). This is because of the lack of a signaling domain and the presence of a repression domain as well as RNA binding activity. RIG-I exhibits strong double-stranded RNA (dsRNA) binding activity *in vitro*. RIG-I selectively binds with poly(rI:rC), poly(rA:rU), and 5′- and 3′-untranslated regions of hepatitis C virus genomic RNA (which are predicted to form a secondary structure) but not with dsDNA, poly(rA), or yeast tRNA (3, 4). RNA binding requires intact helicase and C-terminal autorepression domains (5) (Fig. 1).

Self and Non-self RNA Discrimination by RIG-I

The above results suggest that RIG-I is a specific sensor for dsRNA, which is absent in uninfected cells but known to be accumulated in virus-infected cells; however, influenza A virus infection results in IFN gene activation without detectable dsRNA accumulation (6). In these cells, it is proposed that single-stranded RNA (ssRNA) with 5′-triphosphate functions as a ligand for RIG-I. Actually RIG-I specifically binds with RNA containing 5′-triphosphate but not with RNA containing 5′-di- or 5′-monophosphate (7). These observations led to an interesting hypothesis of how self and non-self RNA species are discriminated. As shown in Fig. 2, host RNA synthesis takes place in the nucleus. Like the viral transcript, cellular primary transcripts contain 5′-triphosphate; however, these RNAs undergo various processes; mRNA acquires a 7-methylguanosine CAP structure at its 5′-end; tRNA undergoes 5′-cleavage and a series of nucleotide base modifications; the primary transcript of ribosomal RNA readily complexes with ribosomal proteins to form ribosomal ribonucleoprotein and undergoes maturation processes, which therefore are masked from detection. Indeed, artificial capping and base modification of 5′-triphosphate ssRNA abrogated detection by RIG-I (7), whereas viral RNA, either freshly introduced by infection or produced by viral replication, contains a non-self marker, 5′-triphosphate. In this regard, 5′-triphosphate RNA generated by DNA virus may well be detected by RIG-I.

Activation Mechanism of RIG-I

It is worth noting that a single amino acid substitution K270A renders RIG-I into a dominant inhibitor (3). Lys-270 is supposed to be a critical motif for ATP binding within the helicase domain, and in the case of other DExH/D helicases, this motif is crucial for its helicase (unwinding dsRNA) activity. As proteolysis is an unlikely mechanism (above) to reverse autorepression, the current de-repression model for RIG-I is illus-

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[2] The abbreviations used are: IFN, interferon; RIG-I, retinoic acid-inducible gene I; RLR, RIG-I-like receptor(s); dsRNA, double-stranded RNA; ssRNA, single-stranded RNA; CARD, caspase recruitment domain; IPS-1, interferon promoter stimulator-1; TRAF3, TNF receptor-associating factor 3; IKK-i, IκB kinase-i; TBK-1, TANK-binding kinase-1; cDC, conventional dendritic cell; NDV, Newcastle disease virus; pDC, plasmacytoid dendritic cell; IL, interleukin; TLR, Toll-like receptor; MyD88, myeloid differentiation factor 88; IRAK1, interleukin-1 receptor-associated kinase 1; IRF, interferon regulatory factor; VSV, vesicular stomatitis virus; JEV, Japanese encephalitis virus; EMCV, encephalomyocarditis virus; RNAi, RNA interference.

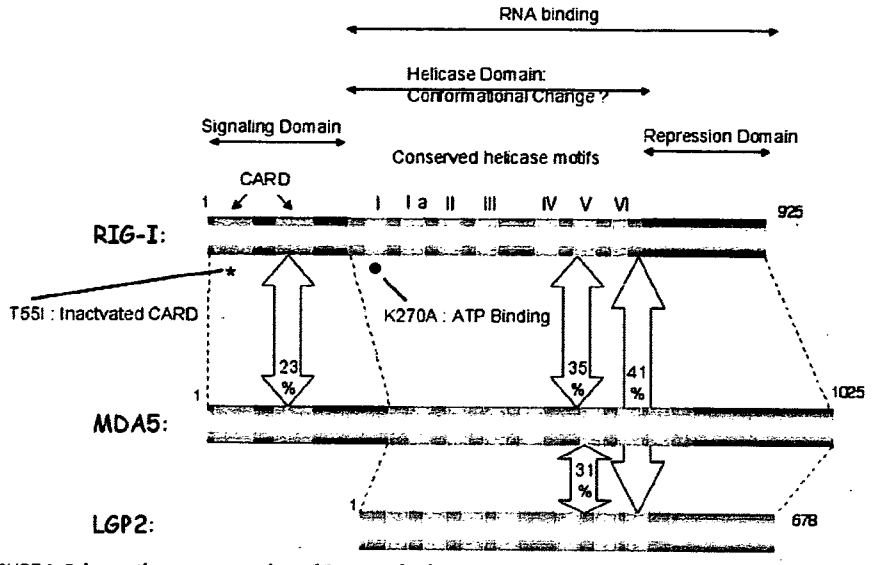

FIGURE 1. **Schematic representation of RIG-I and other RLRs.** Functional domains determined by mutagenesis are indicated. The conserved amino acid sequence of CARD and the helicase domain is indicated (percent identity, between human RLRs).

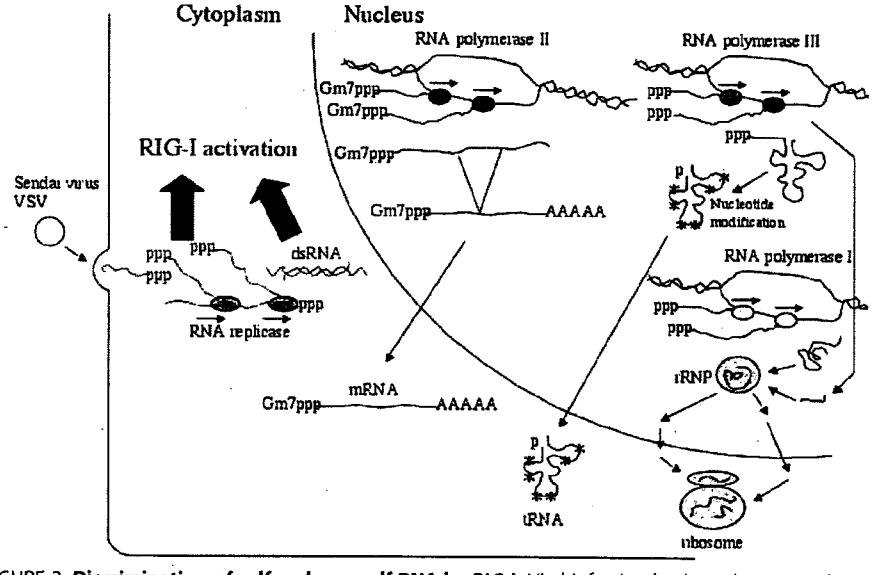

FIGURE 2. **Discrimination of self and non-self RNA by RIG-I.** Viral infection leads to the accumulation of non-self RNAs in the cytoplasm, such as dsRNA and 5′-triphosphate RNA. Cellular transcripts are modified to lack or mask these structures when transported to cytoplasm.

TABLE 1
Differential functions of RIG-I family helicases

RIG-I	Positive regulator	NDV, Sendai virus, influenza virus, VSV, JEV, *in vitro* transcribed dsRNA
MDA5	Positive regulator	Picornavirus, poly (I):poly (C)
LGP2	Negative regulator?	?

trated in Fig. 3. RIG-I exists as a "closed" structure in uninfected cells and therefore CARD is masked. The virus-specific RNA species, dsRNA or 5′-triphosphate ssRNA, specifically binds to RIG-I through its RNA binding domain. This association and ATP binding to the helicase domain change RIG-I conformation to release CARD for relaying signaling to the downstream molecule (another CARD-containing molecule, IPS-1 (alternatively termed MAVS, VISA, and Cardif)) (8–11). IPS-1 is localized on the outer membrane of mitochondria, and this localization is crucial for its function (9, 12–14) although its precise mechanism is not known. MDA5 and RIG-I, which sense a distinct set of viruses (below), commonly transmit signals to IPS-1; thus IPS-$1^{-/-}$ fibroblasts are unresponsive to either set of viruses (15, 16). The signal is branched at IPS-1, resulting in the activation of NF-κB and IRF-3 and -7. The latter involves TNF (tumor necrosis factor) receptor-associating factor 3 (TRAF3) (17) and the protein kinase, IκB kinase-i (IKK-i (ε)) or TANK-binding kinase-1 (TBK-1) (18, 19), which is responsible for the activation of IRF-3 and -7.

LGP2 lacks CARD, suggesting that this helicase is incapable of transmitting a positive signal. Overexpression of LGP2 in cell culture results in the dominant inhibition of virus-induced activation of IFN genes, suggesting its role as a negative regulator; however, its role *in vivo* is not established (2, 20, 21) (Table 1).

RIG-I Acts as a Major Viral Sensor in Fibroblasts and cDCs but Not in pDCs

Analyses of RIG-$I^{-/-}$ fibroblasts and conventional dendritic cells (cDCs) showed that RIG-I is essential in Newcastle disease virus (NDV)-induced IFN production; however, RIG-I is dispensable for virus-induced IFN production by plasmacytoid dendritic cells (pDCs) (22). pDCs adopt a distinct signaling cascade to produce high levels of IFN-α and sense viral infection by TLR7/8 and TLR9, activating signaling cascades MyD88, IRAK1/4, TRAF3/6, IKK-α, and IRF-7 (23, 24). It has been known that dsRNA activates TLR3 in endosome and signals through TIR domain-containing adaptor inducing IFN-β (TRIF)/TIR-containing adaptor molecule-1 (TICAM1) resulting in the activation of kinases (TBK-1 or IKK-i) and transcription factors (IRF-3 and -7 and NF-κB) (23). However, the mice defective in the TLR3-TRIF pathway exhibit normal IFN response upon viral infections (25). When poly(rI:rC) is injected into mice intravenously, IFN-β is strictly produced in a MDA5-dependent manner (below), but the TLR-TRIF pathway is dispensable. However, production of IL-8 and IL-12 p40

requires TRIF in addition to MDA5; IL-12 p40 is particularly largely TRIF-dependent (25). These observations demonstrate that although MDA5 and TLR3 signal a common pathway, different spectrums of cytokine genes are activated.

Specificity of Viral Sensing by RIG-I and MDA5

The overall structural similarity between RIG-I and MDA5 suggests the functional similarity of these proteins. Gene disruption studies revealed that these helicases sense distinct viral species (25). Cytokine production induced by the infection of Sendai virus, NDV, vesicular stomatitis virus (VSV), influenza A virus, and Japanese encephalitis virus (JEV) is markedly impaired in RIG-I$^{-/-}$ cells (Table 1). In contrast, cytokine production by encephalomyocarditis virus (EMCV), Thyler's virus and Mengo virus, all Picornaviruses (genus cardiovirus), is virtually absent in MDA5$^{-/-}$ cells (Table 1). In agreement with these observations, virus challenge experiments using knockout mice revealed that RIG-I$^{-/-}$ and MDA5$^{-/-}$ mice are selectively vulnerable to JEV and EMCV, respectively. It is remarkable that RIG-I/MDA5 deficiency exhibits a severe impact on viral infection *in vivo*, suggesting the critical function of innate immune responses in promoting adaptive immunity and virus eradication. Interestingly, genomic RNA of VSV and poly(rI:rC) selectively activates RIG-I and MDA5, respectively, suggesting that the distinct responses of RIG-I and MDA5 to different viruses are because of the distinct recognition of viral RNA by these sensors.

Virus-encoded Inhibitors of Innate Immune Responses

Viruses evolve to avoid host immune surveillance by producing inhibitors of the IFN system (Table 2). Generally, viral replication takes place in a restricted compartment where the viral genome is protected from detection by host sensors. Mouse hepatitis virus takes this strategy to avoid innate immune responses (26). Viral proteins evolve to counteract RLR functions. NS3/4A of hepatitis C virus inactivates IPS-1 by its protease activity (9, 12–14). Other viral proteins inhibit RLR signaling at various steps for their survival. It is noteworthy that many proteins encoded by DNA viruses also target RLR signaling.

RIG-I Activation by Endogenous RNA?

Although self RNA species are supposed to be tolerant to RIG-I detection (above), various RNAs with a secondary structure exist (known as non-coding and micro-RNA). Therefore, it remains to be established that RLR plays any role in physiological regulation by endogenous RNA. RIG-I$^{-/-}$ mice are embryonic lethal in certain genetic backgrounds, suggesting a role for RIG-I in development. *Caenorhabditis elegans* encodes a DExD/H box helicase, Dicer related helicase-1, which is essential for RNA interference (RNAi) in nematodes (27); however, no evidence has been reported on the role of RIG-I or MDA5 in mammalian RNAi, suggesting that structural similarity may be a consequence of evolution. Apparently, RNAi is independent of the IFN system in mammalian cells (28). It has been reported that experimental gene silencing either by transfection of 21-mer dsRNA or expression of short hairpin RNA has the potential to activate IFN gene and downstream events (28, 29). In this regard, the importance of end struc-

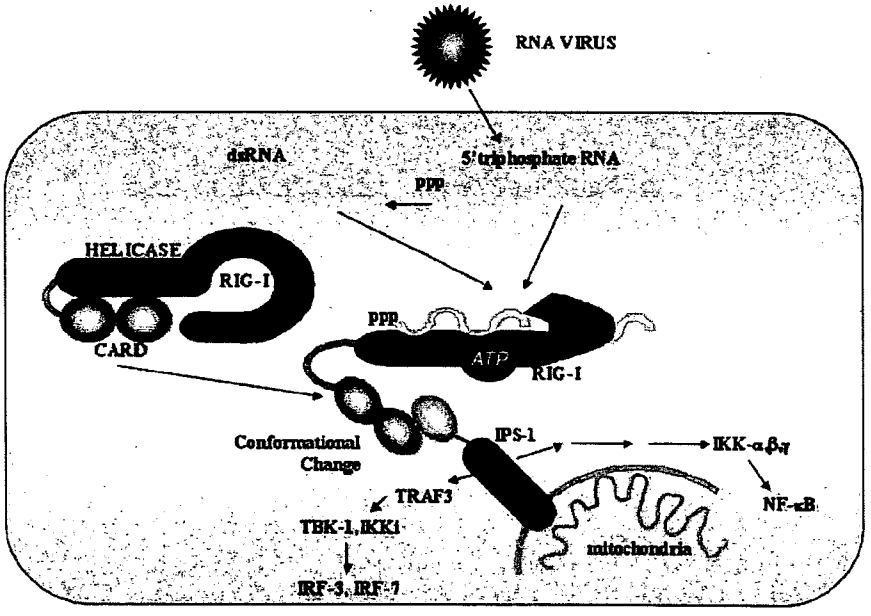

FIGURE 3. **Activation of RIG-I by viral RNA.** Model of RIG-I activation by viral RNA.

TABLE 2
Viral inhibitors of RLR signaling

Virus	Inhibitor	Mode of action	Refs.
Hepatitis C virus	NS3/4A	Cleavage of IPS-1	9, 12–14
Influenza A Virus	NS1	dsRNA binding, binding with RIG-I and IPS-1	6, 31–33
Ebola virus	VP35	dsRNA binding, inhibition of TBK-1, IKK-i	34
Paramyxo virus	V Protein	Binding to MDA5	2, 35, 36
Hanta virus (NY-1)	G1	Inhibition of TBK-1	37
West Nile virus	Unidentified	RIG-I-dependent and -independent pathway	38
Human cytomegalovirus	pp65 (ppUL83)	Inhibition of IRF-3 function	39
Herpes simplex virus	ICP0 and other	Inhibition of IRF-3 function	40
Human papilloma virus 16	E6	Inhibition of IRF-3 function	41
Vaccinia virus	E3L	Inhibition of IRF-3 function	42
Thogoto virus	ML	Inhibition of IRF-3 function	43

ture of substrate RNA is suggested (30); however, strict substrate requirements for the activation of RLR pathway and RNAi remain to be established.

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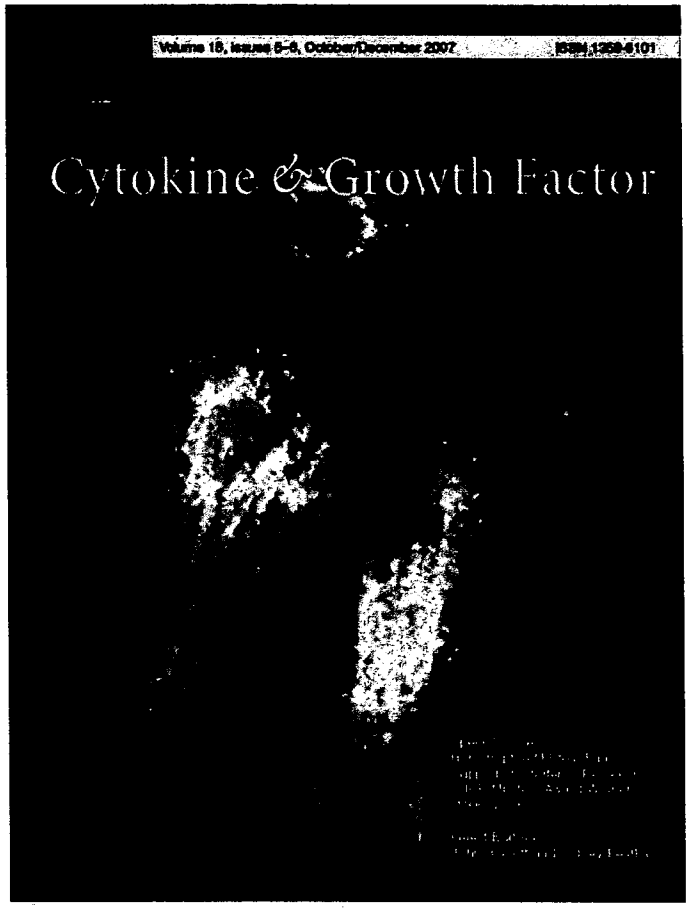

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RIG-I family RNA helicases: Cytoplasmic sensor for antiviral innate immunity

Mitsutoshi Yoneyama [a,b], Takashi Fujita [a,b,*]

[a] *Laboratory of Molecular Genetics, Institute for Virus Research, Kyoto University, Kyoto 606-8507, Japan*
[b] *Laboratory of Molecular Cell Biology, Graduate School of Biostudies, Kyoto University, Kyoto 606-8507, Japan*

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Abstract

Viral infection is detected by cellular sensors as foreign nucleic acid and initiates innate antiviral responses, including the activation of type I interferon (IFN) and proinflammatory cytokines. Recent advances in cytoplasmic virus sensors highlight their essential role in the induction of innate immunity. Moreover, it is intriguing to understand how they can discriminate innate RNA from viral foreign RNA. In this mini-review, we focus on these cytoplasmic virus sensors, termed retinoic acid inducible gene-I (RIG-I)-like receptors (RLRs), and discuss their function in the innate immune system.

Keywords: Type I interferon; RIG-I; RNA helicase; Innate immunity

1. Introduction

Type I IFN is a critical cytokine for antiviral innate immunity [1]. The regulated expression of IFN genes, especially IFN-β, has been analyzed extensively, because of its physiological importance in antiviral immunity, as well as its significance as a model to understand transcriptional mechanisms including the interaction between transcriptional activator and basal transcription machinery. In response to viral infection, transcription factors, ATF/c-*jun*, NF-κB and IFN regulatory factor (IRF), bind to the promoter region of IFN-β gene [2–5], form a transcriptional complex termed "enhanceosome" and activate robust transcription through interaction with the RNA polymerase complex [6]. In the case of IFN-α genes, however, only IRF is known to be an essential regulator [7], indicating an indispensable function of IRF(s) in viral-induced activation of IFN genes. In 1998, it was reported that IRF-3 and -7 are key regulators of IFN gene activation [8–14]. Both IRF-3 and IRF-7, which are specifically phosphorylated by a viral-induced signal, then translocate to the nucleus, thereby binding to the promoter sequence of IFN genes. Analysis of knockout mice with these genes clearly showed their essential role in the activation of antiviral immunity [15,16]. More recently, TANK-binding kinase (TBK1) and IκB kinase (IKK)-i were identified as kinases for the specific phosphorylation of C-terminal Ser-residues of IRF-3 and IRF-7 [17,18], and this observation was confirmed by subsequent reports using gene knockout [19–21]. On the other hand, after the discovery of toll-like receptors (TLRs) as sensors for infection in the innate immunity of higher eukaryotes, analysis of TLR-mediated signaling was dramatically progressed [22]. TLRs recognize microorganism-associated molecular patterns (MAMPs), including lipopolysaccaride (LPS) or viral nucleic acids, and activate the expression of IFN and proinflammatory cytokines, such as interleukin-6 and tumor necrosis factor-α, through the activation of IRFs and NF-κB. Among 10 TLRs in the human genome, TLR3 is characterized as a receptor for double-stranded RNA including polyI:C, which is known as a strong inducer of type I IFNs, and is reported to utilize the TBK-1/IKK-i-IRF-3/7 signaling axis [22]. Therefore, it has been strongly suggested that the TLR system could be a

* Corresponding author. Tel.: +81 75 751 4031; fax: +81 75 751 4031.
E-mail address: tfujita@virus.kyoto-u.ac.jp (T. Fujita).

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main pathway for virus-induced innate immunity, however, the observation that IFN was induced even in TLR3-KO cells after viral infection or polyI:C-transfection indicated the possible involvement of a TLR3-independent, cytoplasmic signaling pathway [23]. Indeed, Nod1 and Nod2 have been identified as cytoplasmic bacterial sensors, which recognize bacterial cell wall components, and induce the expression of proinflammatory cytokines via NF-κB activation [24]. More recently, a bacteria-induced "inflammasome", which activates caspase 1 and leads to the processing of pro-interleukin (IL)-1 and pro-IL-18, has been extensively examined [25]. In 2004, RIG-I was isolated as a cytoplasimic virus sensor, and two other family molecules were identified [23]. Here, we focus on the function and biological significance of these RLRs.

2. RIG-I-like receptors (RLRs)

RLRs consist of three family members: RIG-I, melanoma differentiation-associated gene 5 (MDA5) and the laboratory of genetics and physiology 2 (LGP2). The mouse homolog of MDA5 is also termed Helicard [26]. These are DExD/H-containg RNA helicases and are expressed ubiquitously in cytoplasm. It is noteworthy that these genes are IFN-inducible genes (ISGs). To avoid complications, we propose to re-name RIG-I, MDA5 and LGP2 as RLR-1, -2 and -3, respectively.

2.1. RIG-I (RLR-1)

Although RIG-I was originally identified as the gene induced in retinoic acid-treated cells [27], its physiological function had not been determined. RIG-I is characteristic of the two repeated caspase recruitment domain (CARD) at its N-terminus in addition to C-terminal DExD/H-box RNA helicase domain (Fig. 1). Involvement of RIG-I in antiviral immunity was reported by expression cloning experiments; cDNA library was introduced into mouse L929 cells together with the reporter construct, which is regulated by IRF-binding sites. After stimulation with polyI:C-transfection, the cDNAs which augmented the reporter activity were selected. Among them, one clone, which encoded the N-terminal region of RIG-I, showed constitutive activity to induce the reporter [23]. The physiological importance of RIG-I was determined by analysis of RNA interference and KO mice [28] (see below).

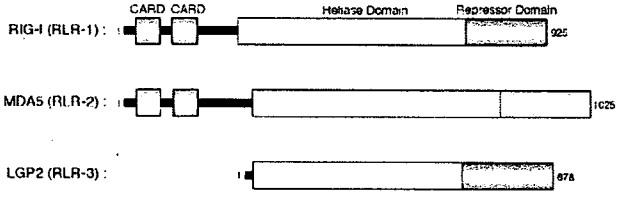

Fig. 1. Primary structure of RLRs. All RLRs are DExD/H-containing RNA helicases (yellow). RIG-I and MDA5 contain tandem CARD at their N-terminal region (pink). Although the C-terminal domain of both RIG-I and LGP2 (orange) has a role as a repressor domain (RD), the homologous region of MDA5 does not show any inhibitory effect.

2.2. MDA5 (RLR-2)

MDA5 was isolated as the gene induced in the IFN- and protein kinase C-activating compound mezerein-treated melanoma cell line, HO-1 cells [29]. The structure of MDA5 protein is closely related to RIG-I with 23% and 35% amino acid similarity in the CARD and helicase domain, respectively. Experiments using both cell culture and KO-mice clearly indicated its essential role in IFN production [30,31] (see below). It was also reported that Helicard, a mouse homolog of MDA5, might be involved in apoptosis [26]. Although the authors showed the cleavage of Helicard between the CARD and helicase domain by caspases, no clear explanation for the cause–result relationship of this cleavage and apoptosis is provided. It is reported that poliovirus infection results in a similar cleavage of MDA5 [32].

2.3. LGP2 (RLR-3)

LGP2 was identified as the gene adjacent to the signal transducer activator of transcription (STAT) 3/5 loci [33]. LGP2 protein showed amino acid similarity with 41% and 31% to helicase domains of RIG-I and MDA5, respectively, but completely lacked CARD. *In vitro* experiments showed that LGP2 plays an inhibitory role in RIG-I/MDA5-mediated signaling [30,34,35]; however, the physiological function of LGP2 remained to be determined.

3. RLR-mediated signal transduction

Overexpression of N-terminal CARD of either RIG-I or MDA5 constitutively activates IRF-3, NF-κB and the subsequent expression of endogenous IFN-β, suggesting the direct involvement of CARD in signaling [23]. Although full-length RIG-I or MDA5 did not show any constitutive activity, virus- or dsRNA-induced activation of signaling was strongly enhanced. Furthermore, the observations that the helicase domain of RIG-I/MDA5 directly interacts with poly I:C and the mutant with inactivated ATP binding of helicase does not work as an inducer, apparently indicated the regulatory role of helicase domains. Recently, it has been reported that the C-terminal region of RIG-I has a role as a repressor domain (RD) to keep RIG-I in the inactivated form in the absence of an activation signal [36]. Therefore, the following model for RLR-mediated signaling is likely (Fig. 2). RIG-I is silenced in cytoplasm by intra-molecular interaction with RD. In the case of MDA5, since the C-terminal domain of MDA5 does not work as RD, an unidentified mechanism could be involved in silencing.

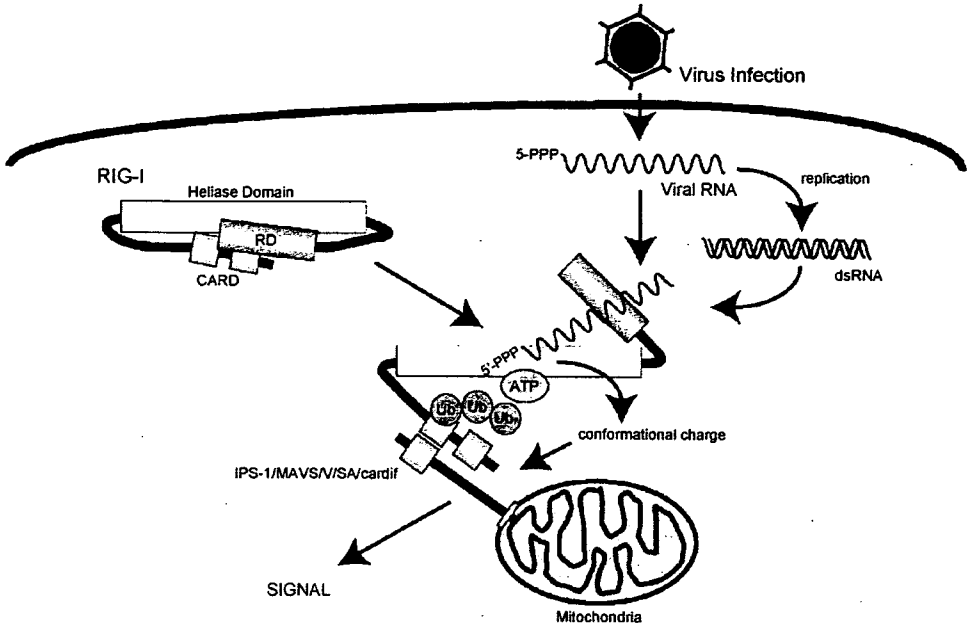

Fig. 2. Schematic representation of RIG-I-mediated signal. RIG-I is expressed in cytoplasm as an inactive form, in which CARD and helicase are masked by intra-molecular interaction with RD. After virus infection, viral RNA with 5′-triphosphate is recognized by the helicase domain of RIG-I. The conformational change induced by interaction with viral RNA allows interaction with the downstream adaptor, IPS-1, on mitochondria and signal transduction, leading to the activation of both IRF-3 and NF-κB.

Once cells are infected with viruses, viral RNA is recognized by the helicase domain of RIG-I or MDA5 with distinct specificity (see below). The recognition of viral RNA induces conformational change of RLRs in an ATP-dependent way and the liberation of CARD from silencing allows signal transduction downstream.

In 2005, four independent groups reported the identification of IFN promoter stimulator-1 (IPS-1) (also termed MAVS/VISA/Cardif) as CARD-containing adaptor molecules for RIG-I/MDA5 [37–40]. Analysis of KO mice with the IPS-1 gene clearly indicated its essential role in virus-induced signaling [41,42]. Interestingly, IPS-1 is localized on the outer membrane of mitochondria using its N-terminal transmembrane domain (TM), and its localization is essential for signaling [40,43–45]. CARD–CARD interaction between activated RLRs and IPS-1 induces the recruitment of downstream signaling molecules, including TBK-1/IKK-i and FADD/RIP1/IKKα/IKKβ, which are involved in the activation of IRF-3/7 and NF-κB, respectively [37]. Recently, it has been demonstrated that direct binding with tripartite motif protein 25 (TRIM25) and Lys63-linked ubiquitination of RIG-I CARD, but not MDA5 CARD, are essential for RIG-I-mediated signaling [46], suggesting differential regulation of the signal. TNF receptor-associated factor 3 (TRAF3) is known to play a role in signaling downstream of IPS-1 [47]. Furthermore, involvement of another CARD-containing molecule, CARD9, in virus-induced activation of the MAPK cascade has been demonstrated, while interaction between RLR/IPS-1 and CARD9 remains to be examined [48].

4. Cell-type specificity of RLRs

Analysis of KO mice with the RIG-I gene clearly showed its cell-type-specific usage in antiviral innate immunity [28]. Virus-induced IFN production was completely abolished in fibroblast and conventional dendritic cells (cDCs) from RIG-I-deficient mice, indicating an essential role of the cytoplasmic virus-sensing system in these cells, whereas RIG-I-deficient plasmacytoid DCs (pDCs), which are known as strong IFN-α inducers, showed no defect (Fig. 3). Identical cell specificity was observed in MDA5-KO mice [31]. Since pDCs derived from MyD88-deficient mice were unresponsive to viral infection, the TLR7/8-MyD88-IRF-7 pathway is indispensable for virus-induced production of type I IFN in pDCs.

5. Substrate specificity of RLRs

As foreign viral MAMP, it has been thought that dsRNA could be a substrate for RLRs, because dsRNA is predicted to be a product as the result of viral replication and polyI:C is a well-known IFN inducer; however, two recent studies have brought a novel insight into substrates for RIG-I [49,50]. They demonstrated that RIG-I recognizes 5′-phospho-structure of viral RNA, rather than dsRNA structure (Fig. 3). Indeed, cytoplasmic viral RNA, which appears after viral infection or replication, contains 5′-triphosphate moiety. On the other hand, 5′-ends of most endogenous innate RNA are modified or masked by specific processing

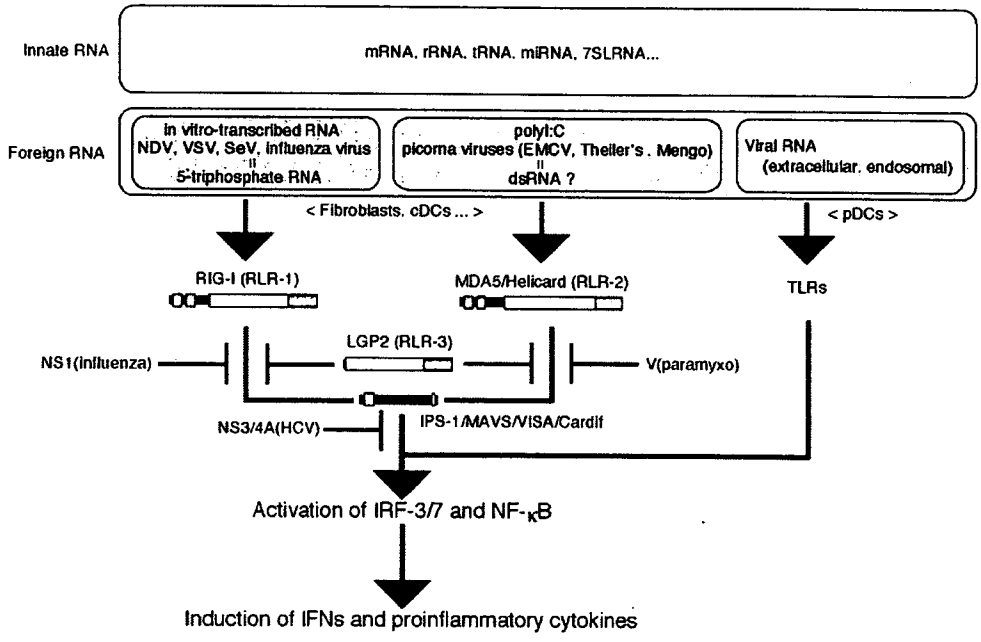

Fig. 3. Specificity of viral RNA recognition in antiviral innate immunity. In most types of cells, RIG-I is essential for the recognition of 5′-triphosphate-containing viral RNA, including NDV, VSV, SeV and influenza virus. MDA5 is a sensor for picornavirus (and polyI:C); however, the determinant of its specific recognition has not been clarified. In pDCs, the TLR system is indispensable for the activation of innate antiviral signaling. These virus sensors strictly discriminate endogenous innate RNA from viral RNA. The viral inhibitors targeting RLR-mediated signal are shown.

steps, such as 7-methyl-guanosine capping for messenger RNA and complex formation as ribosome for ribosomal RNA. Therefore, this discovery highlights a mechanism for discrimination between innate and foreign RNA in innate immunity; however, it remains unclear how 5′-triphosphate-containing self-RNA, such as 7SL RNA, escape detection by RIG-I.

Despite structural and functional similarities between RIG-I and MDA5, the analysis of knockout mice revealed the difference of their substrate specificity [31] (Fig. 3). When RIG-I-deficient embryonic fibroblast cells (MEFs) were transfected with *in vitro* transcribed double stranded (ds)RNA, or infected with Newcastle disease virus (NDV), vesicular stomatitis virus (VSV), Sendai virus (SeV), influenza virus or Japanese encephalitis virus (JEM), production of IFN was strongly impaired, whereas no defect was observed in MDA5-deficient cells. On the other hand, induction of IFN by infection with picornaviruses, such as encephalomyocarditis virus (EMCV), Theiler's virus and Mengo virus, or transfection with polyI:C, was inactivated in MDA5-KO cells, but not in RIG-I-KO cells. These results strongly indicated that these RLRs differentially recognize cytoplasmic viral RNA. Indeed, the substrates for RIG-I, i.e., *in vitro*-transcribed RNA and viruses except for picornavirus, contain a distinctive 5′-triphosphate moiety. In this regard, the 5′-end of picornavirus RNA is masked by viral protein, termed Vpg [51], explaining escape from recognition by RIG-I. Then, what is the structure of substrate for MDA5? A dsRNA structure may be possible candidate for MDA5, because the recent report indicated that dsRNA is accumulated in picornavirus-infected cells, but not in influenza virus-infected cells [49]. Further analysis, including an *in vitro* assay using recombinant proteins, will be needed to clarify this issue.

6. Viral proteins and RLRs

It has been documented that viruses have a strategy to interfere with host immune responses for their survival. Information about viral inhibitors targeting RLRs is also accumulating (Fig. 3). V protein of paramyxoviruses, such as NDV and SeV, specifically interacts with MDA5 and interferes with activity to transmit a signal [30,52,53]; however, the biological significance of this inhibition is not clear, because infection with paramyxovirus is detected by RIG-I, but not by MDA5. Recently, nonstructural protein (NS)1 of influenza A virus was reported to directly bind to RIG-I, and inhibit its function [49,54–56]. On the other hand, NS3/4A of hepatitis C virus is known to be an inhibitor of both RIG-I- and MDA5-mediated signals. In 2003, it was reported that protease activity of NS3/4A specifically inhibits virus-induced activation of IRF-3 [57]. Although the results indicated that RIG-I-mediated signal was inhibited by NS3/4A, direct digestion of RIG-I was not observed [58]. Identification of IPS-1, a downstream adaptor of RLRs, as

the target of NS3/4A protease, suggested that NS3/4A inactivated both RIG-I- and MDA5-mediated signals [40]. Furthermore, the observation that IPS-1 was cleaved just outside of TM by NS3/4A and released from the mitochondrial membrane clearly indicated its essential localization [40,43–45].

These observations suggest that the valance between the inhibition of innate immunity by viral proteins and inactivation of viral growth by RLR-induced immune responses could affect the virulence and pathogenesis of viral infection. Therefore, information about these virus–host interactions would help to bring about a novel therapeutic strategy against infectious diseases.

7. Future perspectives

In recent years, there have been dramatic advances in the understanding of cytoplasmic virus sensors, RLRs, to initiate innate immunity. In contrast to TLRs, whose expression is restricted to a specific cell type, RLRs are expressed ubiquitously and play an essential role in virus-induced IFN expression, suggesting the foremost antiviral defense system in most organs. On the other hand, recent reports documented that IFN system plays a central role in pathogenesis of autoimmune diseases, such as systemic lupus erythematosus (SLE). Although the direct involvement of RLRs in autoimmunity has not been defined, it should be noted that association between the MDA5 gene and the susceptibility locus for type 1 diabetes has been recently reported [59]. In 2006, two studies demonstrated that transfection of dsDNA can induce the expression of type I IFN in a TLR/RLR/IPS-1-independent and TBK-1/IKK-i-IRF-3/7-dependent manner, indicating DNA virus-specific sensor(s) [60,61]. In contrast, it has been reported that infection with DNA virus produced a substantial amount of dsRNA in infected cells [62], suggesting a possible involvement of RLRs, such as MDA5, for DNA virus sensing. It is interesting to clarify how DNA viruses are sensed by the innate immune system. Finally, it has been suggested that RLRs might be involved in mouse development, because RIG-I-KO mice are embryonic lethal with a certain genetic background [28]. This observation provides the interesting possibility that RLRs might interact with endogenous RNA and play an unknown role other than virus sensing.

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Mitsutoshi Yoneyama obtained PhD in Osaka University (Prof. T. Taniguchi's laboratory) in 1993. He worked on molecular mechanism of virus-induced type I IFN activation at The Tokyo Metropolitan Institute of Medical Science, and identified RIG-I RNA helicase as cytoplasmic virus sensor in innate immune system. In 2006, he moved to Institute for Virus Research of Kyoto University as associate professor of Prof. T. Fujita's laboratory.

Takashi Fujita is a professor in the Laboratory of Molecular Genetics, Department of Genetics and Molecular Biology, Institute for Virus Research of Kyoto University. Finished his PhD in Biology in 1982 at Waseda University Tokyo, Japan where he studied the mechanism of IFN priming. During 1982–1984, he worked as a postdoctoral fellow in Cancer Institute (Tokyo). During 1984–1990, he worked as a research associate in Osaka University and studied gene expression of interferon-β and interleukin-2 in Dr. T. Taniguchi's laboratory. From 1990 to 1991 he was a postdoctoral fellow in Whitehead Institute and from 1991 to 1993 he studied gene expression by NF-κB transcription factor family in Dr. David Baltimore's laboratory as a postdoctoral fellow at Rockefeller University. During 1993–2005 he studied the mechanism of IRF-3 activation and identified CARD helicase as sensing molecule for replicating viruses in Tokyo Met. Inst. Med. Sci. From September 2005 to till date he is a Professor in the Institute for Virus Research, Kyoto University.

the development of colitis indistinguishable from that of IL-10-deficient mice[14].

In conclusion, analysis of IL-10–Foxp3 double-reporter mice has shown that Foxp3$^+$ T_{reg} cells can develop either in the thymus or in the periphery to limit inflammation. The new data by Weaver and colleagues[2], moreover, show that both Foxp3$^+$ and Foxp3$^-$ thymic precursors, both of which are negative for *Il10* expression in the thymus, can give rise to peripheral T_{reg} cells expressing IL-10. It seems that production of IL-10 by either Foxp3$^+$ or Foxp3$^-$ T cells requires peripheral signals delivered in response to microbial products abundant in the gut. The 10BiT mice reported here will provide an invaluable new tool for delineating the mechanisms of induction of IL-10 expression in response to different pathogens, in different anatomical locations and at different stages of the immune response.

COMPETING INTERESTS STATEMENT
The authors declare no competing financial interests.

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Cytoplasmic double-stranded DNA sensor

Mitsutoshi Yoneyama & Takashi Fujita

A new study in *Nature* identifies a long-sought cytoplasmic 'sensor' that is responsible, at least in part, for interferon responses induced by double-stranded DNA.

How do cells detect non-self double-stranded DNA (dsDNA) molecules? Several 'sensors' of RNA, including Toll-like receptors (TLRs) and the RNA helicases RIG-I and Mda5, have been shown to be essential for innate immune responses to many types of pathogens. In many contexts, such as autoimmunity and infection by some pathogens, dsDNA also leads to immune responses. Therefore, the existence of a cytoplasmic dsDNA sensor has been proposed, although its actual identification has remained a long-standing quest. New progress has helped to accomplish this quest and to address the longstanding issue of how dsDNA in the cytoplasm is detected by the innate defense system. In *Nature*, Taniguchi and colleagues have identified the DNA-dependent activator of interferon-regulatory factors (DAI) as a candidate cytoplasmic sensor for pathogen DNA[1].

When the body is infected by viral or bacterial pathogens, several types of innate immune responses are initiated. These responses occur within hours of infection even when the infected cells have never been exposed to the pathogen. The main response to viral infection is the activation of cytokine-encoding genes, including those encoding type I interferon (IFN-α/β); these cytokines confer on uninfected cells an antiviral state that inhibits the replication of diverse viruses, including the invading pathogen. Moreover, IFN-α/β and other cytokines promote acquired immunity to specifically eradicate the infecting agents days after infection.

To initiate the innate immune responses to invading pathogens, cells must 'sense' foreign molecules known as 'pathogen-associated molecular patterns' (PAMPs), which are chemical compounds specific to pathogens. For example, lipopolysaccharide is a PAMP associated with Gram-negative bacteria that is detected by TLR4. There are more than ten different transmembrane TLRs that can likewise 'sense' extracellular molecules in mammals and thus function as detectors of various different PAMPs[2].

In the cytoplasm, Nod-like receptors function to detect PAMPs associated with intracellular bacteria[3], whereas RIG-I-like receptors (such as RIG-I and Mda5)[4] function to detect virus-derived RNA. Both Nod-like receptors and RIG-I-like receptors are structurally unrelated to TLRs (**Fig. 1**). RIG-I-like receptors sense double-stranded and 5′-triphosphated RNA, both hallmarks of non-self RNA[5]. In addition, some natural and synthetic RNAs are also 'nonviral inducers' of interferon. In contrast, other than hypomethylated CpG DNA that activates TLR9, leading to the activation of genes encoding IFN-α in plasmacytoid dendritic cells[2], DNA molecules generally do not induce interferon as well as RNA molecules do. Still, some DNA viruses can induce relatively robust interferon production, although the identity of the cytoplasmic DNA sensor required for this response has remained elusive until now.

Data supporting the relevance of DNA-induced interferon production has been provided by studies of DNase II–deficient mice[6]. Macrophages are important during the maturation of mammalian erythrocytes, as they 'engulf' and normally degrade nuclei from erythrocyte precursors; degradation of nuclei occurs in lysosomes. However, DNase II–deficient macrophages are unable to digest nuclei; such macrophages that have engulfed nuclei instead induce IFN-β, which leads to lethal anemia in the developing embryo. Data demonstrating the requirement for IFN-β for this lethality has been provided by studies of mice doubly deficient in both DNase II and the interferon receptor; the lethality is abrogated in these mice.

Relevant to the work by Taniguchi and colleagues discussed here[1], IFN-β production in mice deficient only in DNase II is independent of TLRs and RIG-I-like receptors

Mitsutoshi Yoneyama and Takashi Fujita are in the Laboratory of Molecular Genetics, Institute for Virus Research, and Laboratory of Molecular Cell Biology, Graduate School of Biostudies, Kyoto University, Kyoto, 606-8507, Japan.
e-mail: tfujita@virus.kyoto-u.ac.jp

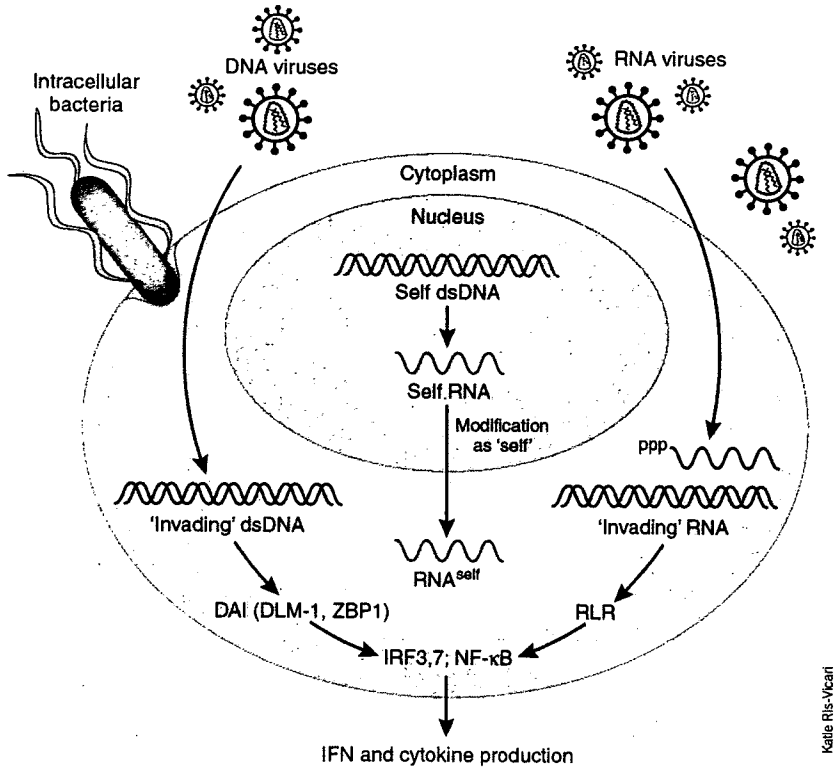

Figure 1 In the innate immune system, cytoplasmic sensor molecules detect invasion of viruses or bacteria, then activate expression of cytokines, including type I interferon, and inhibit their multiplication. A sign of infection is the introduction of pathogen-derived nucleic acids. Advances in this field have now clarified two nucleic acid–sensing systems, with DAI as the sensor of 'invading' dsDNA (blue) and RIG-I-like receptor (RLR) as the sensor of 'invading' RNA (red). Each sensor transmits a signal to induce interferon and cytokines through the activation of IRF3, IRF7 and NF-κB. Because self RNA is abundant in cytoplasm, RIG-I-like receptors must strictly discriminate pathogen-derived RNA (red) versus self RNA (green; RNA^{self}), which was tagged as 'self' by modification[5]. RIG-I, which is a member of the RIG-I-like receptor group, recognizes the 5′-triphosphate moiety (ppp) and dsRNA structures that are specific for viral RNA. In contrast, because self DNA (green) is normally compartmentalized in the nucleus, DAI selectively detects 'invading' dsDNA.

and is triggered by self DNA that cannot be degraded by the DNase II–deficient macrophages. Subsequently, two reports have unequivocally demonstrated that dsDNA introduced directly into the cytoplasm can induce activation of interferon genes independently of TLRs or RIG-I-like receptors but in a way that is dependent on the kinase TBK-1 or the IKKi kinases and interferon-regulatory factor 3 (IRF3)[7,8]. These observations suggest the existence of unique dsDNA sensors in the cytoplasm.

In *Nature*, Taniguchi and colleagues[1] focus on the previously characterized interferon-inducible gene (*Zbp1*) encoding DLM-1, a protein expressed by the peritoneum-lining cells of tumor-bearing mice[9]. DLM-1 is also called 'ZBP1' because it contains two Z-form DNA–binding domains[10]; Taniguchi and colleagues have renamed this 'DAI' (for 'DNA-dependent activator of IRFs'). Because RIG-I-like receptors are interferon inducible, the authors speculated that the gene encoding the dsDNA sensor would also be interferon inducible. They therefore searched for interferon-inducible genes and selected the gene encoding DAI as a possible candidate. They show that DAI has dsDNA-binding properties (**Fig. 1**) and that overexpression of DAI augments interferon production induced by synthetic, bacterial or mammalian dsDNA[1]. Moreover, 'knockdown' of DAI by RNA-mediated interference impairs dsDNA-induced activation of genes dependent on IRF3, transcription factor NF-κB and interferon, indicating involvement of DAI in the dsDNA-mediated interferon response. They also show that the carboxy-terminal portion of DAI directly interacts with the targets TBK1 and IRF3.

Although Taniguchi and colleagues show that 'knockdown' of DAI by RNA-mediated interference impairs dsDNA-induced production of interferon[1], the inhibition is partial, suggesting possible alternative pathways. Tissue-specific expression of human DAI (ref. 11) also suggests this receptor may function in a cell type–specific way. The presence of other sensors, including related families with distinct specificities, is a possibility. Elucidation of the effect of DAI deletion by gene knockout will further the understanding of dsDNA-induced innate immune reaction.

Moreover, unlike the RNA sensors, DAI detects dsDNA, a structure common to both self DNA and non-self DNA, suggesting that the discrimination between these types of DNA is based on subcellular localization rather than a chemical feature of the ligand. It remains to be established how detection of chromosomal DNA is avoided during the mitotic phase when the nuclear membrane disappears. As indicated by studies of DNase II–deficient mice[6], efficient elimination of 'unwanted' homologous DNA by DNase II is critical to avoid toxic production of interferon. Analogously, identification of negative regulators of DAI may be critical for understanding how the discrimination between self DNA and non-self DNA is maintained. In addition, DAI specifically binds to 'left-handed' Z-DNA[10]. This indicates that DAI senses Z-DNA as well as B-DNA; however, the possible physiological relevance of this ability awaits further investigation. This report by Taniguchi and colleagues[1] provides an important clue for elucidating how pathogen-derived dsDNA, or other 'danger signals' such as damaged host DNA, is recognized and how malfunction of this system can lead to autoimmunity. This work may also provide future therapeutic avenues for preventing a host reaction after gene transfer with DNA virus vectors.

COMPETING INTERESTS STATEMENT
The authors declare no competing financial interests.

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Regulation of Antiviral Innate Immune Responses by RIG-I Family of RNA Helicases

K. Onomoto [1] · M. Yoneyama[1] · T. Fujita[1] (✉)

[1]Laboratory of Molecular Genetics, Institute for Virus Research and Laboratory of Molecular Cell Biology, Graduate School of Biostudies, Kyoto University, Kyoto, Japan
tfujita@virus.kyoto-u.ac.jp

Abstract The recognition of viral nucleic acids with pattern recognition receptors (PRRs) is the first step in inducing the innate immune system. Type I interferons (IFNs), central mediators in antiviral innate immunity, along with other cytokines and chemokines, disrupt virus replication. Recent studies indicated at least two distinct pathways for the induction of type I IFN by viral infection. Toll-like receptors (TLRs) are extracellular or endosomal PRRs for microbial pathogens, whereas retinoic acid-inducible gene-I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) are novel intracellular PRRs for the viral dsRNA. In this review, we describe the distinct mechanisms inducing type I IFNs through TLRs and RIG-I/MDA5 pathways.

1 Introduction

Higher organisms including humans are equipped to counteract infecting viruses using two kinds of immune responses: innate and adaptive immunity. Unlike adaptive immunity, which is characterized by its specificity and memory, innate immunity is provoked early in infection and is critical for an initial