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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

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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

antiviral response. The type I interferon (IFN) system plays a major role in antiviral innate immunity (Samuel 2001; Stetson and Medzhitov 2006). Upon viral infection, type I IFN is secreted in body fluid and expands IFN response signals, resulting in the activation of various enzymes that prevent viral replication. In addition to antiviral activity, type I IFN has been known to exert various biological effects such as cell cycle regulation, differentiation, and immune modulation. Furthermore, innate immune responses lead to the activation of specific cells with antigen-presenting functions to facilitate the initiation of adaptive immunity.

The triggering of the IFN system is the activation of IFN genes. Since the initial discovery of type I IFN, the activation mechanism of the type I IFN genes has been a major focus of many researchers. Although several double-stranded (ds) RNA-binding proteins such as protein kinase-activated by RNA (PKR) have been attributed to the detection of replicating viral RNA, gene knockout studies do not support its role (Yang et al. 1995). Recent functional analyses revealed that TLRs function as pathogen receptors including those of viral origin (Takeda and Akira 2005). TLR3 has been identified as a receptor for exogenous dsRNA (Alexopoulou et al. 2001); however, TLR3-deficient cells can still activate type I IFN genes (Diebold et al. 2003; Yoneyama et al. 2004), suggesting the existence of other receptor(s). Screening of an expression cDNA library identified RIG-I as an essential receptor for virus-derived dsRNA (Yoneyama et al. 2004). In this article, we describe the recently identified function of the RIG-I family of RNA helicases in innate immune reactions to infecting viruses.

2
The Role of TLR and RIG-I Family Helicases in Viral Infection

2.1
TLR Detects Extracellular Pathogen-Associated Molecular Patterns

Toll was first identified as a transmembrane receptor regulating insect morphogenesis (Hashimoto et al. 1988). Toll mutation also results in increased sensitivity to fungi in *Drosophila* (Lemaitre et al. 1996), leading to the identification of mammalian Toll-like receptors (TLRs) as sensing receptors of various pathogen-associated molecular patterns (PAMPs) (Medzhitov et al. 1997). Ten members of human TLRs are expressed in a tissue-specific manner and many are expressed in dendritic cells (DCs) and macrophages (Takeda and Akira 2005). Although each TLR detects a distinct set of PAMPs, a common extracellular leucine-rich repeat (LRR) motif is responsible for sensing. When LRR

detects a pathogen, a signal is generated in the cytoplasm, which is mediated by the cytoplasmic domain of the receptor. TLR activation results in the production of various cytokines, leading to the activation of innate immune responses (as described in this volume by Severa and Fitzgerald). Upon TLR activation, macrophages and DCs differentiate into antigen-presenting cells initiating antigen-specific acquired immunity. Viral infection is sensed by three TLRs: TLR3 (Alexopoulou et al. 2001), TLR7/8 (Diebold et al. 2004; Heil et al. 2004; Lund et al. 2004), and TLR9 (Hemmi et al. 2000; Krug et al. 2004; Lund et al. 2003), which are mostly expressed on the endosomal membrane (Fig. 1). Double-stranded RNA (dsRNA), single-stranded RNA, and unmethylated CpG DNA are detected by TLR3, TLR7/8, and TLR9, respectively (Fig. 2). This subset of TLRs activates transcription factors including NF-κB, IRF-3, and

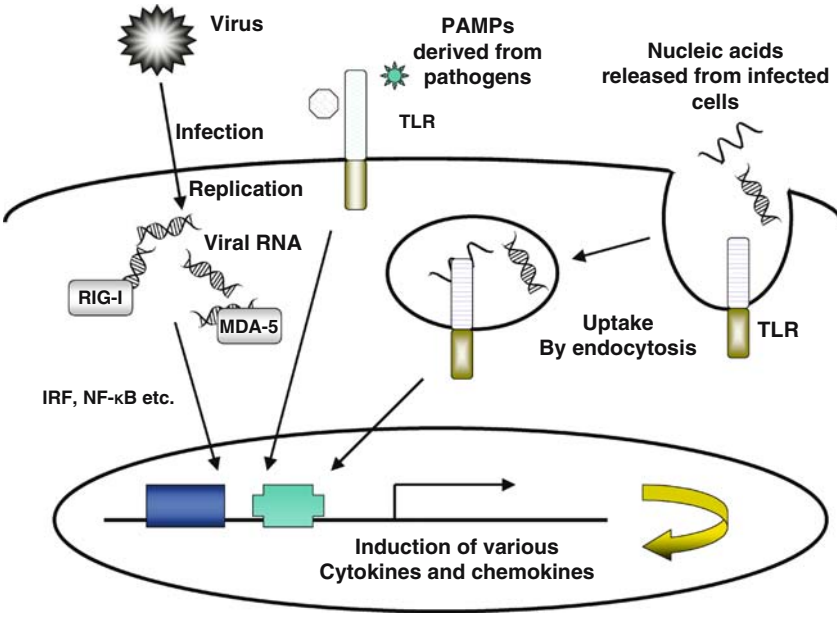

Fig. 1 Recognition of PAMPs by TLR and RIG-I family helicases. Transmembrane receptor TLR is expressed on the plasma or endosomal membranes and senses extracellular PAMPs. RIG-I family helicases detect viral RNA in the cytoplasm. Activation of these receptors transduces signals resulting in overlapping, but in a different set of target genes, including cytokines and chemokines

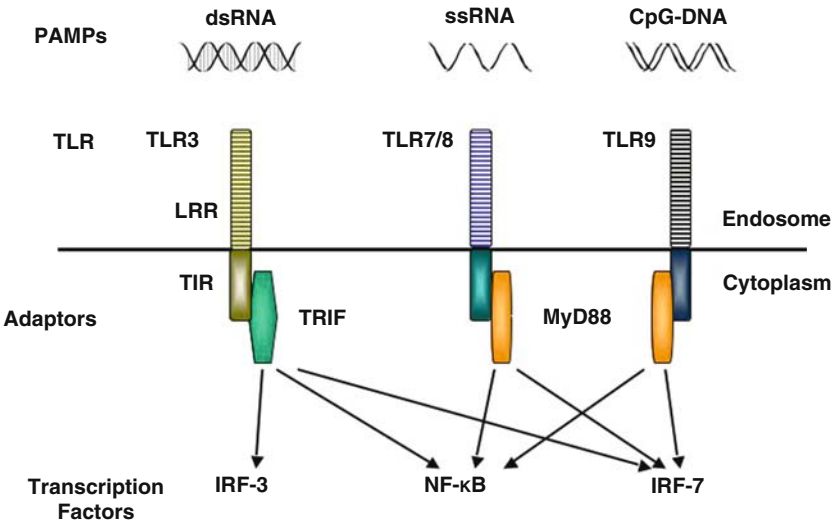

Fig. 2 Recognition of viral nucleic acids by different TLRs. TLR3, TLR7/8, and TLR9 detect dsRNA, ssRNA, and unmethylated CpG DNA. MyD88 adaptor is indispensable for signaling by TLR7/8 and 9, whereas another adaptor TRIF is essential for TLR-3 signaling

IRF-7 through common and distinct cytoplasmic adaptor molecules (Takeda and Akira 2005).

2.2 Cytoplasmic Receptor, RIG-I Helicase Family

Since dsRNA such as polyI:polyC is known to induce IFN synthesis, it is generally accepted that dsRNA is the major viral product responsible for the activation of innate immune responses. TLR3 was first shown to confer responsiveness to exogenously added polyI:polyC in HEK293T cells (Alexopoulou et al. 2001), and is thus hypothesized to function as a physiological sensor of replicating viruses. However, TLR-3-deficient cells are still responsive to viral infection or poly I:poly C transfection (Yoneyama et al. 2004), suggesting an alternative cytoplasmic sensor.

Functional screening identified human RIG-I as putative positive regulator of IFN genes (Yoneyama et al. 2004). RIG-I is a putative RNA helicase containing two repeats of caspase recruitment domain (CARD) at the N-terminal region and a DExH/D box helicase homology region at its C-terminal region (Fig. 3). RIG-I exhibits specific binding activity to dsRNA. Overexpression of

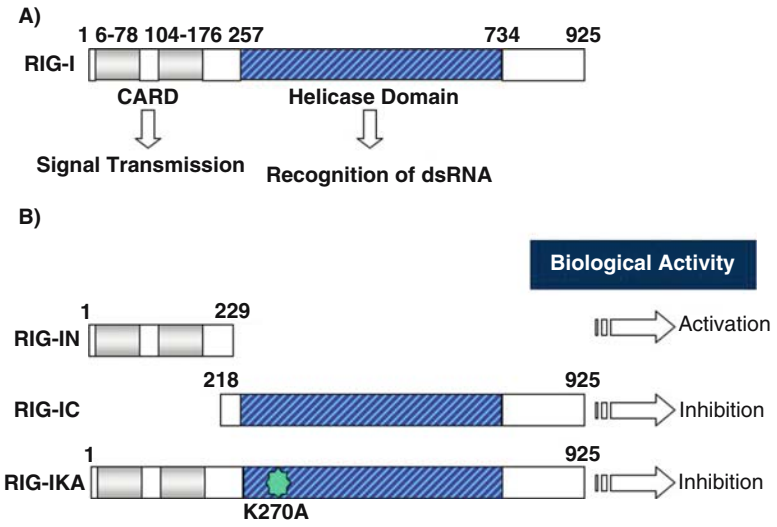

Fig. 3 A, B Structure–function relationship of RIG-I. **A** Structure of RIG-I. **B** Biological activity of RIG -I mutants

RIG-I in cultured cells did not significantly activate the IFN promoter; however, overexpression of the N-terminal region containing two CARD repeats alone constitutively activated the IFN promoter. This suggests that CARD is essential and sufficient for signaling, and is under negative regulation by the C-terminal region. Full-length RIG-I is present as an inactive form; however, it can be activated by viral infection or transfection of dsRNA. This supports the speculation that inhibition of CARD by the C-terminal region is reversed by dsRNA. Interestingly, RIG-I lacking CARD acts as a dominant-negative inhibitor of virus-induced activation of IFN-β promoter. Furthermore, K270A mutant, which has disrupted ATP binding motif within the conserved helicase domain, also functions as a dominant inhibitor. These observations suggest that, in addition to dsRNA binding, ATP hydrolysis is necessary for the induced unmasking of CARD.

In the human genome database, there are two other genes encoding RIG-I-related helicases, MDA5 and LGP2 (Yoneyama et al. 2005). MDA5 exhibits a similar domain structure as RIG-I, characteristic of two repeat CARDs and the helicase domain (Fig. 4). The third helicase LGP2 lacks CARD. Functional analyses of these helicases, using cell culture, revealed that MDA5 functions as a positive signaling regulator, similar to RIG-I. Recent studies using gene disruption of RIG-I and MDA5 revealed that these helicases detect different viruses (Gitlin et al. 2006; Kato et al. 2005, 2006). MDA5 was essential for detection of picorna virus infection,

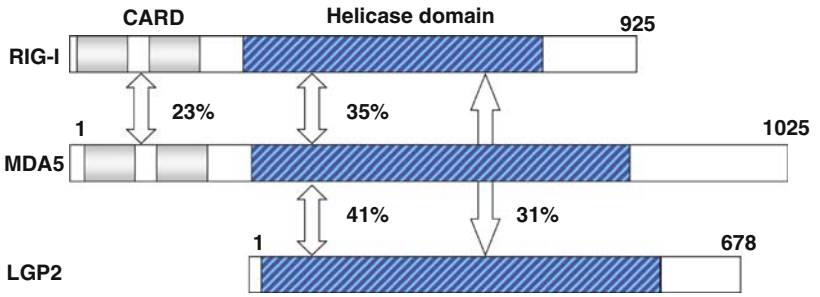

Fig. 4 Structure of RIG-I family helicases. Human and mouse RIG-I family consists of RIG-I, MDA5, and LGP2. Percentage indicates amino acid identity between corresponding domains

while RIG-I was critical for detection of other viruses types tested. Interestingly, this virus specificity likely reflects different RNA species generated by respective viruses. At present, the chemical basis of this difference is not known. Functional analyses of LGP2 in cell culture revealed that LGP2 dominantly inhibits the virus-induced activation of IFN genes (Rothenfusser et al. 2005; Yoneyama et al. 2005). Since LGP2 is transcriptionally induced by autocrine IFN, its function as a feedback negative regulator has been suggested.

3 Signaling Cascades of Antiviral Innate Responses

A comparison of signaling cascades initiated by the detection of dsRNA by TLR3 and RIG-I/MDA5 is illustrated in Fig. 5. TLR3 activation by dsRNA occurs in the endosome and the signal is transmitted through TRIF (Hoebe et al. 2003; Oshiumi et al. 2003; Yamamoto et al. 2002, 2003), TBK-1 (NAK, T2K)/IKKi (IKK-ε) kinases (Fitzgerald et al. 2003; Hemmi et al. 2004; McWhirter et al. 2004; Perry et al. 2004; Sharma et al. 2003). The latter kinases are responsible for a specific phosphorylation and activation of IRF-3. It was shown that TBK-1/IKKi kinases are under positive and negative regulation by NAP1 and SIKE, respectively (Huang et al. 2005; Sasai et al. 2005). RIG-I/MDA5 activates a novel adaptor IPS-1 (MAVS, Cardif, VISA) containing a single copy of CARD (Kawai et al. 2005; Kumar et al. 2006; Meylan et al. 2005; Seth et al. 2005; Sun et al. 2006; Xu et al. 2005). Interestingly, IPS-1 is anchored on the outer membrane of mitochondria via its C-terminal transmembrane domain (Seth et al. 2005). Although a mitochondrial association is critical for the signaling, its mechanism is elusive. IPS-1 apparently activates the

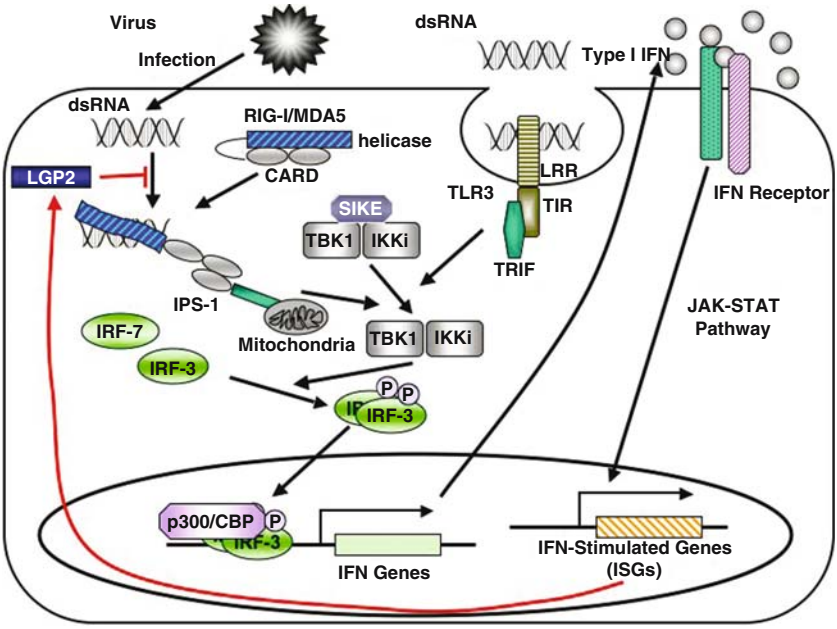

Fig. 5 Signaling cascade induced by dsRNA. TLR3 recognizes extracellular dsRNA in endosomes. Upon dsRNA binding, the cytoplasmic domain of TLR3 transmits a signal to an adaptor, TRIF. Cytoplasmic dsRNA is recognized by RIG-I and MDA5. CARD of these helicases interacts with an adaptor, IPS-1, which localizes on the outer membrane of mitochondria. Signals mediated by TRIF and IPS-1 activate common protein kinases TBK-1 and IKK-I, resulting in phosphorylation-mediated activation of transcription factor IRF-3. IRF-3, as a complex with co-activator CBP or p300, activates target genes including type I IFN genes. Secreted IFN activates secondary signals through IFN receptor and JAK-STAT pathway to activate ISGs

IRF-3 kinases TBK-1/IKKi. Thus, RIG-I/MDA5 activates a distinct signaling cascade from TLR3 and the signal is converged at TBK-1/IKKi (Fig. 5).

4 Cell-Type-Specific Function of TLRs and the RIG-I Family

As mentioned earlier, TLR7/8 and TLR9 detect distinct viral PAMPs and activate signaling cascades, MyD88, IRAK1, and IRF-7 (Takeda and Akira 2005). So far, this signaling has been showed to be specific for plasmacytoid DCs (pDCs), which are responsible for the production of high levels of serum IFN-α (Fig. 6).

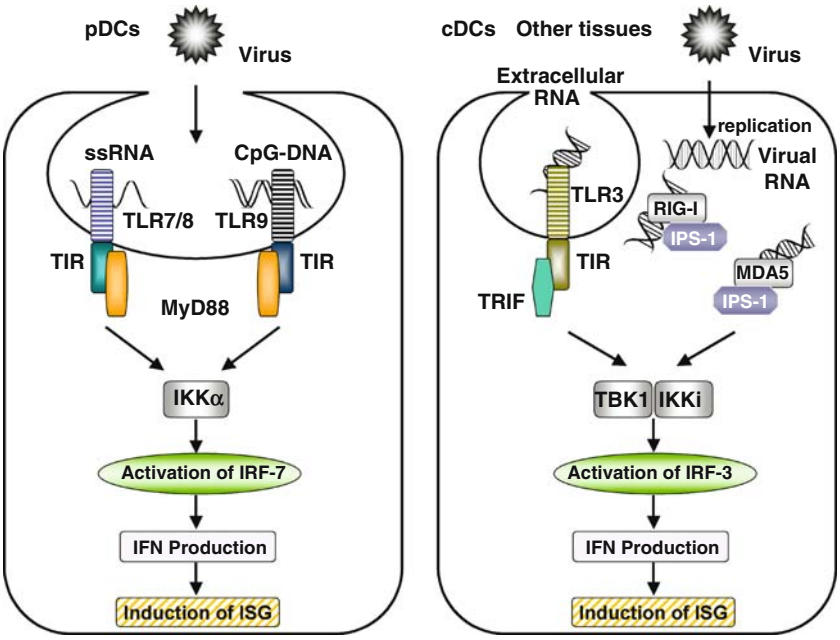

Fig. 6 Tissue-specific signaling cascade for IFN gene activation. Virus infection triggers a distinct signaling cascade in pDCs and other cell types including cDCs. TLR7/8 and TLR9 are specifically expressed in pDCs, whereas TLR3 is expressed in cDCs. IFN induction in pDC is dependent on MyD88, IKKα, and IRF-7, whereas these adaptors are dispensable in other cell types

pDCs and other cell types, including cDCs, use distinct pathways in a mutually exclusive manner to sense viral infections. As revealed by analysis using knockout mice, MyD88 but not RIG-I is essential in pDCs, and RIG-I but not MyD88 is critical in cDCs (Kato et al. 2005, 2006) (Fig. 6). The biological significance of TLR3 function in viral infection is not well established.

5 Viral Evasion Strategies for Antiviral Responses

With the elucidation of host antiviral response mechanisms, it has become evident that replication-competent viruses are equipped to counteract the antiviral mechanisms. It is well known that acutely infecting viruses, which undergo a lytic infection, selectively inhibit host macromolecular syntheses collectively

known as shut off. Apart from this nonspecific blockade, viruses encode inhibitory proteins, which target specific processes of the antiviral signaling.

V proteins of paramyxoviruses bind to MDA5 and inhibit its signaling (Andrejeva et al. 2004; Yoneyama et al. 2005). V protein of Sendai virus specifically binds to MDA5 but neither interaction nor blockade was observed with RIG-I; however, in light of the fact that paramyxoviruses are specifically detected by RIG-I, as evidenced by RIG-I knockout mice, its physiological relevance is controversial.

Hepatitis C virus (HCV) is known to be poorly adaptable to tissue culture for replication. One reason is its high sensitivity to IFN-mediated reaction: HCV replication requires host cell mutations that inactivate RIG-I signaling (Sumpter et al. 2005). HCV encodes a protein complex, NS3/4A, which acts as RNA helicase and protease. NS3/4A protease cleaves IPS-1 at its cytoplasmic domain, thus releasing it from mitochondria (Lin et al. 2006; Loo et al. 2006; Meylan et al. 2005). As IPS-1 is an essential adaptor for both RIG-I and MDA5 signaling and its association with mitochondria is obligatory, this cleavage completely blocks RIG-I/MDA5 signaling. Indeed, IPS-1 mutation at the cleavage motif or NS3/4A protease inhibitor restores the activation cascade stimulating the IFN genes.

NS1 protein of influenza A virus has been implicated in the inhibition of IFN gene activation. Using influenza A virus with NS1 mutation and RIG-I knockout mice, it was shown that NS1 blocks the signaling cascade triggered by RIG-I (Kato et al. 2006). NS1 is a dsRNA binding protein, thus sequestration of RIG-I from its ligand is one mechanism; however, the dsRNA-binding-deficient mutant of NS1 remains inhibitory (Donelan et al. 2003), suggesting multiple actions of this protein.

Ebola virus VP35 protein is another dsRNA binding protein inhibiting RIG-I-mediated signaling (Cardenas et al. 2006). Like NS1 of influenza A virus, VP35 may have dual inhibitory functions: in addition to dsRNA sequestration, it may be inhibiting steps downstream of IPS-1and IRF-3 kinases.

Since RIG-I and MDA5 are IFN-inducible and positive feedback is an important trait of the system, inhibition of IFN action, including IFN-R, by the JAK-STAT pathway remotely inhibits RIG-I and MDA5. In this regard, viral proteins that target IFN action are also inhibitory for IFN production.

6 Ligands for RIG-I and MDA5

In vitro binding studies revealed that RIG-I exhibits a specific binding activity to dsRNA, such as poly I:C, poly A:U, 5′ or 3′ non-coding genomic RNA of HCV synthesized in vitro, but not to poly A, tRNA, single-stranded region of HCV genomic RNA and dsDNA (Sumpter et al. 2005; Yoneyama et al. 2004).

MDA5 exhibits a much weaker binding activity to poly I:C. Inconsistent with the in vitro binding, functional analysis using knockout mice and cells deficient in either RIG-I or MDA5 revealed that dsRNA produced by in vitro transcription and poly I:C are specifically detected by RIG-I and MDA5, respectively (Kato et al. 2006). Furthermore, the RNA viruses tested were classified into two groups; picorna viruses (including EMCV) are specifically sensed by MDA5 and other viruses (including VSV, influenza virus and Sendai virus) by RIG-I. The specificity arises from different classes of RNA structure, as suggested by the results that RNA extracted from VSV and EMCV viral particles activated RIG-I and MDA5, respectively. This result includes noteworthy facts: VSV genomic RNA is unlikely to be highly double-stranded; under certain circumstances, viral replication may not be necessary to activate RIG-I. For dsRNA recognition, one report suggests the importance of end structure for selective activation of IFN genes or RNA interference (Marques et al. 2006). The search and elucidation for true ligands present in virus-infected cells for RIG-I and MDA5 is absolutely necessary to further our understanding of how self and non-self is recognized at the RNA level. At present there is no reasonable explanation to satisfy all these observations.

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