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Structural Mechanism of RNA Recognition by the RIG-I-like Receptors

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Cytoplasmic nonself RNA, such as that generated by invading viruses, is recognized by a family of sensory molecules termed RIG-I-like Receptors (RLRs). Here, we discuss the mechanism of the RLRs' sensing of non-self RNA. Our findings define three functional domains of RLRs and provide insights into how RLRs function as a molecular switch through interactions with virus-specific RNA ligands.

A wide variety of cells in the body are capable of exhibiting antiviral innate immunological responses upon viral infection. Infected viruses are detected by sensory molecules, including Toll-like receptors (TLRs) and RIG-I-like receptors (RLRs: retinoic acid-inducible gene I, RIG-I; melanoma differentiation-associated gene 5, MDA5; and laboratory of genetics and physiology 2, LGP2). Whereas TLRs detect viral components in specific cells, such as dendritic cells and macrophages, RLRs sense the infected viruses in the cytoplasm of most cell types. RLRs sense viral RNA and result in immunological responses, including the production of type I interferon (IFN) and inflammatory cytokines (Akira et al., 2006; Yoneyama et al., 2005; Yoneyama et al., 2004).

Sensing of Specific Viruses by RLRs

Double-stranded (ds) RNA can mimic the induction of the classical antiviral response by being a potent inducer of type I IFN (Samuel, 2001). Analyses of gene-deleted mice and their cells reveal that RIG-I and MDA5 sense specific types of viruses (Gitlin et al., 2006; Kato et al., 2005; Kato et al., 2006): whereas MDA5 senses picornaviruses, RIG-I senses many other viruses, including influenza A, Sendai, vesicular stomatitis, and Japanese encephalitis. This differential recognition is based on the distinct nonself RNA patterns generated by the viruses, as shown by the observation that RIG-I is selectively activated by transfection of in vitro-transcribed RNA, whereas MDA5 is selectively activated by transfection of poly I:C (Kato et al., 2006) (see below). Viral RNA molecules harboring a 5′ppp end, such as in vitro-transcribed RNA, were discovered as a new class of nonself RNA that selectively activates RIG-I (Hornung et al., 2006; Pichlmair et al., 2006). The 5′ppp end of host transcripts (self) is either removed or masked by the attachment of a "cap" before their transport to the cytoplasm. In contrast, although cells infected with influenza virus do not accumulate detectable amounts of dsRNA, viral 5′ppp RNA is produced during infection, leading to the ability of host cells to sense the virus via RIG-I (Pichlmair et al., 2006).

Recognition of the Ligand by the RIG-I ATPase Helicase

Functional analyses revealed that both RIG-I and MDA5 contain an effector domain (CARD: caspase recruitment domain) and a regulatory domain (helicase homology and C-terminal repressor domain) (Saito et al., 2007; Yoneyama et al., 2005; Yoneyama et al., 2004). However, it is not well understood how nonself RNA is physically recognized; for example, neither of these helicases contains a known RNA-binding motif. Recently, a biochemical study using recombinant RIG-I revealed that RIG-I specifically binds to dsRNA, including relatively short (∼25 bp) species, or 5′ppp RNA through an interaction with the C-terminal domain (CTD, aa. 792-925) of RIG-I (Takahasi et al., 2008) (see below). Another report demonstrated that a similar C-terminal fragment (aa. 802-925) binds to 5′ppp RNA but not to dsRNA. The reason for this discrepancy is unknown. The failure to detect RIG-I-dsRNA complex may be due to the nonconventional method used (Cui et al., 2008).

As predicted from its primary structure, RIG-I is a ligand-dependent ATPase (Cui et al., 2008; Gee et al., 2008; Takahasi et al., 2008). Mutation of the ATP-binding site (K270A) inactivates RIG-I to trigger antiviral signaling (Yoneyama et al., 2004). However, RIG-I K270A recognizes both dsRNA and 5′ppp RNA (Takahasi et al., 2008). Artificial removal of the CARD rendered it as a constitutive active ATPase (Gee et al., 2008). However, physiological significance of this observation is unknown because excision of the CARD under physiological conditions has not been observed. In vitro helicase assay revealed that the full-length RIG-I selectively unwinds dsRNA harboring 3′-terminal overhang (> 5 nt) in the presence of ATP (Takahasi et al., 2008). Interestingly, although dsRNA resistant to the helicase activity (with either blunt or 5′-terminal overhang end) efficiently induces IFN production in cell culture, those types with 3′-terminal overhang, which are susceptible to being unwound, failed to activate IFN genes. These results indicate that unwinding of dsRNA is not a critical step for triggering signals, as suggested by the observation that single-stranded RNA with a 5′ppp end is a functional ligand for RIG-I. These observations raise the question of what role ATP plays. It is evident that unwinding of dsRNA by RIG-I through ATP hydrolysis does not trigger antiviral signal. Thus, stable formation of a complex between RIG-I and 5′ppp RNA or dsRNA, independent of ATP, may be a prerequisite step for RIG-I activation. If so, the function of ATP is likely the conversion of this precomplex into active conformation through the motor-like function of the DExD/H-box helicase. It remains to

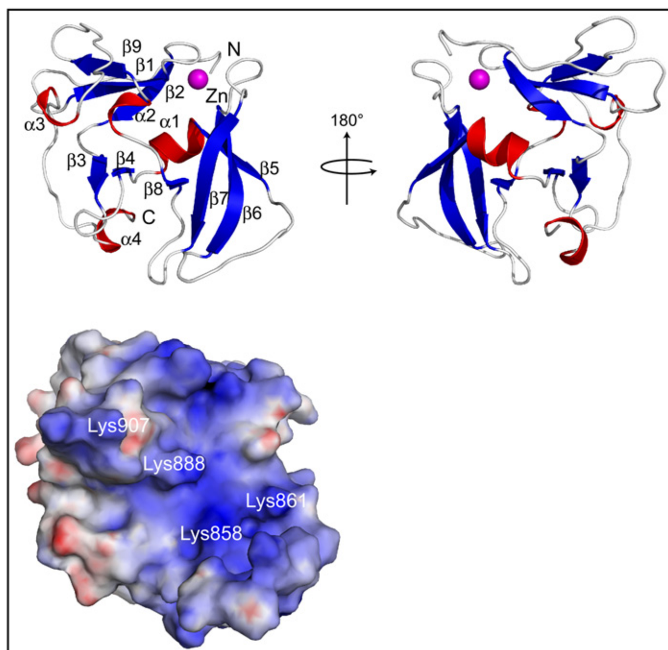

Figure 1. Solution Structure of the RIG-I CTD
Upper panel: ribbon diagram of the RIG-I CTD. The zinc atom, which stabilizes the structure, is indicated as a purple sphere.
Lower panel: Electrostatic surface potential of the RIG-I CTD. One side of the CTD (upper left) displays a cleft lined with basic amino acids (lower). Indicated are the residues critical for dsRNA and 5′ppp-RNA binding and biological activity, as revealed by alanine replacements. (F. Inagaki and K. Takahasi, personal communication).

be shown whether ATP hydrolysis is involved in the conformational change.

RNA-Recognition Domain of RIG-I

Limited protease digestion of the RIG-I-RNA complex revealed that RNA binding induces a digestion-resistant RIG-I fragment (Takahasi et al., 2008). This fragment (aa. 792-925, 17 kD) corresponds to the CTD and is sufficient to recognize dsRNA or 5′ppp RNA. Interestingly, the CTD nearly overlaps with a previously identified repressor domain (aa. 723-925) (Saito et al., 2007). The repressor domain is capable of interacting with the CARD and a helicase domain (helicase linker region, aa. 420-627), and overexpression of the repressor domain blocked RIG-I-mediated signaling; therefore, an autorepression model is proposed, in which the CARD is masked through intramolecular interactions mediated by the repressor domain. The CTD exhibits limited conservation among the RLR family members. However, the repressor function is shared by RIG-I and LGP2.

The atomic structure of the CTD has been determined by nuclear magnetic resonance (NMR) (Takahasi et al., 2008) and X-ray crystallography (Cui et al., 2008), and an essentially identical structure was determined in both crystals and solution. Although the amino acid sequences exhibit little homology to other proteins, the RIG-I CTD is structurally similar to the mammalian suppressor of Sec4 (Mss4), and both structures are stabilized by Zn^{2+} within the molecule (Figure 1). Nevertheless, it is unlikely that RLR and Mss4 are functionally related, because Mss4 is a guanine nucleotide exchange factor and is not known to function as an RNA-binding protein. One side of the CTD exhibits a large cleft with positive surface charges, and the opposite side contains acidic patches. The addition of either dsRNA or 5′ppp RNA specifically titrated the NMR signal of the basic cleft, suggesting that the cleft is an RNA-recognition surface. Consistent with this observation, mutagenesis on the basic cleft specifically reduced both RNA binding and the signaling capacity of RIG-I. In addition, Cui et al. showed via gel-filtration analysis that the RIG-I CTD recognizes 5′ppp RNA as a CTD dimer (Cui et al., 2008). Similarly, recombinant LGP2 binds to dsRNA and forms a large complex (Murali et al., 2008). It is likely that a single dsRNA molecule can simultaneously bind to multiple CTD molecules. Alternatively, it is possible that dsRNA can induce protein-protein association to form a dimer (oligomer), as in the case of TLR3 recognition of dsRNA (Liu et al., 2008); therefore, structural determination of the complex is necessary to address this issue.

Functional studies suggest that the CTD retains two distinct functions: RNA recognition and signal repression. Mutagenesis on the basic concave surface inactivated RNA recognition; however, none of the mutations rendered RIG-I constitutively active, suggesting that RNA-recognition surfaces and RNA-repression surfaces do not overlap. Furthermore, ATP binding or hydrolysis is not required for the recognition of RNA by RIG-I, suggesting a model for inactive RIG-I, as depicted in Figure 2, in which the repressor domain mediates a closed structure through intramolecular interactions but the RNA-binding domain is available. When viruses produce dsRNA or 5′ppp RNA, these types of nonself RNA bind to the concave RNA-recognition surface and induce conformational change in the presence of ATP, then the CARD is exposed. The released CARD forms complexes with either other RIG-I molecules or downstream adaptor Interferon Promoter Stimulator-1 (IPS-1, also known as MAVS, VISA, and Cardif) to transduce biological signals.

Long versus Short dsRNA

As mentioned above, RIG-I and MDA5 differentially recognize nonself RNA. However, the underlying mechanism of this recognition is not clear. Although the CTDs of both RIG-I and MDA5 share common residues, their surface charges diverged (our unpublished observation). Poly I:C is the only defined synthetic RNA ligand for MDA5. Commercial poly I:C is made by annealing poly I and poly C with 5′pp (approximately 1000 bases or longer). Although commercial poly I:C (> 3 kbp) selectively activates MDA5, short poly I:C generated by enzyme digestion (~300 bp) is unable to activate MDA5 but acts as a potent ligand for RIG-I (Kato et al., 2008). In vitro assay using recombinant RIG-I and MDA5 also showed that short and long poly I:C preferentially induced ATPase activity for RIG-I and MDA5, respectively. Consistent with these observations, short dsRNA species (1.2~1.4 kbp) of the Reovirus genome selectively activated RIG-I but longer dsRNA (3.4 kbp) was capable of activating MDA5. These results strongly suggest that RIG-I and MDA5 discriminate long and short dsRNA (Figure 3); however, the mechanism underlying the sensing of nucleotide length is unknown.

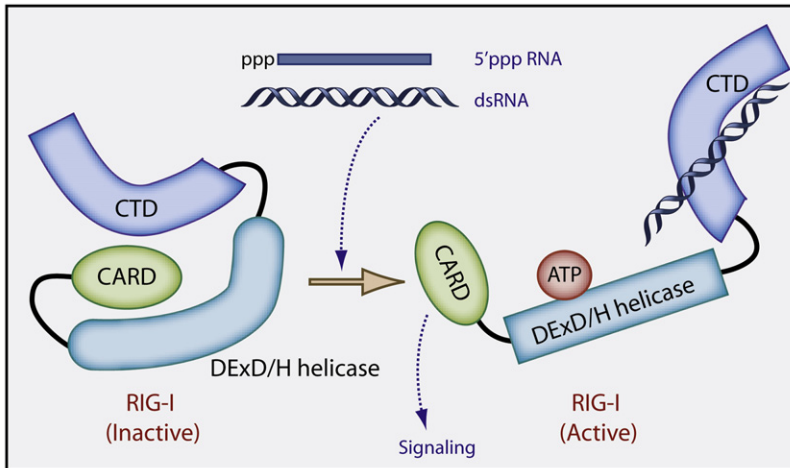

Figure 2. Model of RIG-I Activation by Nonself RNA
The CTD coincides with the functionally defined repressor domain and interacts with both the helicase domain and the CARD in the absence of its ligand. When viruses produce dsRNA or 5′ppp-RNA, these types of nonself RNA bind to the RNA-recognition cleft on the CTD and induce conformational change in the presence of ATP, then the CARD is exposed. The released CARD forms complexes with either other RIG-I molecules or downstream adaptor IPS-1 to transduce biological signals.

Why long poly I:C does not activate RIG-I despite its high affinity to poly I:C, particularly in MDA5 deficient cells, is unclear. One clue is the observation that although short dsRNA and 5′ppp RNA induce protease-resistant 30 kD and 17 kD fragments of RIG-I, poly I:C induces a 60 kD fragment (Takahasi et al., 2008). This result suggests that poly I:C binds to RIG-I but induces an abortive conformation of RIG-I, rendering it incapable of signaling downstream. Evidently, this issue requires further structural analyses.

Viral Strategy for Evading Host Recognition

Different viruses produce distinct classes of RNA patterns in infected cells. So far, three types of RNA patterns that can be recognized by host cells have been identified: 5′ppp RNA, short dsRNA, and long dsRNA. Naturally, viruses try to avoid detection from host antiviral mechanisms, and they have developed various strategies for escaping detection. As reported recently, several negative-strand RNA viruses, including Hantaan virus, Crimean-Congo hemorrhagic fever virus, and Borna disease virus, manage to cleave off the 5′ppp structure of viral gemonic RNA (Habjan et al., 2008) (Figure 3). In addition, picornaviruses covalently link VPg protein at the 5′ end of viral RNA by disrupting the 5′ppp structure (Paul, 2002). Picornaviruses also accumulate long replicative form (RF) dsRNA in infected cells, which may block the function of RIG-I. However, MDA5, with its ability to sense longer dsRNA, may provide a secondary checkpoint to counter this viral hiding strategy. In addition, viruses encode protein inhibitors, such as nonstructural (NS) proteins NS1 (influenza), NS3/4A (hepatitis C), V (paramyxoviruses), and VP35 (ebola) that disrupt the antiviral-signaling cascade (Yoneyama and Fujita, 2007), and these could work in concert to block the host antiviral response. These results highlight the offense and defense between host mechanism of nonself RNA detection and viral evolution.

Conclusion and Future Perspective

The understanding of how foreign RNA species are recognized by RLRs has advanced substantially. It is now evident that in addition to the DExD/H helicase domain, the CTD and the CARD, which sense foreign RNA and execute signaling, respectively, are essential for RIG-I function. The current model suggests that these domains may interact with each other and that ligand RNA interaction may induce critical conformational changes to trigger biological signals. In this regard, it will be important to determine the structure of full-length RIG-I at the atomic level with

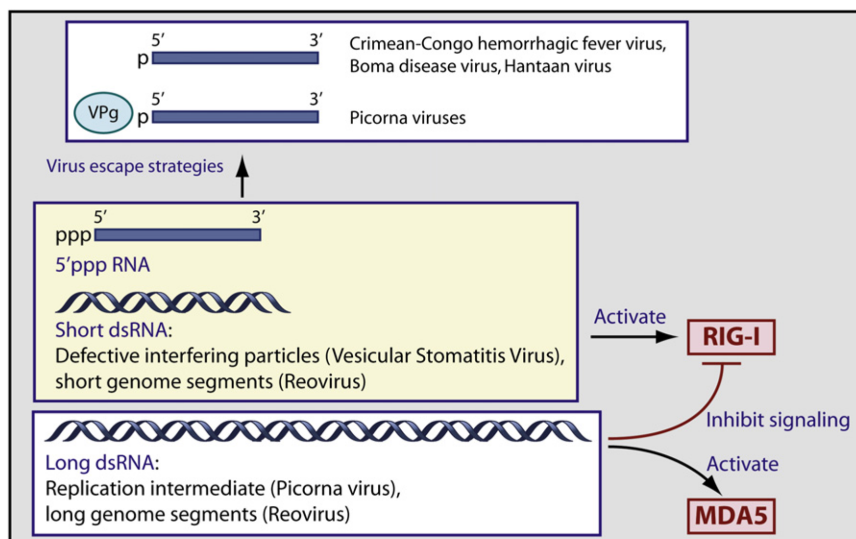

Figure 3. Nonself-RNA-Recognition Specificity of RIG-I and MDA5
5′ppp RNA and short dsRNA are specifically recognized by RIG-I. Although long dsRNA may block RIG-I for signaling, it efficiently activates MDA5. Some viruses are known to modify viral 5′ppp residues to escape detection by RIG-I.

and without its ligand in order to finalize our understanding of this process. Furthermore, this will provide clues for understanding functions of other, numerous DExD/H helicases.

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