J. Biochem. 2016;159(3):279–286 doi:10.1093/jb/mvv122

THE JOURNAL OF BIOCHEMISTRY

JB Review

Regulation of antiviral innate immune signaling by stress-induced RNA granules

Received August 31, 2015; accepted September 24, 2015; published online January 8, 2016

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Activation of antiviral innate immunity is triggered by cellular pattern recognition receptors. Retinoic acid inducible gene-I (RIG-I)-like receptors (RLRs) detect viral non-self RNA in cytoplasm of virus-infected cells and play a critical role in the clearance of the invaded viruses through production of antiviral cytokines. Among the three known RLRs, RIG-I and melanoma differentiation-associated gene 5 recognize distinct non-self signatures of viral RNA and activate antiviral signaling. Recent reports have clearly described the molecular machinery underlying the activation of RLRs and interactions with the downstream adaptor, mitochondrial antiviral signaling protein (MAVS). RLRs and MAVS are thought to form large multimeric filaments around cytoplasmic organelles depending on the presence of Lys63-linked ubiquitin chains. Furthermore, RLRs have been shown to localize to stress-induced ribonucleoprotein aggregate known as stress granules and utilize them as a platform for recognition/activation of signaling. In this review, we will focus on the current understanding of RLR-mediated signal activation and the interactions with stress-induced RNA granules.

Keywords: innate immunity/retinoic acid inducible gene-I-like receptor/RNA/stress response/viral infection.

Abbreviations: CARD, caspase recruitment domain; CTD, C-terminal domain; eIF2α, eukaryotic translation initiation factor 2α: EMCV, encephalomyocarditis virus; G3BP1, Ras-GAP SH3 domain binding protein-1; GCN2, general control non-derepressible 2; HCV, hepatitis C virus; HGNC, HUGO Gene Nomenclature Committee; IAV, influenza A virus; IFN, interferon; IFI16, IFN-γ-inducible protein 16; IRF, IFN regulatory factor; ISG, IFN-stimulated gene; LGP2, laboratory of genetics and physiology 2; MAM, mitochondria-associated endoplasmic reticulum membrane; MAVS, mitochondrial antiviral signaling protein; MDA5, melanoma differentiation-associated gene 5; NF, nuclear factor; NS1, non-structural protein 1; OAS, 2′-5′ oligoadenylate synthetase; PB, processing body; PKR, double-stranded RNA-dependent protein kinase; PRR, pattern recognition receptor; RBP, RNA binding protein; RIG-I, retinoic acid inducible gene-I; RLR, RIG-I-like receptor; SG, stress granule; ss, single-stranded; STING, stimulator of IFN genes; TIA1, T-cell restricted intracellular antigen-1; TIAR, TIA1-related protein; TLR, Toll-like receptor; TRAF, tumor necrosis factor receptor-associated factor; TRIM25, tripartite motif protein 25; USP, ubiquitin-specific protease; Ub, Ubiquitin chain; VSV, vesicular stomatitis virus.

In higher vertebrates, invasion of viruses is detected by host pattern recognition receptors (PRRs), which transiently induce the production of type I and III interferons (IFNs) and pro-inflammatory cytokines (*1*, *2*). The expressed cytokines can activate the Janus kinase (Jak)/signal transducer and activator of transcription signaling pathway through interactions with their cognate receptors on both infected and surrounding uninfected cells, thereby provoking an antiviral state via expression of large numbers of IFN-stimulated genes (ISGs), such as double-stranded (ds) RNA-dependent protein kinase (PKR) and 2′–5′ oligoadenylate synthetase (OAS) (*3*). In addition to these transient effects, ISG proteins are known to be responsible for regulating the subsequent adaptive immune responses, suggesting a critical role of the IFN system in antiviral immunity (*4*).

Viral infection is detected by several groups of PRRs (*1*, *5*). The Toll-like receptors (TLRs) family members, TLR3, 7/8 and 9, detect viral dsRNA, single-stranded (ss) RNA and CpG DNA, respectively, on the plasma membrane of the endosomal compartment of dendritic cells and macrophages. DNA sensors, such as DNA-dependent activator of IFN regulatory factors (IRFs), IFN-γ-inducible protein 16 (IFI16) and cyclic GMP-AMP synthase, detect cytoplasmic DNAs produced by infection with DNA viruses or engulfment of dead cells, and activate stimulator of IFN genes (STING)-dependent signaling (*6–8*). Retinoic acid inducible gene-I (RIG-I)-like receptors (RLRs) are ubiquitously expressed PRRs that participate in the detection of cytoplasmic non-self RNAs generated by infection with RNA viruses. Among these PRRs, RLRs are critical for activation of the IFN system in response to most of RNA viruses in most of cell types.

In this review, we will focus on the molecular machinery underlying RLR-mediated signal transduction. Notably, as recent reports have demonstrated that RLRs utilize stress-induced RNA granules as a

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scaffold for signal activation, we will discuss the relationship between RLR signaling and RNA granules.

RIG-I-Like Receptors

Mammalian cells encode three RLR members, RIG-I (gene symbol from the HUGO Gene Nomenclature Committee [HGNC]: *DDX58*), melanoma differentiation-associated gene 5 (MDA5; HGNC symbol: *IFIH1*) and laboratory of genetics and physiology 2 (LGP2; HGNC symbol: *DHX58*). All members are DExD/H box-containing RNA helicases, and RIG-I and MDA5, but not LGP2, have an N-terminal tandemly repeated caspase recruitment domain (2CARD) that is responsible for interaction with the adaptor molecule, mitochondrial antiviral signaling protein (MAVS, also known as IPS-1, VISA and Cardif; Fig. 1) (*1*). As MAVS is primarily expressed on the outer membrane of mitochondria, peroxisomes and/or the mitochondria-associated endoplasmic reticulum membrane (MAM), interactions between RLRs and MAVS and the subsequent recruitment of downstream signaling molecules, such as tumor necrosis factor receptor-associated factor (TRAF)3/6 and inhibitor of nuclear factor (NF)-κB (IκB) kinase (IKK) family members (*i.e*. IKKα/β/γ, IKKε and TANK-binding kinase 1 [TBK1]), occur on the surface of these membrane structures. The resulting 'signalsome' induces nuclear translocation of transcription factors, including NF-κB and IRF-3/7, leading to transcriptional activation of IFNs and pro-inflammatory cytokines. Recently, Liu *et al*. (*9*) reported that the phosphorylation of MAVS by TBK1 is required for the subsequent phosphorylation and activation of IRF-3. On the other hand, peroxisomal MAVS selectively transmits type III IFN-inducing signal in which another IRF family member, IRF-1, is involved (*10*).

Forced expression of the 2CARD constitutively activates endogenous IFNs, indicating that RIG-I and MDA5 act as positive regulators of viral-induced IFN production (*11*). When viral infection is not present, the 2CARD of RIG-I is masked by an intramolecular interaction between the second CARD and a portion of the RNA helicase domain (Hel2i, see below) (*12*). In the case of MDA5, the basal activity of 2CARD is regulated by phosphorylation and dephosphorylation at Ser88 (*13*); however, the specific mechanisms that negatively regulate the 2CARD of MDA5 remain unclear. Interestingly, a link between gain-of-function mutations of RLRs and several disorders in both mouse and human system, including Aicardi–Goutieres syndrome and Singleton–Merten syndrome, was demonstrated (*14–18*), indicating that strict control of RLR activity under steady-state condition is critical for maintenance of homeostasis. Mutant of RIG-I, in which the 2CARD is deleted, strongly inhibits virus-induced signaling, suggesting that the C-terminal region has a regulatory function. Indeed, the RNA helicase domain (Hel-1, Hel-2 and insert of Hel-2 (Hel-2i)) and C-terminal domain (CTD) are responsible for RNA recognition (*19–21*), and a subsequent ATP-depending conformational change allows the 2CARD to interact with the CARD of MAVS (see below) (*22*). A recent report proposed that RIG-I and MDA5 can also function as antiviral effector-like proteins by displacement of functional viral proteins from viral dsRNA (*23*, *24*).

Another RLR, LGP2, which has no N-terminal CARD, cannot directly activate MAVS-mediated signaling, and *in vitro* analyses have clearly indicated the role of LGP2 in the negative regulation of RIG-I/MDA5-mediated signaling (*25*). However, studies using *LGP2*-knockout mice have shown that LGP2 can function as positive regulator in response to several types of RNA viruses, including encephalomyocarditis virus (EMCV) and vesicular stomatitis virus (VSV) (*26*, *27*). Although the mechanisms through which LGP2 mediates these signaling events are unclear, recent reports have demonstrated that LGP2 can help MDA5 to recognize substrate non-self RNA and assemble functional filaments (*28*). In contrast, IFN activation in response to influenza A virus (IAV) infection is significantly upregulated in LGP2-deficient cells, suggesting that LGP2 negatively regulates anti-IAV responses (*29*).

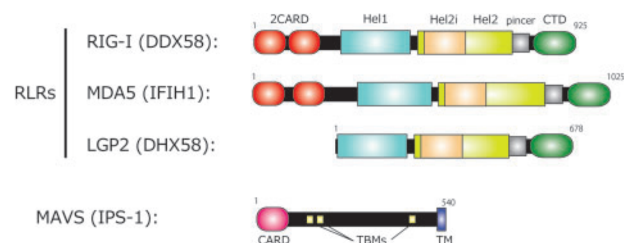

Fig. 1 Structure of RLRs and MAVS. The three RLRs contain a CTD, DExD/H box-containing RNA helicase domain (Hel-1, Hel-2i and Hel-2) and pincer domain. RIG-I and MDA5 have N-terminal tandem CARDs (2CARD). MAVS has a single CARD at its N-terminal region, three TRAF-binding motifs (TBMs) and a C-terminal transmembrane domain (TM). Modified from Yoneyama *et al*. (2015), Viral RNA detection by RIG-I-like receptors, *Curr Opin Immunol*, 32, 48–53, Copyright (2015), with permission from Elsevier (*81*).

Non-Self RNA Substrates for RLRs

RIG-I detects infection of a variety of RNA viruses, including IAV, VSV, hepatitis C virus (HCV) and several DNA viruses. The non-self RNAs that can be specifically recognized by RIG-I are short ssRNA or blunt-ended panhandle dsRNA (<1,000 bp) with 5′-triphosphate (5′ppp) moiety (*30*). The 5′-diphosphate RNA (5′pp), which is observed in genomic dsRNA of reovirus and the polyU/UC region of HCV, can also be recognized by RIG-I (*31*, *32*). A recent study demonstrated that 2′-*O*-methylation at the 5′-penultimate residue of cellular mRNA is a critical determinant of self mRNA and that the His residue at position 830 in the CTD of RIG-I is responsible for exclusion of 2′-*O*-methylated self-mRNA from viral unmethylated RNA (*33*).

MDA5 mainly detects non-self RNA generated by picornaviruses, such as poliovirus, EMCV and Theiler's murine encephalomyelitis virus, in which the 5′-teriminal of the viral RNA is covalently attached to the viral protein genome-linked (*34*). The non-self

signature of these viruses comprises long dsRNAs (>1,000 bp) produced during viral replication (*35*). Several RNA viruses, including reovirus, West Nile virus and Dengue virus, can be detected by both MDA5 and RIG-I.

Regulation of RLR-Mediated Signaling

Activation of RLRs by oligomerization

When substrate viral RNAs are present in the cytoplasm, RLRs recognize the non-self RNA structure and activate downstream signaling. Analysis of the crystal structure of the C-terminal region of RIG-I with substrate RNA clearly demonstrated that the Hel-1, Hel-2i, Hel-2, and the CTD wrap around the 5′-end of substrate RNA (*19*, *20*) and that the 5′ppp moiety is covered by the CTD, which adopts a cleft-like structure with basic amino acids (*35–38*). Recent *in vitro* studies have shown that the RNA-bound RIG-I forms multimolecular aggregates on the substrate RNA in an ATP-dependent and 2CARD-independent manner (*40*, *41*). Because RIG-I can bind to and move on substrate dsRNA, this ATP-dependent sliding activity may help to form RIG-I aggregates on substrate RNA (*42*). The 2CARD of activated RIG-I is released from autorepression and forms a helical tetrameric 'lock washer' structure (see below), which allows the 2CARD to interact with the CARD of MAVS (*43*).

In contrast to RIG-I, MDA5 has no preference for the end structure of substrate RNA because the CTD of MDA5 does not have an end-capping loop, as is observed in RIG-I CTD (*44*). Indeed, analysis of the crystal structure of MDA5 with substrate dsRNA has shown that the helicase domains and CTD surround dsRNA, similar to that observed in RIG-I; however, the CTD of MDA5 interacts with the dsRNA stem, but not the 5′-end, forming a ring-like structure in the middle of the dsRNA (*21*). This structural difference could explain the differential substrate specificities of RIG-I and MDA5. As is the case for RIG-I, MDA5 can also form a helical filamentous structure on dsRNA; however, *in vitro* experiments have revealed that ATP hydrolysis of MDA5 enhances the dissociation of MDA5 from the substrate dsRNA (*45*). In the subsequent report, Peisley *et al*. demonstrated that the ATP-driven dissociation is critical for discrimination between long non-self dsRNA and short self RNA by MDA5 (*46*, *47*).

Upon the formation of filamentous oligomers of RIG-I and MDA5, MAVS also forms prion-like filamentous aggregates on the surface of cytoplasmic organelles, such as mitochondria, peroxisomes and MAM (*48*, *49*). A recent crystal structure analysis of

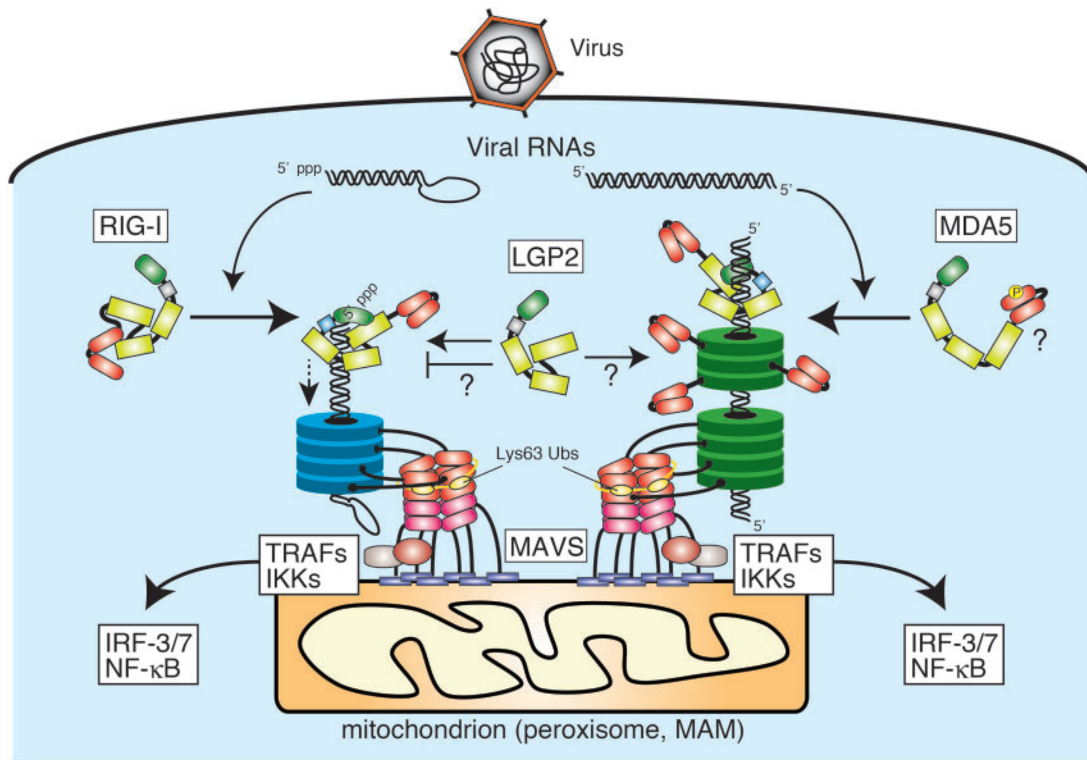

Fig. 2 Molecular mechanisms of RLR activation. RIG-I and MDA5 are localized in the cytoplasm in an inactive configuration. In response to viral infection, RIG-I and MDA5 recognize viral non-self RNAs, i.e. 5′-ppp-containing panhandle dsRNA and long dsRNA, respectively, and induce ATP-dependent conformational changes to form filamentous oligomers on the substrates. The C-terminal half of RIG-I preferentially recognizes the 5′-ppp structure using the basic cleft of CTD, whereas MDA5 does not have an end-preference. The released N-terminal 2CARDs form a 'lock-washer'-like tetramer in a Lys63-linked Ubs-dependent manner. The CARD of MAVS on the mitochondria forms a filament along the 2CARD tetramer, resulting in recruitment of downstream signaling molecules, such as TRAFs and IKKs, which activate the transcription factors, IRF-3/7 and NF-κB. Modified from Yoneyama *et al*. (2015), Viral RNA detection by RIG-I-like receptors, *Curr Opin Immunol*, 32, 48–53, Copyright (2015), with permission from Elsevier (*81*).

the CARD of MAVS with or without the 2CARD of RIG-I proposed a model for nucleation of the CARD of MAVS; this model suggested that the 'lock-washer'-like RIG-I 2CARD-tetramer may serve as a template for the CARD of MAVS, resulting in formation of the similar 'lock-washer'-like filament of the MAVS CARDs on the RIG-I 2CARD tetramer (*50*, *51*). However, the mechanisms through which signal-competent signalsomes form on these filamentous oligomers of 2CARDs and CARDs are still unknown. Importantly, artificial oligomerization of a CARD-defective MAVS mutant can activate IFN-inducing signal, suggesting that aggregation of the CTD of MAVS, which contains TRAF-binding motifs, could be necessary for downstream signaling (*52*) (Fig. 2).

Regulation by polyubiquitin chains

Ubiquitin chains (Ubs) are required for the oligomerization of RIG-I and MDA5 and for the regulation of signal activation (*53*). Initially, RIG-I was reported to be polyubiquitinated (Lys63-linked Ubs) by the E3-ligase, tripartite motif protein 25 (TRIM25) at Lys172 in the 2CARD (*54*). However, the subsequent reports have demonstrated that unanchored Lys63-Ubs are associated with the 2CARD and are required for signal activation by RIG-I using *in vitro* reconstitution assay (*55*). Furthermore, a similar mechanism has been observed in the case of MDA5 (*56*). A recent crystal structure and biochemical analysis showed that Lys63-Ubs directly associate with RIG-I 2CARD tetramers at the outer rim, thereby stabilizing the tetrameric conformation of 2CARDs (*43*). However, RIG-I has also been shown to form filaments independent of Ubs, suggesting that requirement for Lys63-Ubs for 2CARD activation may vary according to assay conditions or cell types (*40*). Further studies are needed to fully elucidate the physiological roles of Ubs.

In addition to TRIM25, Ub conjugation has also been shown to regulate RLR-mediated signaling. The E3-ligases RING finger protein leading to RIG-I activation (Riplet; also known as RNF135 and REUL), mex-3 RNA binding family member C (MEX3C) and TRIM4 directly conjugate Lys63-Ubs at Lys788, Lys99/169 and Lys154/164/172 of RIG-I, respectively, and positively regulate RIG-I-mediated signaling (*57–59*). A recent systematic functional analysis of TRIM family members demonstrated that several TRIMs could positively regulate RLR-mediated signaling (*61*). In contrast, TRIM13 plays a negative role in MDA5-mediated signaling, but not in RIG-I-mediated signaling (*61*). Moreover, the TRAF family E3-ligases TRAF2, 5 and 6 are involved in the regulation of RLR-mediated signaling (*62*). Ubiquitin-specific protease (USP) family members have also been shown to be involved in RLR-mediated signaling. For example, USP3 and USP21 cleave Lys63-Ubs conjugated on the 2CARD and inhibit RIG-I-mediated signaling (*63*, *64*), whereas USP4 and USP15 positively regulate RIG-I-mediated signaling by deubiquitination

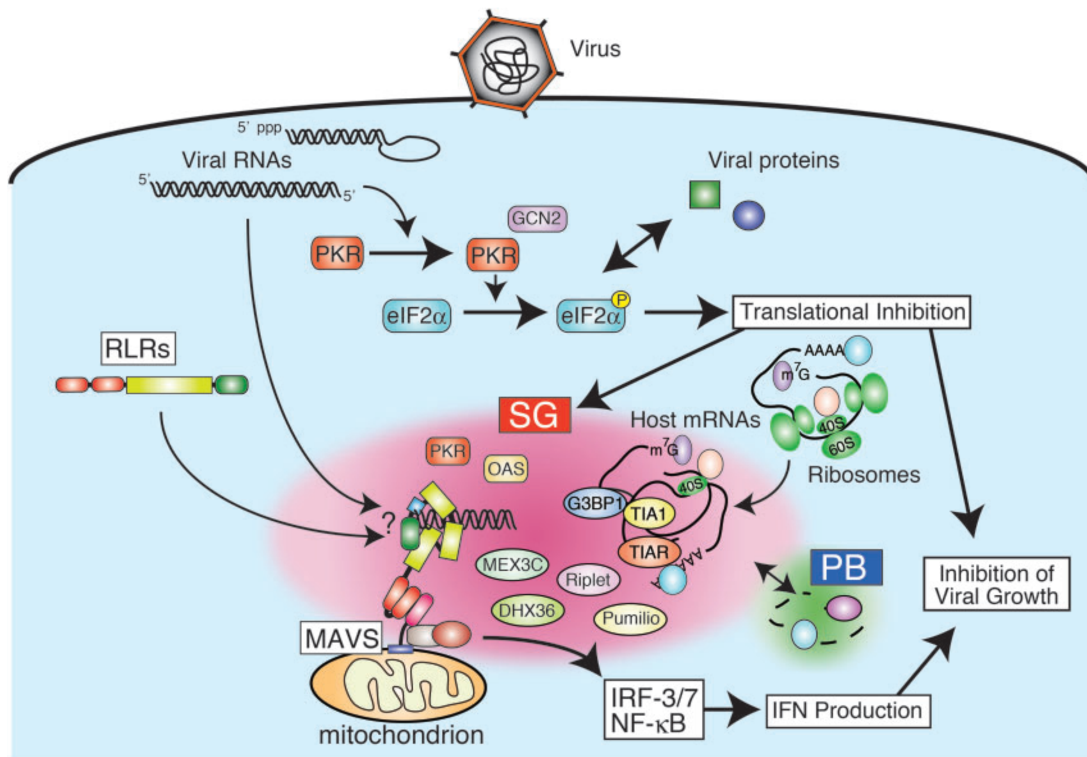

Fig. 3 Interaction between RLR-mediated signaling and RNA granules. In response to viral infection, viral dsRNAs can activate not only RLRs but also PKR. PKR phosphorylates eIF2α at Ser51 residue and terminates initiation of cellular translation, resulting in inhibition of viral replication. The translation-stalled cellular mRNAs and RBPs transiently accumulate in SGs. Although SGs are known to communicate with PBs, the precise mechanisms remain unclear. In virus-induced SGs, viral dsRNA and viral proteins are localized with RLRs and other antiviral proteins, suggesting that SGs may function as a platform for antiviral signaling. Some viruses have viral proteins that positively inhibit the formation of SGs to escape from antivirus activities, whereas some exploit SGs for their efficient growth.

of the Lys48-Ubs from RIG-I and TRIM25, respectively (*65*, *66*).

Regulation of RLR-Mediated Signaling by Stress-Induced RNA Granules

Viral infection induces cellular stress responses, including the formation of cytoplasmic membrane-less RNA granules, termed stress granules (SGs) (*67*). A recent study revealed that RLRs are localized in virus-induced SGs, together with cellular mRNAs, 40S ribosomes and RNA binding proteins (RBPs), in response to infection by several types of viruses. Interestingly, the artificial inhibition of virus-induced SG formation significantly impairs IFN production, suggesting that SGs play an important role in antiviral innate immunity (*68*) (Fig. 3).

SGs

SGs are ribonucleoprotein aggregates that are transiently formed under the cellular stress conditions, such as heat, hypoxia, oxidation and osmotic shock. SGs have been shown to function as a depository, storing translation-stalled inert mRNAs in order to avoid unwanted protein synthesis and/or delivering mRNAs to other RNA granule, processing bodies (PBs), where mRNAs are degraded by exonucleases (*69*). However, the physiological significance of this process is still controversial. SG formation is predominantly induced by phosphorylation of eukaryotic translation initiation factor 2α (eIF2α) at Ser51 residue by mammalian eIF2α kinases, *i.e.* PKR, general control non-derepressible 2 (GCN2) and heme-regulated eIF2α kinase. The phosphorylation of eIF2α terminates the initiation of translation, and the resulting translation-stalled mRNAs and RBPs accumulate in SGs through unknown mechanisms. Although several essential marker proteins of SGs have been defined and examined, the biochemical analysis of SGs has been limited owing to the nature of SGs as membrane-free and heterogeneous aggregates.

RLR signaling and SGs

PKR, a well-known ISG, is activated by viral dsRNA and exhibits antiviral activity through eIF2α-phosphorylation-mediated inhibition of translation. Infection by many types of RNA viruses induces SG formation (*70*); this process is predominantly regulated by PKR. Several viruses are known to utilize GCN2 for SG formation (*71*). In the case of IAV, although wild-type IAV can barely activate SGs, the mutant IAV in which viral non-structural protein 1 (NS1) is deleted (IAVΔNS1) has been shown to significantly induce PKR-dependent SG formation (*68*). Importantly, during IAVΔNS1 infection, the viral RNAs and nucleocapsid proteins are colocalized in SGs together with RIG-I, PKR and SG markers, such as Ras-GAP SH3 domain binding protein-1 (G3BP1) and T-cell restricted intracellular antigen 1 (TIA1)-related protein (TIAR). The siRNA-induced gene knockdown of the G3BP1 or PKR genes abrogates IAVΔNS1-induced IFN production concomitant with defects in SG formation. These observations strongly suggest that the formation of SGs is critical for IAV-induced antiviral innate immunity and that SGs may function as a scaffold for viral RNA recognition by RLRs. Recent reports have also revealed the functional importance of SGs and have identified several SG-localized regulatory molecules, such as MEX3C, Riplet, DHX36 and Pumilios involved in IFN production (*57*, *58*, *72*, *73*). In contrast, PKR-dependent localization of MDA5 in SGs is dispensable for IFN responses (*74*); therefore, further investigations will be necessary to elucidate the details of these mechanisms.

As is the case for NS1 of IAV, several viruses have strategies to inhibit SG formation in order to promote their propagation. For example, poliovirus, Coxsackievirus and EMCV, which all belong to the family *Picornaviridae*, encode 3C proteases to terminate SG formation by cleavage of G3BP1 (*75–77*). The expression of the 3C-resistant G3BP1 mutant induces the prolonged formation of EMCV-induced SGs and enhances the production of IFNs, suggesting that viruses may have developed countermeasures to protect against SG-mediated antiviral activity. However, several viruses have been shown to utilize SGs for their replication. For example, viral RNA of HCV (*Flaviviridae*) can induce PKR-dependent SG formation and localize to SGs together with viral proteins and SG components. The formed complex distributes to the ER-derived membranous web, where the HCV replicates, indicating exploitation of SGs by HCV (*78–80*). Similarly, the SG proteins G3BP1 and TIA1 are responsible for viral assembly or egress of HCV (*79*). Further studies are needed to determine whether SGs and/or SG components are responsible for antiviral or proviral activities for different viral species.

Concluding Remarks

Since the initial identification of RLRs in 2004, the molecular machineries underlying non-self RNA recognition by RLRs and RLR/MAVS-mediated signal activation have been extensively investigated. Recent advances have indicated that the activated RLRs form filaments along substrate RNAs. The resulting exposed 2CARDs are accumulated as the Lys63 Ubs-dependent tetramers, which induces the formation of functional heteromeric oligomers with MAVS CARDs. Additionally, PKR-dependent SG formation may be responsible for potent activation of RLR-mediated signaling in response to infection of several types of viruses. However, as direct evidence linking RLR oligomers and SGs has not been provided, additional studies are needed in order to elucidate the details of this relationship. More work is also required to address the following questions: What are the mechanical differences in oligomer formation between RIG-I and MDA5? How is LGP2 involved in the oligomerization of RIG-I/MDA5? How do CARD filaments recruit downstream signaling molecules? How is the balance between antiviral and proviral activities of SGs regulated? Is viral RNA actually detected by RLRs in SGs?

Funding
This work was supported by Grants-in-Aid for Scientific Research on Innovative Areas (15H01252), (B) (26293101) and Young Scientists (B) (15K19103) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan and the Takeda Science Foundation.

Conflict of Interest
None declared.

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