RNA sensing: the more RIG-I the merrier?

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Helicases translocate along and unwind double-stranded nucleic acids coupled to ATP hydrolysis, which provides the required energy. They are essential to life and have important roles in, for example, DNA replication and repair, transcription and RNA interference. In 2004, an essential function of helicases was discovered in the innate immune response [1]. Retinoic acid inducible gene I (RIG-I) encodes a multidomain protein that contains a superfamily 2 DExD/H-Box RNA helicase domain (Fig 1A). It specifically recognizes viral RNAs in cells infected with many RNA viruses—such as influenza A—and initiates a host response that leads to the production of type I interferons (IFNs), a family of secreted cytokines with potent antiviral effects. Despite the progress in our understanding of RIG-I activity, as detailed below, the role of ATP hydrolysis by the helicase domain remains poorly understood, although it is crucial for downstream signalling [1]. Whether RIG-I forms oligomers on RNA is also unclear. Two studies in this issue of *EMBO reports* by the Garcia-Sastre and Pyle groups provide new insights into these issues [2,3].

The types of RNA that activate RIG-I, the signal transduction pathway and the viral antagonists that target it have been intensively studied [4]. Moreover, crystal structures of RIG-I, with and without RNA, have provided detailed molecular insights into its activation [5]. RIG-I surveys the cytosolic RNA content for molecules bearing a 5'-triphosphate (5'PPP) group, which is present on the RNA genome of several RNA viruses but is not found on most cellular cytosolic RNAs [4]. RIG-I first recognizes the 5'PPP group on target RNAs with its carboxy-terminal domain (CTD) and then binds to the RNA—which must be base-paired to a complementary stretch of RNA—with the helicase domain. RNA binding induces major structural rearrangements that displace the two caspase recruitment

domains (CARDs; Fig 1A) from their interaction with the helicase domain [5], enabling CARD interaction with the adaptor protein mitochondrial antiviral signalling protein (MAVS), which relays the signal to transcription factors that induce IFN.

The Pyle group reports, in this issue, the crystal structures of RIG-I bound to a 10 base pair (bp) double-stranded RNA (dsRNA), with an empty ATP-binding

pocket and with ADP-Mg²⁺, and compares these with a previously published structure containing sulphate [2]. Depending on the occupancy of the ATP-binding site, the helicase domain makes contact with various nucleotides of the bound RNA. This suggests that the helicase domain scans the RNA in a manner dependent on ATP binding and hydrolysis. In these and previous structures of RIG-I, the

Fig 1 | RIG-I activation by short and long stem-loop RNAs. (A) RIG-I has two caspase recruitment domains (CARDs) at its aminoterminal, a central helicase domain divided into subdomains Hel1, Hel-2i and Hel2, and a carboxyterminal domain (CTD, also called regulatory domain). (B) First, the RIG-I CTD recognizes RNA 5'PPP groups. It then binds to the RNA with its helicase domain, inducing a conformational change that exposes the CARDs and enables their interaction with MAVS to activate IFN gene transcription. The RNA loops—shown as dashed lines—are largely irrelevant to RIG-I activation. In addition, RIG-I might oligomerize on long stretches of base-paired RNA requiring ATP hydrolysis [3]. The resulting RIG-I cluster might trigger stronger MAVS activation. 5'PPP, 5'-triphosphate; IFN, type I interferon; MAVS, mitochondrial antiviral signaling protein; RIG-I, retinoic acid inducible gene I.

CTD caps the RNA by interacting with the 5'-end [2,5], thereby anchoring RIG-I and limiting helicase scanning to a few nucleotides. This predicts that stem-loop RNAs having only one 5'-end bind to one RIG-I molecule, irrespective of the length of the base-paired stem. Indeed, Kohlway *et al* [2] demonstrate a 1:1 stoichiometry between RIG-I and RNA hairpins ranging from 10 bp to 30 bp. Two RIG-I molecules bind to a dsRNA of 22 bp, consistent with the presence of two 5'-ends. Furthermore, 'short' poly I:C more potently stimulates RIG-I ATPase activity than 'long' poly I:C when an equal mass is used. However, an equal number of short or long poly I:C molecules induces similar ATPase activity, indicating that the availability of ends—and not the length of the RNA—determines RIG-I binding and ATPase activity. Notably, the RNA hairpins that form 1:1 complexes with RIG-I stimulate IFN production in cells, implying that monomeric complexes containing a single RIG-I molecule bound to the 5'-end of a base-paired RNA are sufficient to initiate signalling.

However, does this monomeric signalling unit allow for maximal induction of IFN gene transcription? The study from the Garcia-Sastre group suggests that oligomerization of RIG-I on longer base-paired stretches of RNA further boosts signalling [3]. This conclusion is based on an analysis of RIG-I activation and IFN induction triggered by a Sendai virus defective-interfering (SeV-DI) RNA. This single-stranded RNA is predicted to form a base-paired, 94 bp long stem and a loop of 358 nucleotides. SeV-DI RNA was prepared by *in vitro* transcription, resulting in the presence of a 5'PPP group. Transfection of this RNA into reporter cells potentially triggered RIG-I-dependent IFN induction, and shortening of the stem to 46 bp or 25 bp reduced IFN induction. Why might this be? Oligomerization of RIG-I on dsRNA has been observed [6] and Patel and colleagues found that it also occurs on SeV-DI RNA. Indeed, purified RIG-I protein forms not only a 1:1 complex with SeV-DI RNA, but also complexes of higher molecular weight. Oligomerization of RIG-I is markedly reduced on SeV-DI RNAs with a shortened stem. Thus, the length of the base-paired region in SeV-DI RNA and RIG-I oligomerization correlate with IFN induction. The authors also show that RIG-I oligomerization on SeV-DI RNA requires a 5'PPP-moiety on the RNA and a base-paired 5'-end, ATP and an intact RIG-I ATPase domain. Given also that RIG-I

oligomers do not form in the presence of a non-hydrolysable ATP analogue, the authors propose a model for maximal RIG-I activation: after initial binding to the 5'-end, ATP hydrolysis allows RIG-I movement further down the base-paired RNA, and additional RIG-I molecules load onto the 5'-end (Fig 1B). In this model, a longer base-paired region would accommodate a larger number of RIG-I molecules, bringing together more CARDs for enhanced downstream signalling.

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This model might seem contradictory to the data presented by the Pyle group. However, the two studies use RNAs with different lengths of base-paired stems: SeV-DI RNAs with stems ranging from 94 bp to 25 bp and *in vitro*-transcribed RNAs with stems between 30 bp and 8 bp. Indeed, both studies are consistent in that they find little or no RIG-I oligomerization on RNAs with a stem of 25–30 bp. Furthermore, in both studies, the RNAs that fail to oligomerize RIG-I induce IFN reporters. Therefore, oligomerization of RIG-I on RNA is not necessary for IFN induction, but could enhance the signal (Fig 1B). Notably, after RNA binding and CARD exposure, multiple complexes containing RIG-I and RNA interact through the CARDs to facilitate the activation of MAVS [4].

The model proposed by the Garcia-Sastre group posits internal binding of RIG-I to base-paired RNA, which could be achieved either by release from the 5'-end and re-binding or by translocation on the RNA. Indeed, RIG-I can translocate along dsRNA in a 5'PPP-dependent and an ATP-dependent manner [7], and behaves similarly to a helicase in an unwinding assay [8]. Patel *et al* [3] find that both long and short SeV-DI RNAs induce similar RIG-I ATPase activity, which might argue against translocation of RIG-I on long base-paired RNA, assuming it involves multiple rounds of ATP hydrolysis. Structural work failed to provide evidence for unwinding of duplex RNAs and showed that the RIG-I CTD caps the 5'-end of bound RNAs, which is predicted to block translocation of RIG-I away from the 5'-end [5]. For translocation or release/re-binding to occur, the tight

interaction between the CTD and the 5'PPP would need to be broken and RIG-I would need to change its conformation, moving the CTD away from the 5'-end (Fig 1B). Although no structural evidence for this has been reported, the related helicase melanoma differentiation-associated protein 5 (MDA5) adopts such a conformation [4].

Many viruses that trigger the RIG-I pathway have RNA genomes with complementary 5'- and 3'-ends, predicted to form a base-paired stem often called a 'panhandle'. A comparison of putative panhandle structures [9] with the footprint of RIG-I [6] suggests that the length of the 'handle' is often too short to accommodate many RIG-I molecules. For example, the flu panhandle is 12–13 bp and thus unlikely to allow for RIG-I oligomerization. Nevertheless, flu RNA genomes activate RIG-I in infected cells [4]. The contribution of RIG-I oligomerization to IFN induction in infected cells depends on whether RNAs with longer base-paired stretches are generated naturally during the virus life cycle, which will depend on the virus. For example, paramyxovirus defective interfering RNAs (such as SeV-DI RNA) are typically produced only when viruses are passaged at high multiplicities of infection [10]. It is thus possible that oligomerization of RIG-I boosts IFN induction at later stages of an *in vivo* infection, when RNAs with long base-paired regions might be generated. Further work is required to understand the structural biology of RIG-I oligomerization and its contribution to virus-sensing under physiological conditions.

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