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Sensing viral RNAs by Dicer/RIG-I like ATPases across species

Simona Paro[1], **Jean-Luc Imler**[1,2], and **Carine Meignin**[1,2]

[1]Antiviral Immunity Group, CNRS-UPR9022, Institut de Biologie Moléculaire et Cellulaire, Strasbourg, France

[2]Faculté des Sciences de la Vie, Université de Strasbourg, Strasbourg, France

Abstract

Induction of antiviral immunity in vertebrates and invertebrates relies on members of the RIG-I-like receptor and Dicer families, respectively. Although these proteins have different size and domain composition, members of both families share a conserved DECH-box helicase domain. This helicase, also known as a duplex RNA activated ATPase, or DRA domain, plays an important role in viral RNA sensing. Crystallographic and electron microscopy studies of the RIG-I and Dicer DRA domains indicate a common structure and that similar conformational changes are induced by dsRNA binding. Genetic and biochemical studies on the function and regulation of DRAs reveal similarities, but also some differences, between viral RNA sensing mechanisms in nematodes, flies and mammals.

Introduction

Viral infections represent a major threat for all living organisms. Viruses consist in their most basic form of a nucleic acid encapsulated in a protein shell and their replication depends on the molecular machineries of their host cells. Both viral and host components are present in infected cells, which makes the distinction between self and non-self very challenging to the innate immune system. In addition, the error-prone viral nucleic acid polymerases enable viruses to adapt rapidly and suppress their host's defence mechanisms. It is valuable to compare antiviral immune responses in a wide range of organisms, to understand their strategies to counter viral infections.

Although studies on antibacterial and antifungal defences revealed that important innate immunity pathways (e.g. Toll/interleukin-1 and TNF receptor pathways) have been conserved through evolution, things are more complex for antiviral immunity. In invertebrates (and in plants), RNA interference represents a major pathway of antiviral host-defence. In vertebrates, however, the response to viral infections is dominated by the

Correspondence: Carine Meignin, CNRS UPR9022, IBMC, 15 rue Rene Descartes, 67000 Strasbourg, France, c.meignin@unistra.fr, Tel: 00 33 388 417 115.

interferon (IFN) system, and the induction of IFN stimulated genes (ISGs) [1]. In spite of major differences in the effectors deployed, the antiviral responses of multicellular eukaryotes are triggered by the sensing of foreign nucleic acids in the cytosol.

In invertebrates, double-stranded viral RNA generated during replication is processed into 21-23bp small interfering (si) RNA duplexes by Dicer family RNase III nucleases. These si-RNA duplexes are then loaded onto Argonaute (AGO) family nucleases within the RNA-induced silencing complex (RISC), where one of the strands will guide the RISC complex to target homologous viral RNA sequences [2]. In mice, Dicer can process viral RNA into siRNAs in some cell types [3,4]. In addition, some endogenous micro (mi)RNAs produced by Dicer can counter viral infection (e.g. [5]). However, in most tissues, viral RNA is sensed by receptors of the RIG-I-like receptor (RLR) family [6]. Upon RNA-binding, the RLRs activate a signalling cascade leading to transcription of type I and type III IFN genes (Figure 1).

Both Dicer nucleases and RLR receptors share an evolutionarily conserved DECH box "helicase" domain, which plays an important role in RNA sensing [7,8]. Here we review the structure and function of the DECH box proteins involved in the antiviral immune response, in vertebrates, *Caenorhabditis elegans* and *Drosophila melanogaster*.

Dicers and RLRs define a family of duplex RNA activated ATPases

Consistent with their differing functions as small RNA processing enzymes and signalling receptors, the Dicer and RLR family proteins have distinct domain organizations. Whereas Dicer enzymes contain PAZ, RNase III and dsRNA binding (dsRBD) domains, the RLRs (with the exception of LGP2) contain two amino-terminal CARD domains (Figure 2). Yet, Dicer and RLR family members share the critical DECH-box helicase, which consists of three tandem motifs, HEL1, HEL2i and HEL2 [9–12]. The HEL1 and HEL2 components of this tripartite core domain represent modified RecA domains, classically found in superfamily2 (SF2) RNA helicases, each containing a specialized motif discriminating the top (5′ to 3′) strand of the RNA duplex [13]. Furthermore, HEL1 presents an helical bridging, or pincer, domain, which is not present in SF2 helicases. This bridging domain provides an interface for interaction with the two α-helices connecting HEL2 to the C-terminal domain (CTD) [14]. The HEL1 and HEL2 domains are separated by the HEL2i domain, which sequesters the CARD2 domain in the absence of ligand (Figure 3a). Binding of dsRNA, which is facilitated by HEL2i, triggers a major conformational change of the DECH-box domain, leading to expulsion of CARD1 and CARD2 and activating signalling [7,8].

Metazoan Dicer enzymes are complex multi-domain proteins, typically larger than 200kDa, and difficult to crystallize. However, the characteristic HEL1, HEL2i and HEL2 motifs are conserved in most Dicers [7]. Furthermore, electron microscopy (EM)-based 3D reconstructions of human Dicer and *Drosophila* Dicer-2 reveal "L"-shaped particles composed of three distinct regions [15] (Figure 3b). The PAZ domain, which binds the extremity of the dsRNA helix, is located at the head of the structure. The RNase III domains are in the long arm body of the L. Finally, the tripartite "helicase" domain extends along the

base of the L (Figure 3b). The crystal structure of the RIG-I DECH-box helicase can be mapped to fit into the homologous region of Dicer [15]. The RIG-I helicase domain binds dsRNA which then appears to be clamped by the ligand-induced conformational change [15]. Similar conformational changes following dsRNA binding may occur in both protein families (Figure 3), although this remains to be determined directly for Dicer.

Importantly, neither Dicer nor RIG-I has been shown to function as a *bona fide* helicase. Thus, the generic acronym DRA has been proposed to include both these families of proteins that sense and respond to viral RNA [13]: DRA corresponds to **D**uplex **R**NA activated **A**TPases, (or alternatively, **D**icer/**R**IG-I like **A**TPases). In metazoa, two groups of DRAs participate in antiviral immunity: the signalling sDRAs and the catalytic (RNase III) cDRAs. While flies and other insects, lack sDRAs, they have two cDRAs, one of which (Dicer-2) is dedicated to antiviral immunity. *C. elegans* and mammals, on the other hand, have a single cDRA and multiple sDRAs (Figure 2). Interestingly, sDRAs participate in different antiviral pathways in *C. elegans* and mammals.

An ancient role of sDRAs in sensing viral RNA

In mammals, differences in the CTD domain account for the different binding specificities of RIG-I and MDA5. The RIG-I CTD domain accommodates the terminal 5′ tri- or di-phosphates of dsRNA [6,16]. By contrast, the MDA5 CTD binds to the internal segments of long dsRNAs, rather than at their extremities [17] (Table I). This is consistent with critical role of MDA5 in sensing of picornaviruses, which produce long dsRNAs that lack terminal 5′-di or tri- phosphates. [18]. In addition, the LGP2 sDRA lacks terminal CARD domains (Figure 2) and functions as a coreceptor together with MDA5 [19,20].

In *C. elegans*, Dicer-1 functions in competing RNAi pathways, while specificity is determined by associated RNA binding proteins. The alternative co-factors recruit Dicer-1 to form complexes specific for viral replication intermediates, aberrant endogenous RNA (endoRNAi), or exogenous dsRNA (exoRNAi). The identification of a natural viral pathogen infecting *C. elegans* has opened the way for the genetic characterization of antiviral resistance [21]. One major locus associated with sensitivity to virus infection is the sDRA Dicer-related helicase-1 (DRH-1), which senses viral RNAs and recruits Dicer-1 to the antiviral RNAi pathway [22,23]. Interestingly, DRH-1 is not required for processing exogenous dsRNAs into siRNAs, indicating that it discriminates something more than the double-strandedness of viral RNAs. The requirement of the CTD domain for the antiviral activity of DRH-1 suggests, by analogy with RIG-I, that DRH-1 may recognize 5′-di or triphosphates to improve viral RNA recognition and processing by Dicer-1.

The antiviral function of DRH-1 requires not only the DRA homology domain and the CTD, but also the N-terminal domain (NTD), which is not conserved in RIG-I. This NTD domain may function to recruit effector molecules, as do the CARD domains of RLRs [22,23]. A second member of the DRH family, DRH-3, interacts with Dicer-1 in silencing aberrant endogenous RNAs during spermatogenesis and embryogenesis (the ERI pathway) [24]. DRH-3 also potentiates the antiviral response, but in a Dicer-1 independent manner (see below) [23].

In summary, in both worm and vertebrate sDRAs, homologous DRA and CTD domains interact with viral RNAs while species-specific NTD domains activate different responses. In *C. elegans* a sDRA mediates recruitment of Dicer-1 to viral RNA substrates, raising the question of the substrate specificity of the human and fly Dicer enzymes.

Sensing RNAs by cDRAs

In *Drosophila*, two Dicer orthologues produce miRNAs (Dcr-1) and siRNAs (Dcr-2) [25]. Dcr-1 lacks conserved residues in the modified HEL1 domain (Figure 2), which may be critical to process dsRNAs. Although purified Dcr-2 can cleave pre-miRNAs *in vitro*, this activity is blocked by the R2D2 protein and inorganic phosphate [26]. R2D2 contains a dsRNA binding domain (dsRBD) and associates with Dcr-2 (see below), while inorganic phosphate occupies a pocket in the PAZ domain of Dcr-2, precluding binding of the 5′ phosphate extremity of pre-miRNAs [27,28].

The DRA domain of Dcr-2 appears to play at least two roles. Firstly, it initiates dicing of blunt-ended dsRNAs, perhaps by unwinding the first dsRNA bases and allowing positioning of a 3′-OH extremity in the PAZ domain [29]. Secondly, the DRA domain is required for translocation of Dcr-2 along the dsRNA molecule. Consequently, the DRA domain allows Dcr-2 to generate many siRNAs from a single long dsRNA substrate [26,29]. Thus, the DRA domain converts a distributive double–strand nuclease into a processive enzyme.

The single mammalian Dicer orthologue has the same domain organization *as Drosophila Dcr-2* (Figure 2), but processes pre-miRNAs rather than dsRNAs. Human Dicer binds to both substrates with the same affinity [30], but pre-miRNAs trigger specific repositioning of the DRA domain, which confers substrate specificity [31] (Figure 3b). Accordingly, DRA domain deleted hDicer can process dsRNA *in vitro* [32]. DRA truncated isoforms of Dicer, with enhanced dsRNA activity, are found in rodent oocytes and nematodes [33,34]. These truncated isoforms may participate in the control of viral infections (e.g. [3,4]). Importantly, full-length hDicer can also process dsRNA, when associated with TRBP.

dsRBD cofactors of cDRAs

In mammals and invertebrates, cDRAs function in protein complexes with dsRBD cofactors (Figure 2). The human TRBP and PACT cofactors, are both composed of three contiguous dsRBDs, which associate with the DRA domain of hDicer. TRBP binding triggers repositioning of the DRA domain, allowing efficient processing of dsRNA [31] (Figure 3b). *C. elegans* has a unique TRBP/PACT orthologue, RDE4 [35]. The nuclease activity of Dicer-1 requires RDE4 for viral RNA and exogenous dsRNA substrates, but not for endogenous dsRNAs or pre-miRNAs [23,36].

The situation is more complex in flies, possibly reflecting the absence of sDRAs. The fly orthologue of the genes *TRBP* and *RDE4* is *loquacious* (*loqs*) [37]. Two isoforms, Loqs-PB and Loqs-PD, have been characterized. Loqs-PB binds specifically to Dcr-1 through its dsRBD3 domain, and is important for the production of miRNAs. The Loqs-PD isoform, lacks the dsRBD3 domain and acts as a cofactor of Dcr-2 in the production of exogenous and endogenous siRNAs [38,39]. Surprisingly, however, Loqs is not required for the

resistance to viral infections, or the production of virus-derived siRNAs by Dicer-2 [40]. This implies that Dcr-2 may have an intrinsic capacity to sense and engage viral RNAs. In insects, a third dsRBD-containing co-factor, R2D2, participates in antiviral RNAi. However, R2D2 acts downstream of Dcr-2 processing, playing a critical role in the loading of siRNAs onto AGO2 [39].

From cell intrinsic to systemic antiviral immunity

Given the rapid propagation of most viruses, an essential aspect of antiviral immunity is the capacity to trigger paracrine signalling, leading to antiviral immunity in non-infected cells. This task is achieved by IFNs in vertebrates, which induce the production of antiviral molecules such as PKR. Upon infection, this dsRNA-dependent protein kinase phosphorylates eIF2α to shut down translation, thus ensuring an immediate response of the cell. Interestingly, TRBP and PACT interact with PKR and regulate its activity [41,42] (Figure 1).

In invertebrates, the systemic antiviral response utilises different mechanisms [43]. In *C. elegans*, the RNAi response is divided into primary and secondary steps and two types of siRNAs are produced. Primary siRNAs are produced by Dicer-1 and have a size of 23nt and a monophosphate at their 5′ extremities. These siRNAs are transferred to non-infected cells by poorly characterized mechanisms, where they trigger the synthesis of 5′ triphosphorylated 22nt-long secondary siRNAs by an endogenous RNA-dependent RNA polymerase (RdRP). Secondary siRNAs, which have a strong bias for a G at the 5′ position, appear to be loaded directly onto members of an expanded clade of worm specific AGOs, without Dicer-1 processing [44] (Table I). Importantly, the sDRA, DRH-3, is essential for the biogenesis of RdRP-derived small RNAs in *C. elegans*, and plays a critical role in antiviral immunity [22,23]. This indicates first that secondary siRNAs contribute to host defence by amplifying the antiviral response and, second, that a sDRA can function in antiviral immunity in a Dicer-independent manner in nematodes.

In insects, two mechanisms appear to contribute to the amplification of the antiviral response. The first is the dsRNA uptake pathway [45], which allows uninfected cells to sample their environment for the presence of dsRNA and trigger a preventive RNAi response [46]. The second mechanism involves the inducible expression of cytokines, such as Unpaired or Vago, following cellular stress or the detection of dsRNA. Such cytokines contribute to host-defence by ill-defined mechanisms [47–49]. The *Drosophila* genome does not encode RdRPs that could mediate signal amplification. However, the recent discovery that viral RNAs can be reverse transcribed into DNA in persistent viral infections raises the possibility that spreading of antiviral silencing might involve DNA- rather than RNA-dependent polymerases [50].

Conclusion and perspectives

Important knowledge has been gained in recent years on the function and regulation of DRAs in antiviral immunity across species. Furthermore, structural studies on DRAs suggest common mechanisms for binding viral dsRNA and in substrate-induced conformational changes in activity. These data pave the way for more detailed structure-function studies on

the discrimination between viral RNAs of different origins by DRAs. In this regard, the available collection of *Dcr-2* alleles with point mutations in the DRA domain represents a powerful resource for studies in *Drosophila* [25].

The conserved set of s-cDRAs and dsRBD cofactors forms a core viral dsRNA sensing and response mechanism in Metazoans, although these proteins intervene at different levels in the antiviral responses across species. Such modifications probably reflect the constant arms race between viruses and their hosts, requiring rapid and constant evolution of antiviral innate immunity pathways (e.g. [51,52]). Thus, the nature of the ancestral response to viral infection remains uncertain. The importance of RNAi in antiviral immunity in worms and insects points to the ancient origin of this antiviral pathway. However, sDRAs with CARD domains are present in the genomes of oysters and sponges, suggesting that *bona fide* RLRs are present in invertebrates, but were lost in nematodes and insects [53]. Functional characterization of antiviral innate immunity in other groups of invertebrates will shed light on the ancestral function of DRAs and the complex interrelationship between signalling and catalytic members of the family.

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Highlights

- Dicers and RLRs are important for antiviral immunity in Metazoans.
- Dicers and RLRs share a conserved Duplex RNA activated ATPase (DRA) domain.
- DRAs from Dicers and RLRs undergo similar conformational changes upon dsRNA binding.
- Signaling DRAs trigger different pathways upon sensing viral RNA in worms and mammals.
- dsRNA binding cofactors regulate activity of catalytic DRAs.

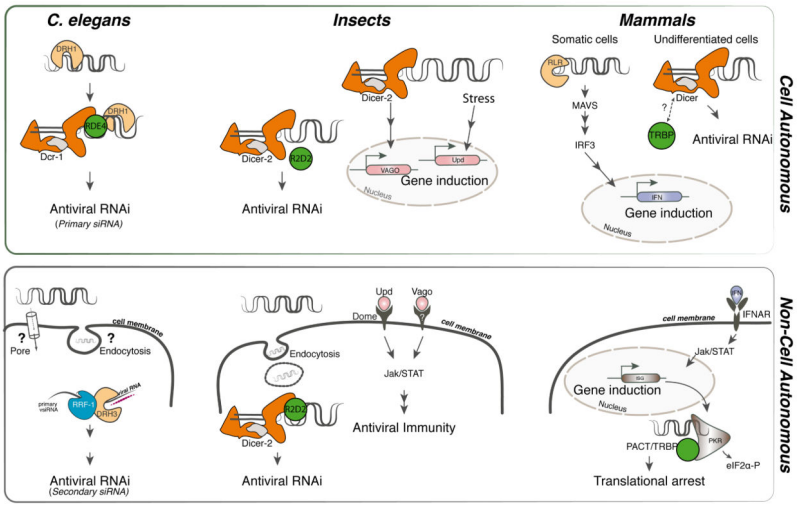

Figure 1. Antiviral innate immune pathways across species

Schematic representation of antiviral pathways in the nematode *C. elegans*, insects and mammals. Signalling DRAs are shown in light orange, catalytic DRAs in dark orange (RNaseIII domains highlighted in grey) and dsRBD cofactors in green. Cell autonomous primary responses are illustrated in the top panel, and secondary responses amplifying antiviral immunity are shown in the bottom panel. In *D. melanogaster*, the cytokines Upd2 and Upd3 activate the Jak/STAT pathway through the receptor Domeless [49], whereas in cells from the mosquito *Culex fasciculatus*, Vago triggers activation of the pathway through an unknown receptor [48]. Although also associated with antiviral activity in *Drosophila*, the exact function of Vago in this organism is still unclear. In undifferentiated mouse cells, virus-derived siRNAs contribute to antiviral immunity [32–33].

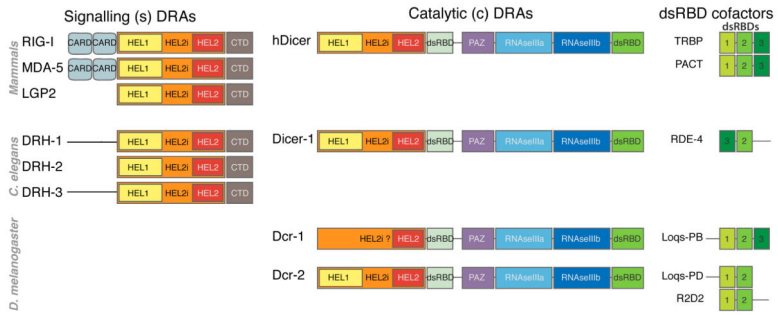

Figure 2. Conservation of s- and c-DRAs and their dsRBD cofactors

The domain organization of DRAs from *Homo sapiens*, *Caenorhabditis elegans* and *Drosophila melanogaster* is shown. In RIG-I and MDA5, the CARD domains function as homotypic protein-protein interaction domains to recruit and activate the signal transducer MAVS. In Dicer enzymes, the two RNase III domains form the catalytic core of the enzyme, the PAZ domain contains a pocket anchoring the 3′OH extremity of the substrate RNA, and the dsRNA Binding Domain enhances the affinity of the enzyme for its substrate. The dsRBD cofactors contain two to three evolutionarily conserved dsRNA Binding Domains (shown with different shades of green).

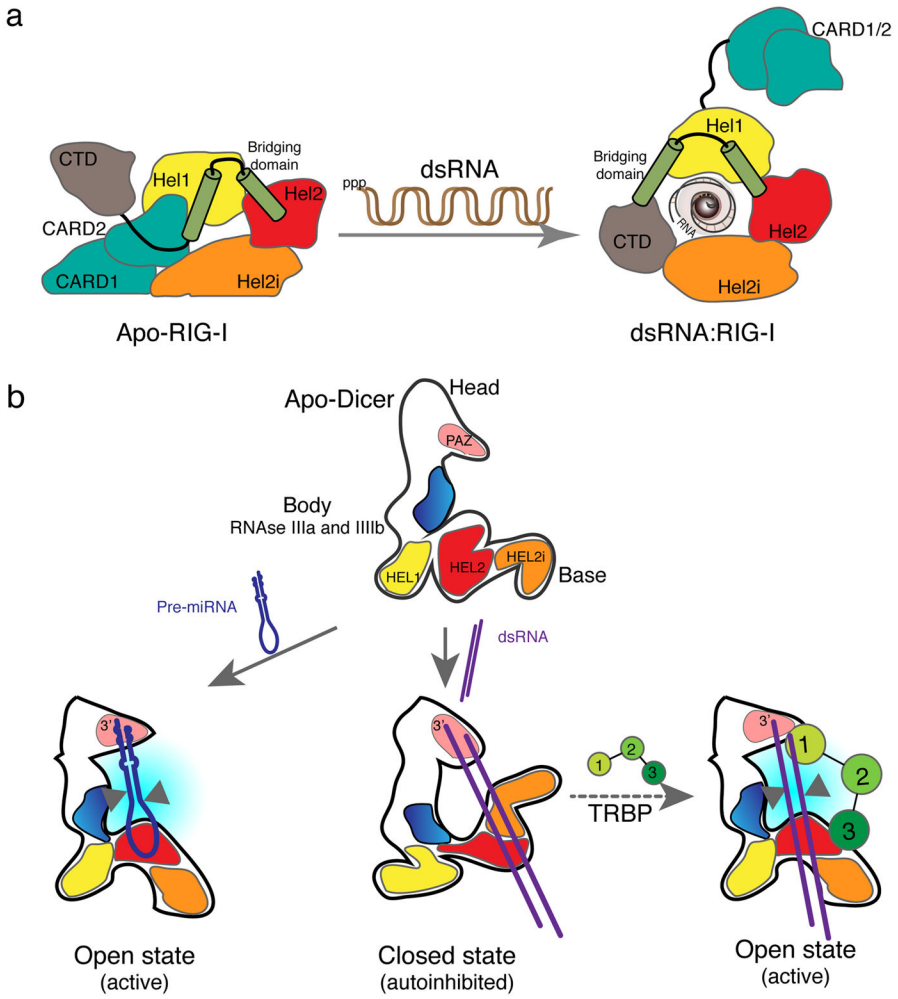

Figure 3. RNA induced conformational changes in sDRAs and cDRAs

(a) In non-infected condition, the signalling CARD2 domain of RIG-I is sequestered by the motif HEL2i, which is not present in other SF2 helicases. Binding of dsRNA containing 5′ triphosphate extremities triggers a major conformational change, with HEL1, HEL2 and HEL2i wrapping around the dsRNA stem. The CARD domains are expelled, making them available for signalling. (b) The DRA domain of hDicer rearranges differently in the presence of pre-miRNAs or dsRNA. Binding of the pre-miR *let7* triggers a bending of the base branch away from the platform, opening the conformation of the enzyme, and positioning the RNA next to the RNase III domains (blue) for cleavage. In the case of dsRNA, the binding of the free end of the duplex to the PAZ domain (pink) and of the stem to the DRA domain triggers an inward bending of the base branch. This closed conformation maintains the dsRNA at a distance from the RNase III domains, explaining the poor efficiency of the cleavage as well as the autoinhibitory function of the DRA domain. TRBP interacts with the DRA domain to trigger a different conformation, allowing processing of dsRNA. Arrowheads point to RNAse III processing. Redrawn with modifications from references [8] and [31].

Table I

General RNA structure of DRA ligands and products

Species	cDRA	sDRA	RNA structure
C. elegans	Dicer-1		5′p-23 nt siRNA duplexes with 2 nt 3′ overhang (*product*)
		DRH1	viral dsRNA, uncharacterized features (*ligand*)
		DRH3	5′ppp-22nt secondary siRNAs with 5′ G (*product*)
D. melanogaster	Dcr-2		viral dsRNA, uncharacterized features (*ligand*)
	Dcr-2		5′p-21 nt siRNA duplexes with 2 nt 3′ overhang (*product*)
M. musculus	Dicer		5′p-21 to 23 nt siRNA duplexes with 2 nt 3′ overhang (*product*)
		RIG-I	5′ppp-or 5′pp- short viral dsRNA *(ligand)*
		MDA5	long viral dsRNA *(ligand)*