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Dicer's role as an antiviral: still an enigma

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Abstract

Dicer is a multifunctional protein that is essential across species for the generation of microRNAs, a function that is highly conserved across the plant and animal kingdoms. Intriguingly, Dicer exhibits antiviral functions in lower organisms including *Drosophila melanogaster* and *Caenorhabditis elegans*. Antiviral activity occurs via small interfering RNA production following cytoplasmic sensing of viral dsRNA. Notably, such antiviral activity has not yet been clearly demonstrated in higher organisms such as mammals. Here, we review the evidence for Dicer as an innate antiviral across species.

Introduction

The discovery of Toll in *Drosophila melanogaster* and the subsequent characterization of the mammalian Toll-like receptors illustrated how ancient and highly conserved the pathways of innate immunity truly are. Orthologous pattern recognition receptors have since been found in species as evolutionarily divergent as plants and humans, highlighting the importance and evolutionary age of innate immune proteins. Likewise, Dicer and RNA interference (RNAi) are crucial components of a conserved antiviral response in a diverse array of eukaryotic organisms including *D. melanogaster* (reviewed in [1]). Dicer is a large, multi-domain enzyme whose RNase III activity is responsible for producing small RNA species from larger precursors. Specifically, Dicer is the enzyme responsible for cleaving pre-miRNAs into mature miRNAs and dsRNAs into siRNAs, and is thus a crucial component of the RNAi gene regulatory network.

Dicer is an indispensable antiviral protein in many organisms including plants and invertebrate animals. In these lower eukaryotes, Dicer is directly involved in binding and cleaving foreign dsRNA, which includes virus-derived RNA, and loading it into the host organism's RNAi pathway and thereby mediating viral suppression. In this way, Dicer acts as a sensor of viral RNA, capable of inducing a specific and directed antiviral response.

The structure of Dicer is highly conserved across animals [2] (Figure 1). Yet relatively little is known about Dicer's antiviral capacity in mammals. In this review, we discuss how Dicer functions as a cytoplasmic sensor of viral RNA in the model organisms *Caenorhabditis elegans* and *D. melanogaster*, and discuss recent evidence that suggests that mammalian Dicer may act similarly.

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Small RNA antiviral immunity in invertebrates

Worms and insects can detect, process, and target foreign RNA using their RNAi pathways. While some important differences exist between these species, the general RNAi pathway is well conserved. Foreign RNA is detected in the cytoplasm and cleaved by the RNA-processing enzyme Dicer into small RNAs. These Dicer-derived small RNAs are then incorporated into RNA-induced silencing complexes (RISCs) and used to produce a direct and specific RNAi response against other RNA from the same virus.

In *C. elegans*, virus-derived dsRNA is cleaved by Dicer to create primary viral small interfering RNAs (vsiRNAs). Dicer's DEXD/H helicase domain appears to be important for the processing of siRNA from a dsRNA precursor, while being dispensable in the processing of pre-miRNAs into miRNAs [3,4]. These primary vsiRNAs are then thought to recruit RNA-dependent RNA polymerase (RdRP) complexes to viral RNA [5–7]. RdRP complexes produce large amounts of single-stranded anti-sense secondary vsiRNA, which are then incorporated in RISCs, where they can target viral RNA for silencing [8]. Worms lacking an active Dicer-dependent RNAi pathway are susceptible to infection by several natural and non-natural viral pathogens, including single-stranded RNA viruses such as insect Flock house virus, mammalian vesicular stomatitis virus (VSV), and Orsay virus, which naturally infects *C. elegans* [9–13].

In contrast to *C. elegans*, *D. melanogaster* possesses two homologous yet functionally distinct Dicer molecules, Dicer-1 and Dicer-2. Dicer-1, which lacks a functional DEXD/H helicase domain, is responsible for processing hairpin-structured pre-miRNAs, while Dicer-2, which has a functional DEXD/H helicase domain, processes dsRNA substrates and therefore selectively handles antiviral RNAi processing [14,15]. In addition, production of vsiRNAs in *D. melanogaster* occurs without RdRP and secondary vsiRNAs. The overall antiviral pathway of Dicer-2 in *D. melanogaster* closely resembles that of *C. elegans*. Viral dsRNA is cleaved by Dicer-2 to create vsiRNAs, which are incorporated into RISCs, where they then can target viral RNA for inhibition [16,17]. Dicer-2 has been shown to induce the expression of certain antiviral genes following infection in flies [18].

Without a functional Dicer-2, flies succumb rapidly to infection by a number of viral pathogens, demonstrating the importance of this Dicer-2-mediated antiviral response [15–17,19,20].

Dicer proteins clearly function as important mediators of the cytoplasmic antiviral response in both *C. elegans* and *D. melanogaster*. In both organisms, an active helicase domain allows Dicer to process non-pre-miRNA substrates, i.e., RNA molecules missing the 2-nt 3' overhang characteristic of pre-miRNAs, while also allowing it to function processively, cleaving off small RNA molecules as it moves along the length of dsRNA [3,4]. Collectively, these studies indicate that Dicer's helicase domain is important for Dicer to function in a dsRNA-specific reaction for antiviral responses in both worms and flies.

Dicer's role in mammalian innate immunity against viruses

Evidence that mammalian Dicer may function as a viral RNA sensor has been steadily increasing. Mammalian Dicer is closely related to the Dicer enzymes in *C. elegans* and *D. melanogaster* (Figure 1). As in these other species, the mammalian Dicer enzyme processes both pre-miRNAs and dsRNAs into mature miRNAs and siRNAs, respectively [21]. Mammalian Dicer has a DEXH/D helicase domain, a Piwi Argonaute Zwiille (PAZ) domain, a Domain of Unknown Function 283 (DUF283), a double-stranded RNA binding domain (dsRBD), and two RNase III domains. The RNase III domains are each responsible for cleaving a strand of substrate RNA, while the PAZ domain binds to the 5' phosphate and 3'

end of these substrate RNAs and positions them properly for cleavage within the enzyme [22]. The DUF283 and dsRBD domains are likely important in binding to RNA substrates [23,24]. Even though the RNA helicase domain appears to be dispensable for pre-miRNA processing, it is essential for binding and processing dsRNA substrates and for binding the Dicer-partner proteins TAR RNA-binding protein (TRBP) and protein kinase RNA activator (PACT) [4,25].

How can mammalian Dicer act as an antiviral?

Many herpesviruses produce miRNAs that can regulate host or viral gene expression. In addition, viral infection induces significant changes in host miRNA expression (reviewed by [26,27]). These miRNAs may have direct antiviral functions or may target the antiviral immune response. Following infection, specific miRNA expression patterns have been observed, suggesting virus-specific and tissue-specific miRNA responses to both DNA and RNA viruses [28,29]. Furthermore, insertion of endogenous miRNA target sites into a viral genome can attenuate virus replication, demonstrating that host miRNAs have the capacity to interfere with viral RNA [30]. RNA viruses can also be engineered to express virus-derived cytosolic pri-miRNA which are processed into functional miRNA via a non-canonical, Dicer- and Drosha-dependent mechanism [31,32]. Both virus-derived and host-derived miRNAs have the potential to regulate viral RNA levels. Thus, by carrying out its canonical and non-canonical functions as the mature miRNA-processing enzyme, mammalian Dicer does affect the expression of viral and antiviral proteins (Figure 2A).

Mammalian Dicer may also exhibit a miRNA-independent antiviral function. Dicer has the potential to generate small virus-derived RNAs (Figure 2B). In fact, RNA molecules resembling Dicer-cleavage products have been found in mammalian cells infected with a wide array of human viruses [28,33–36]. However, levels of viral small RNAs (vsRNAs) are often very low and sRNA silencing of viruses has not been observed in mammalian cells suggesting that sRNA-mediated gene silencing as an anti-viral strategy is limited to non-chordates (reviewed in [26]). Two exciting recent reports demonstrate that Dicer-cleavage products of viral RNA are produced at significant levels in certain virus-infected mammalian cells. Li *et al.* found significant amounts of vsRNAs in baby hamster kidney cells and suckling mice infected with a mutant strain of Nodamura virus that lacks B2, a suppressor of RNAi [37]. Many of these small RNAs mapped to the ends of the viral RNA genome and had the length and 2-nt overhang hallmarks of Dicer products. Maillard *et al.* showed a similar accumulation of vsRNAs following the infection of mouse embryonic stem cells (mESC) with encephalomyocarditis virus or the same mutant Nodamura virus [38]. These RNAs were generated in a Dicer-dependent manner, and were found to associate with RISC proteins. Upon differentiation, mESC lost the capacity to accumulate these virus-derived small RNAs. Further studies will reveal the function and effect of these small RNAs and elucidate whether they represent a cell-type specific mammalian antiviral RNAi response.

Mammalian Dicer could also directly interact with the RIG-I-like receptor (RLR) viral-RNA sensing pathway (Figure 2C). Mammalian Dicer possesses a conserved and functional DExH/D helicase domain that is evolutionarily related to the RNA helicase domains found on RLRs [39] (Figure 3). These RLRs are a family of cytoplasmic antiviral sensors – retinoic acid-inducible gene 1 (RIG-I), melanoma differentiation-associated gene 5 (MDA5), and laboratory of genetics and physiology-2 (LGP2) – each of which is critical in detecting and responding to foreign RNA within the cytoplasm (reviewed by [40]). Recent evidence suggests that Dicer can differentially bind dsRNA and pre-miRNA, with dsRNA being an important byproduct of infection by RNA and DNA viruses [41]. One potential way in which Dicer acts as an antiviral is as part of a dsRNA-detection complex interacting with

one or all of the RLRs through their binding to viral RNAs. For example, by forming a complex with LGP2, Dicer could promote MDA5 or RIG-I signaling, thus enhancing interferon (IFN) induction following viral infection. LGP2 has been shown to directly interact with mammalian Dicer. Proteomic analysis of human type I IFN inducing pathways in a HEK293 cell over-expression system has revealed that human Dicer does associate with LGP2 following stimulation with the dsRNA analogue polyI:C [42].

Interestingly, Dicer is known to interact with orthologous DExD/H helicases in *C. elegans*, which are critical components of the antiviral RNAi response [43]. In *C. elegans*, the RLR orthologue and Dicer-associated protein, Dicer-related helicase 1 (DRH-1), is essential for the Dicer-dependent cleavage of viral dsRNA into primary vsiRNAs. Guo *et al.* recently demonstrated that worms lacking DRH-1 are unable to mount an antiviral RNAi response against Orsay virus [44]. However, the antiviral RNAi response in these worms can be rescued with a chimeric DRH-1 protein consisting of the helicase and C-terminal domains of human RIG-I and a worm-specific N-terminal domain [44]. These data suggest that DRH-1 may act as a viral sensor.

Further investigation is needed to determine if human Dicer interacts with, recruits, or requires different partner proteins during its processing of dsRNA when compared to pre-miRNAs, and/or whether its interaction with known viral RNA sensors like LGP2 is dependent upon a dsRNA ligand or other proteins as part of a larger complex. Numerous viral proteins have been found to directly inhibit Dicer and other members of the RNAi pathway (reviewed in [45]). Together these findings suggest that Dicer does more than simply process miRNAs during the course of a viral infection.

What models are available for studying mammalian Dicer?

Investigating the role of Dicer in mammalian innate immunity has been complicated by the fact that Dicer is an essential gene required for embryogenesis and cellular homeostasis. Knocking out *Dicer* in mice results in embryonic lethality due to a defect in angiogenesis [46]. Likewise, knocking out *Dicer* in cells leads to senescence [47]. This can be overcome by SV40 transformation of Dicer knock-out cell lines [48]. Such knockout cell lines have been used in seminal studies to understand Dicer function [28,30,49]. However SV40 encodes its own miRNAs that could complicate results [50]. For these reasons, Dicer's specific contribution to the antiviral response in mammals has been difficult to investigate.

Otsuka *et al.* developed and characterized a unique Dicer hypomorphic mouse that is able to escape embryonic lethality. Created by inserting a gene-trap element into the *Dicer* gene, this Dicer hypomorphic mouse expresses Dicer during embryogenesis. However, once full-grown, the gene-trap mice exhibit significantly reduced levels of Dicer expression in all tissues examined – apart from the testes – making it a useful tool for probing the function of Dicer in the context of viral infection [51]. Dicer hypomorphic mice come with several limitations. First, they are not a true knockout since cells retain the ability to express Dicer, albeit at reduced levels. Second, the gene-trapped allele has the potential to express a truncated N-terminal form of the Dicer protein – despite lacking an active RNase domain, this could still retain other functionalities of the N-terminal helicase and/or DUF domains.

Dicer hypomorphic mice have been found to be more susceptible to infection by several viruses. These mice exhibit decreased survival to infection with VSV and produce higher titers of virus in comparison to wild-type mice [51]. Macrophages collected from the Dicer hypomorphic and wild-type mice were shown to produce equivalent levels of type I IFN following infection with VSV. Type I IFN levels in the animals following VSV infection were not shown [51]. Like VSV, murine cytomegalovirus (mCMV) results in increased mortality in Dicer hypomorphic mice along with elevated levels of viral replication.

However, infection of Dicer hypomorphic mice with mCMV induced significantly lower levels of IFN- β protein in the serum and interferon-stimulated genes (ISG) in the spleen compared to wild-type mice [52]. Further analysis of splenocytes from Dicer hypomorphic mice showed a substantial dysregulation of many ISGs, even before infection [52]. The different type I IFN findings in these two infection models might be due to different levels of expression of wild-type Dicer from the gene-trapped allele in the cell types examined. Alternatively, Dicer could have a tissue- or cell-specific effect on the IFN response.

Knockdown studies in cell lines have also demonstrated a role for mammalian Dicer in controlling virus infections. A study by Matskevich and Moelling investigated the effect of Dicer knockdown on influenza A viral replication in interferon-unresponsive, monkey-derived Vero cells and in A549 human lung adenocarcinoma cells. Knockdown of Dicer increased viral replication in both Vero cells and also in A549 cells following antibody neutralization of their type I IFN response [53]. Similarly, knockdown of Dicer in Huh7 cells resulted in increased replication of dengue virus [1,54]. Whether the antiviral effect in Dicer knockdown cells is mediated by miRNA processing or a more direct antiviral functionality needs additional clarification.

Conclusion

Collectively, a number of important studies demonstrating altered viral susceptibility in Dicer-deficient systems suggest that mammalian Dicer plays a crucial role in the antiviral response by directly curbing viral replication and/or inducing a type I IFN response. Dicer's role in miRNA processing is well known and vitally important for regulating essentially every pathway within a given cell. Yet Dicer still remains an enigmatic protein, and any roles of mammalian Dicer beyond miRNA production are difficult to discern. Recent studies have begun to elucidate a role for Dicer in antiviral pathways that is distinct from its miRNA-processing functionality. Mammalian Dicer was found to interact with dsRNA in a different way than it interacts with miRNA precursors, further suggesting that Dicer may interact uniquely with virus-derived RNA. Evidence also suggests that Dicer and RLR pathways intersect at the protein level, and that Dicer may participate in the assembly and/or function of the RLR antiviral sensor complex.

Future studies will reveal if mammalian Dicer indeed functions as a direct protein-mediator of antiviral innate immunity.

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Highlights

1. Dicer has important antiviral functions in *D. melanogaster* and *C. elegans*
2. Dicer binds to dsRNA
3. Models of mammalian Dicer are in development
4. Mammalian Dicer interacts with homologous antiviral RLRs
5. Dicer is highly conserved among organisms

C. elegans ***D. melanogaster*** ***H. sapiens***

Figure 1. Conservation of Dicer domains across species

The domain organization of Dicer from *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Homo sapiens*. The domain structure of Dicer proteins from each of the species is highly conserved. *D. melanogaster* has two Dicer molecules: Dicer-1 lacks a functional helicase domain and is responsible for processing pre-miRNAs into mature miRNAs, and Dicer-2 has a functional helicase domain and is responsible for processing dsRNAs into siRNAs. For this reason, Dicer-2 selectively handles antiviral RNAi processing. DEXD/H Helicase, a highly conserved helicase domain; PAZ, Piwi Argonaute Zwillie domain; DUF283, Domain of Unknown Function 283; RNase III; dsRBD, double-stranded RNA-Binding Domain.

Figure 2. Three potential mechanisms by which Dicer could be involved in the innate antiviral response

A. Dicer is the key miRNA-producing enzyme and affects innate signaling pathways via its role in miRNA production.

B. Dicer can process virus-derived RNA and feed it into the RNAi pathway, resulting in direct interference of viral transcripts or viral genomes. Dicer is known to cleave dsRNA and Dicer-cleavage products have been found in cells infected with various viruses.

C. Dicer functions as part of larger viral-RNA bound detection complex along with the RLRs. These proteins all have helicase domains that bind to dsRNA. By forming a complex with RLRs, Dicer may enhance their antiviral effects.

RISC, RNA-induced silencing complex; LGP2, laboratory of genetics and physiology-2; IFN, interferon.

Figure 3. The domain organization of the human retinoic-acid-inducible-gene-1-like receptors (RIG-I-like receptors, or RLRs) and human Dicer

Mammalian Dicer shares a DExD/H helicase domain with the RLR family of cytosolic RNA sensors. The RLR family consists of RIG-I (retinoic acid-inducible gene 1), MDA5 (melanoma differentiation-associated gene 5), and LGP2 (laboratory of genetics and physiology-2). CARD, caspase activation and recruitment domain; Helicase, DExD/H helicase domain; PAZ, Piwi Argonaute Zwillie domain; DUF283, Domain of Unknown Function 283; RNase III; dsRBD, double-stranded RNA-binding domain.