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Loquacious-PD removes phosphate inhibition of Dicer-2 processing of hairpin RNAs into siRNAs

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ABSTRACT

Drosophila Dicer-2 processes RNA substrates into short interfering RNAs (siRNAs). Loquacious-PD (Loqs-PD), a dsRNA-binding protein that associates with Dicer-2, is required for processing of a subset of RNA substrates including hairpin RNAs into siRNAs. Inorganic phosphate—a small molecule present in all cell types—inhibits Dicer-2 from processing precursor of microRNAs (pre-miRNAs), which are processed by Dicer-1. Whether or how Loqs-PD modulates the inhibitory effect of inorganic phosphate on Dicer-2 processing of RNA substrates is unknown. To address this question, I performed in vitro hairpin RNA processing assay with Dicer-2 in the presence or absence of Loqs-PD and/or inorganic phosphate. I found that inorganic phosphate inhibits Dicer-2 alone, but not Dicer-2 + Loqs-PD, from processing blunt-end hairpin RNAs into siRNAs. Thus, Loqs-PD removes the inhibitory effect of inorganic phosphate on Dicer-2 processing of blunt-end hairpin RNAs, allowing siRNA production in the presence of inorganic phosphate.

1. Introduction

Dicer enzymes produce microRNAs (miRNAs) and small interfering RNAs (siRNAs). In *Drosophila*, Dicer-1 processes precursor of miRNAs (pre-miRNAs) into miRNAs and Dicer-2 processes long dsRNAs into siRNAs [1].

Endogenous RNA substrates of Dicer-2 to make siRNAs (endo-siRNAs) include partially self-complementary hairpin RNA transcripts, transposon RNAs, and dsRNAs derived from convergent transcription of mRNAs (cis-natural antisense transcripts, cis-NAT) [2–6]. The most abundant endo-siRNA in vivo is esi-2.1, a hairpin-derived endo-siRNA. This suggests that the esi-2.1 precursor hairpin RNA is a predominant substrate of Dicer-2. Dicer-2 also processes exogenous long dsRNAs derived from viral RNA genomes or intermediates of replication and those introduced artificially into exogenous siRNAs (exo-siRNAs) [1,7].

Dicer-2 has an N-terminal helicase domain, a central dsRNA-binding domain (dsRBD), a platform domain, a PAZ domain, two RNase III domains, and a C-terminal dsRBD (Fig. 1A). The Dicer-2 helicase domain binds and hydrolyzes ATP for processive siRNA production [8,9]. The Dicer-2 PAZ domain has a phosphate-binding pocket important for high-fidelity production of 21 nt siRNAs, which is important for efficient RNA silencing [10]. Each RNaseIII domain has an RNaseIII active site, and the two RNase active sites cleave dsRNA. The C-terminal dsRBD is crucial for efficient and high-fidelity production of siRNAs [11]. Recent cryo-electron microscopy structures of Dicer-2 showed that Dicer-2 adopts L-shape structure, in which the helicase domain forms the shorter arm and the PAZ, platform, and RNase III domains form the longer arm [12].

Dicer-2 can be bound by a cofactor dsRNA-binding protein Loquacious-PD (Loqs-PD) [13]. Loqs-PD has two dsRBDs (Fig. 1A). The C-terminal region of Loqs-PD binds the Dicer-2 helicase domain [14,15]. Loqs-PD is required for efficient production of a subset of siRNAs in vivo [7,16,17]. Loqs-PD is required for efficient production of hairpin-derived endo-siRNAs (esi-1.1, esi-1.2, and esi-2.1), cis-NAT-derived endo-siRNAs, and exo-siRNAs derived from an inverted repeat transgene [16]. In contrast, Loqs-PD is dispensable for production of transposon-derived endo-siRNAs [16,17] and virus-derived exo-siRNAs from certain RNA viruses [7]. It is unknown why Loqs-PD is crucial for production of only a subset of siRNAs. It is also unknown what distinguishes the substrates that require Loqs-PD for efficient processing and those that do not.

We previously showed that inorganic phosphate—a small molecule found in all cell types—specifically inhibits Dicer-2 from processing precursor of microRNAs (pre-miRNAs) and short

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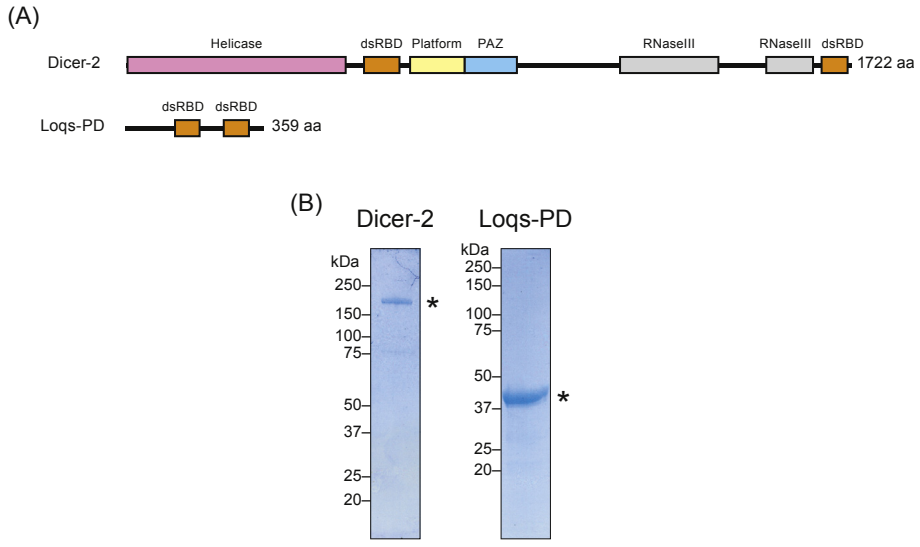

Fig. 1. Recombinant proteins of Drosophila Dicer-2 and Loqs-PD.
(A) Domain structures of *Drosophila* Dicer-2 and Loqs-PD.
(B) Coomassie-stained SDS-PAGE gels of purified recombinant Dicer-2 and Loqs-PD proteins.

dsRNAs [8,18]. Recombinant Dicer-2 alone can efficiently process pre-miRNAs into miRNA-like dsRNA products in vitro. Notably, these miRNA-like dsRNA products produced by Dicer-2 are shorter than the biologically relevant miRNAs produced by Dicer-1. Such miRNA-like dsRNA products should not be produced in vivo. In fact, a physiological concentration of inorganic phosphate inhibits Dicer-2 from processing pre-miRNAs and short dsRNAs [8,18]. Phosphate inhibition of Dicer-2 is dose-dependent and specific; inorganic phosphate inhibits neither Dicer-2 from processing long dsRNAs into siRNAs nor Dicer-1 from processing pre-miRNAs into miRNAs, and other anions do not inhibit Dicer-2 from processing pre-miRNAs. These studies suggest that inorganic phosphate binds the phosphate-binding pocket in the Dicer-2 PAZ domain and inhibits access of pre-miRNAs and short dsRNAs to the Dicer-2 PAZ domain, inhibiting their cleavage [18]. However, how inorganic phosphate affects Dicer-2 processing of hairpin RNAs with an intermediate length remains unknown. Whether or how the inhibitory effect of inorganic phosphate on Dicer-2 is modulated by Loqs-PD is also unknown.

To address these questions, I performed in vitro esi-2.1 precursor hairpin RNA processing assay with Dicer-2 in the presence or absence of Loqs-PD and/or inorganic phosphate (Dicer-2 ± Loqs-PD ± inorganic phosphate). I tested hairpin RNA substrates with distinct end structures (5′ monophosphorylated vs 5′ hydroxyl and blunt end vs 3′ overhang end), considering that RNA substrate end structures play a crucial role in processing by Dicer-2 [9,12,18,19]. I found that inorganic phosphate inhibits Dicer-2 alone, but not Dicer-2 + Loqs-PD, from processing esi-2.1 precursor hairpin RNAs with a blunt end. Thus, Loqs-PD allows Dicer-2 to process blunt-end hairpin RNAs in the presence of inorganic phosphate, which may explain the in vivo requirement of Loqs-PD for production of hairpin-derived endo-siRNAs.

2. Materials and methods

2.1. Recombinant protein purification

Recombinant Dicer-2 and Loqs-PD proteins were purified from Sf9 cells and *E. coli* cells, respectively, as previously described [8,16,18].

2.2. RNA substrates preparation

^{32}P-body-labeled hairpin RNAs were prepared using in vitro T7 transcription system in the presence of α-[^{32}P]ATP (800 Ci/mmol; PerkinElmer) and were gel purified, as previously described [8,16].

2.3. In vitro dicing assays

In vitro RNA processing reactions by Dicer-2 were performed using 100 nM ^{32}P-body-labeled hairpin RNAs, 8 nM Dicer-2 ± Loqs-PD in the presence or absence of 1 mM ATP and/or 25 mM inorganic phosphate at 25 °C and analyzed as described [8,16,18,20]. Aliquots of the reaction time course were run on denaturing urea-PAGE gels. Dried gels were exposed to image plates and analyzed with an FLA-9000 and ImageGauge 3.0 software (Fujifilm, Tokyo, Japan).

To determine rates of reaction, substrate processed versus time was fit to $y = y_0 + A(1 - e^{-kt})$, where $dy/dt = Ake^{-kt}$ using Igor Pro 6.31 (WaveMetrics, Lake Oswego, OR, USA). When $t = 0$, $dy/dt = Ak$; k gives the initial rate of reaction [21].

2.4. Statistical test

Statistical tests were performed using unpaired two-tailed Student's t-test using data obtained from three independently performed experiments. P-value <0.05 was used as a threshold for statistical significance.

3. Results

3.1. Dicer-2 requires ATP to process hairpin RNAs

To perform in vitro hairpin RNA processing assay with Dicer-2 ± Loqs-PD ± inorganic phosphate, I purified recombinant Dicer-2 protein from Sf9 cells and recombinant Loqs-PD protein from *E. coli* cells (Fig. 1B). For substrates, I prepared four esi-2.1 endo-siRNA precursor hairpin RNAs with 64 or 62 bp long stems [6]. The hairpin RNAs tested have one of four chemically distinct structures: (1) a 5′ monophosphorylated blunt end, (2) a 5′ hydroxyl blunt end, (3) a 5′ monophosphorylated, 3′ 2-nt overhang end, and (4) a 5′ hydroxyl, 3′ 2-nt overhang end (Fig. 2). I performed in vitro RNA

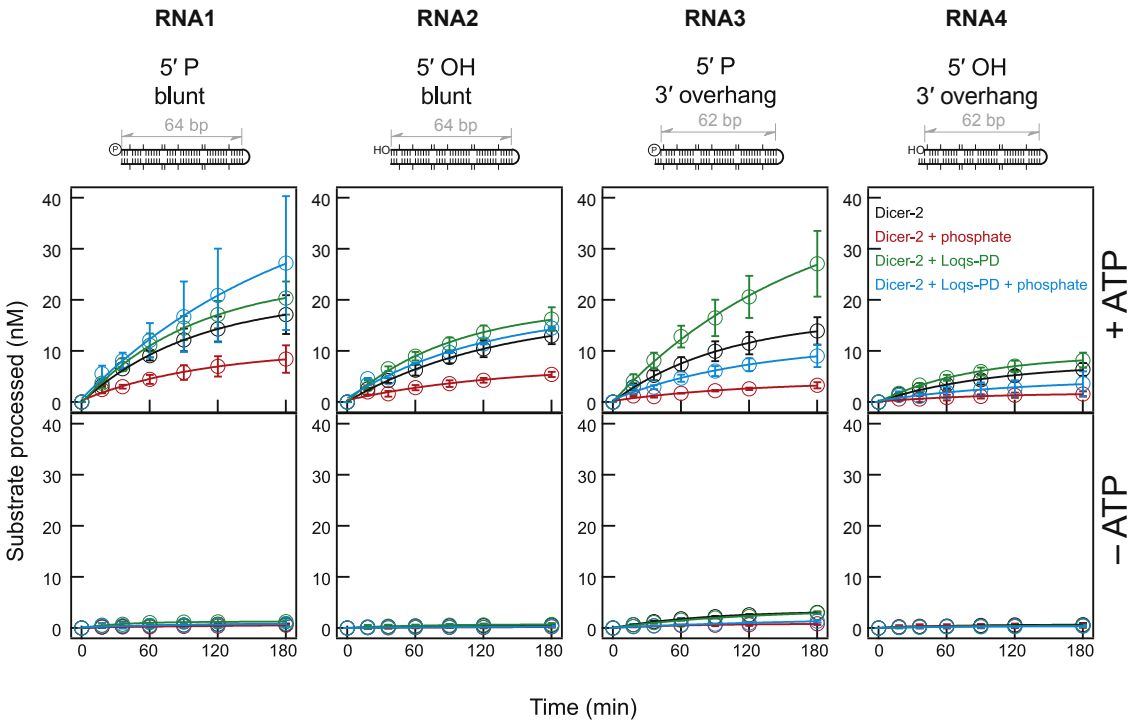

Fig. 2. Time course of in vitro processing assay of hairpin RNAs by Dicer-2 ± Loqs-PD.
Hairpin RNAs (100 nM, uniformly ^{32}P-radiolabeled) with (1) a 5′ monophosphorylated blunt end, (2) a 5′ hydroxyl blunt end, (3) a 5′ monophosphorylated, 3′ 2-nt overhang end, or (4) a 5′ hydroxyl, 3′ 2-nt overhang end were incubated with 8 nM Dicer-2 ± Loqs-PD in the presence or absence of 1 mM ATP and/or 25 mM inorganic phosphate. Data are mean ± s.d. for three independent trials.

processing assay using the purified recombinant proteins (Dicer-2 alone and Dicer-2 + Loqs-PD) and these four RNAs in the presence or absence of ATP and/or inorganic phosphate.

In the absence of ATP, neither Dicer-2 alone nor Dicer-2 + Loqs-PD cleaved any of the RNA substrates appreciably with or without inorganic phosphate (Fig. 2). In contrast, Dicer-2 and Dicer-2 + Loqs-PD cleaved RNAs in the presence of ATP. I concluded that both Dicer-2 and Dicer-2 + Loqs-PD require ATP to process these hairpin RNA substrates.

3.2. Inorganic phosphate inhibits Dicer-2, but not Dicer-2 + Loqs-PD, from processing blunt-end hairpin RNAs

In the presence of ATP, the RNA processing rates differed among Dicer-2 ± Loqs-PD ± inorganic phosphate (Fig. 2). To better compare processing rates among the different conditions, using the time course data (Fig. 2), I determined initial velocities of the processing reactions (Fig. 3).

Inorganic phosphate inhibited Dicer-2 from cleaving RNA1 with a 5′ monophosphorylated blunt end (0.21 ± 0.01 min^{-1} vs 0.10 ± 0.01 min^{-1}. P-value = 0.000091). Interestingly, however, inorganic phosphate did not inhibit Dicer-2 + Loqs-PD from cleaving RNA1 (0.24 ± 0.05 min^{-1} vs 0.25 ± 0.04 min^{-1}. P-value = 0.85). Similarly, inorganic phosphate inhibited Dicer-2 (0.13 ± 0.02 min^{-1} vs 0.06 ± 0.02 min^{-1}. P-value = 0.014), but not Dicer-2 +Loqs-PD (0.21 ± 0.02 min^{-1} vs 0.17 ± 0.03 min^{-1}. P-value = 0.18) from cleaving RNA2 with a 5′ hydroxyl blunt end. Thus, Loqs-PD enables Dicer-2 to cleave blunt-end hairpin RNAs even in the presence of inorganic phosphate.

Inorganic phosphate inhibits both Dicer-2 and Dicer-2 + Loqs-PD from processing 3′ overhang-end hairpin RNAs.

Unlike RNAs 1 and 2, inorganic phosphate inhibited both Dicer-2 (0.17 ± 0.03 min^{-1} vs 0.04 ± 0.01 min^{-1}. P-value = 0.0028) and Dicer-2 + Loqs-PD (0.24 ± 0.05 min^{-1} vs 0.11 ± 0.01 min^{-1}. P-value = 0.0083) from cleaving RNA3 with a 5′ monophosphorylated, 3′ overhang end. Inorganic phosphate also inhibited Dicer-2 + Loqs-PD from cleaving RNA4 with a 5′ hydroxyl, 3′ overhang end (0.11 ± 0.02 min^{-1} vs 0.05 ± 0.01 min^{-1}. P-value = 0.0063). Inorganic phosphate also showed a trend to inhibit Dicer-2 from cleaving RNA4 while it did not reach a statistical significance (0.08 ± 0.02 min^{-1} vs 0.03 ± 0.03 min^{-1}. P-value = 0.064). Thus, unlike blunt-end hairpin RNAs, Loqs-PD cannot enable Dicer-2 to cleave 3′ overhang-end hairpin RNAs in the presence of inorganic phosphate.

4. Discussion

My studies here show that Loqs-PD enables Dicer-2 to cleave blunt-end hairpin RNAs in the presence of inorganic phosphate. Similarly, previous studies showed that Loqs-PD enables Dicer-2 to cleave RNA substrates normally refractory to cleavage, such as dsRNA with blocked, structured, or frayed ends [15,19].

Based on the results in this study and past studies, I propose a model of how Loqs-PD enables Dicer-2 to process blunt-end hairpin RNAs in the presence of inorganic phosphate (Fig. 4). Previous biochemical and structural studies revealed that Dicer-2 processes blunt-end RNA substrates and 3′ overhang-end RNA substrates differently [9,12,15,18,19]. Blunt-end hairpin RNA substrates are bound by the Dicer-2 helicase domain (Fig. 4A, top). The blunt-end hairpin RNA substrates are threaded through the Dicer-2 helicase domain until their ends reach the Dicer-2 PAZ domain. Then the blunt-end hairpin RNA substrates are cleaved by the Dicer-2 RNase III active sites. In contrast, 3′ overhang-end hairpin RNA substrates are bound by the Dicer-2 PAZ domain and then the 3′ overhang-end hairpin RNAs are aligned to the RNase III active sites for cleavage (Fig. 4A, bottom).

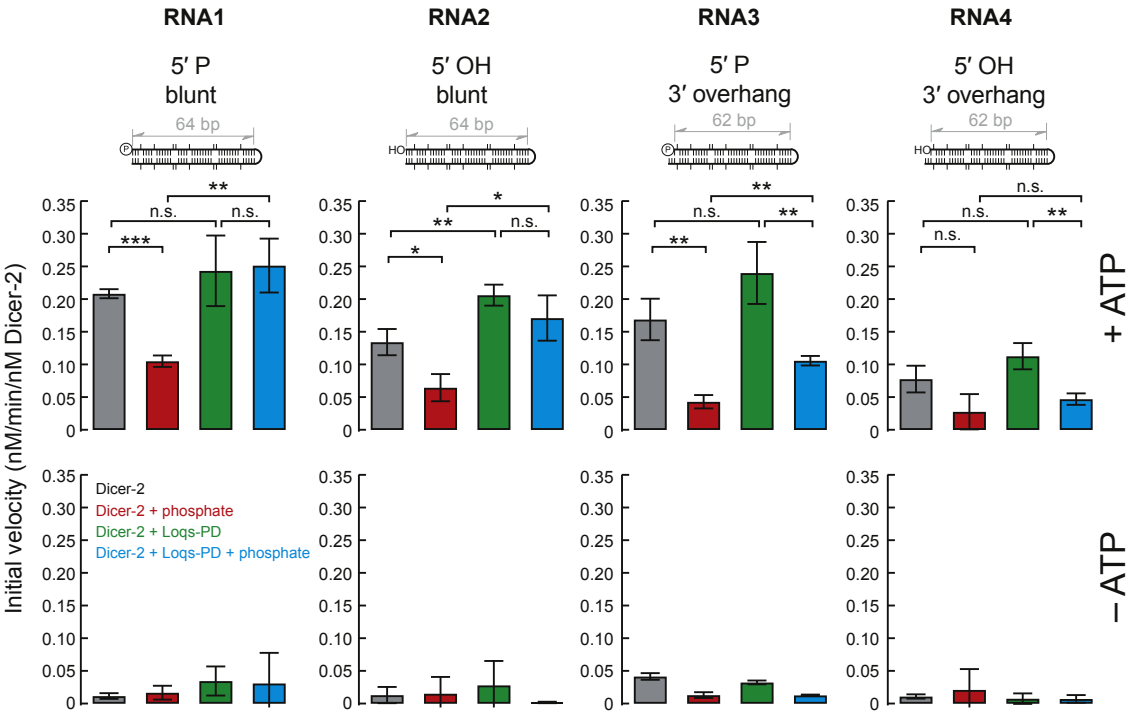

Fig. 3. Initial velocities in the in vitro processing assay of hairpin RNAs by Dicer-2 ± Loqs-PD.
Initial velocities determined by the time course assay results shown in Fig. 2. Data are mean ± s.d. for three independent trials. P-value <0.05, <0.01, and <0.001 are indicated by *, **, and ***, respectively (Student's t-test).

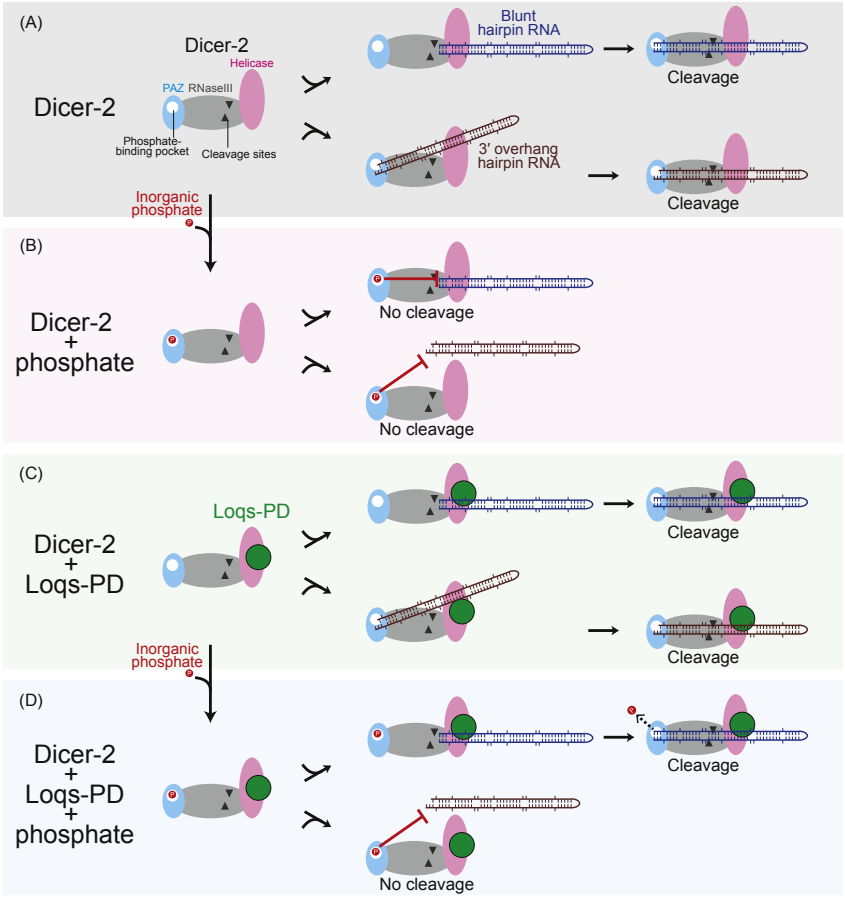

Fig. 4. Model.
(A–D) Models how inorganic phosphate inhibits Dicer-2 from processing hairpin RNAs and how Loqs-PD removes this inhibitory effect. Models are shown for (A) Dicer-2 alone, (B) Dicer-2 + phosphate, (C) Dicer-2 + Loqs-PD, and (D) Dicer-2 + phosphate + Loqs-PD. The background colors of the panels (A–D) correspond to the colors used in Fig. 2 and 3. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

In vivo at a physiological concentration of inorganic phosphate, inorganic phosphate binds the phosphate-binding pocket in the Dicer-2 PAZ domain and inhibits access of RNA substrates to the PAZ domain, inhibiting their cleavage (Fig. 4B) [18]. The inhibition occurs to both blunt-end hairpin RNAs and 3′ overhang-end hairpin RNAs.

Loqs-PD binds the Dicer-2 helicase domain [14,15]. A blunt-end long dsRNA substrate and a 3′ overhang-end long dsRNA substrate are bound by the Dicer-2 helicase domain differently [12]. Therefore, Loqs-PD bound to the Dicer-2 helicase domain is also expected to interact blunt-end hairpin RNA substrates and 3′ overhang-end hairpin RNA substrates differently (Fig. 4C).

Loqs-PD increases the binding affinity of a blunt-end long dsRNA substrate to Dicer-2 [15]. The binding affinity increase of hairpin RNA substrates by Loqs-PD allows blunt-end hairpin RNAs to displace a bound inorganic phosphate from the binding pocket in the Dicer-2 PAZ domain, enabling efficient cleavage of the blunt-end hairpin RNAs (Fig. 4D, top). In contrast, due to a different binding mode of Loqs-PD between blunt-end hairpin RNAs and 3′ overhang-end hairpin RNAs, Loqs-PD cannot help 3′ overhang-end hairpin RNAs to displace a bound inorganic phosphate from the Dicer-2 PAZ domain (Fig. 4D, bottom). Thus, Loqs-PD enables Dicer-2 to process blunt-end hairpin RNAs, but not 3′ overhang-end hairpin RNAs, in the presence of inorganic phosphate.

Loqs-PD is required for efficient production of a subset of siRNAs in vivo [7,16,17]. Different RNA substrate end structures may determine if Loqs-PD can enable Dicer-2 to process substrate RNAs in the presence of inorganic phosphate. For some RNA substrates, including blunt-end hairpin RNAs, processing by Dicer-2 is inhibited by phosphate while processing by Dicer-2 + Loqs-PD is not. Such RNA substrates require Loqs-PD for siRNA production. In contrast, for other RNA substrates, phosphate does not inhibit Dicer-2 from processing. Such RNA substrates do not require Loqs-PD for siRNA production.

This may explain partially their different dependencies on Loqs-PD in siRNA production in vivo.

Regulation of Dicer activities by inorganic phosphate and its modulation by Loqs-PD orthologue may be widely conserved. Inorganic phosphate activates *Arabidopsis* Dicer-like 3 (DCL3) activity while it inhibits *Arabidopsis* Dicer-like 4 (DCL4) activity to process a 3′ overhang-end 50 nt dsRNA into siRNAs [22]. Phosphate deficiency in *Arabidopsis* seedlings inhibits DCL3 activity in a cell-free extract assay system [23]. DCL3 activity in these phosphate-deficient seedling extract is activated directly by adding inorganic phosphate in the cell-free assay system [23]. DCL3 binds a dsRNA-binding (DRB) protein cofactor DRB3 [24] while DCL4 binds DRB4, both of which are *Drosophila* Loqs-PD orthologues [25]. These *Arabidopsis* Dicer cofactor DRB proteins may modulate the effects of inorganic phosphate on the DCL3 and DCL4 activities. Similarly, inorganic phosphate may regulate human Dicer activity as well considering that crystal structures showed that inorganic phosphate binds the human Dicer PAZ domain [26]. Human Dicer cofactor dsRNA-binding proteins TRBP and PACT (Loqs orthologues) may modulate effects of inorganic phosphate on human Dicer activity to generate small silencing RNAs.

Author contributions

Conceptualization, Methodology, Investigation, Writing, Funding Acquisition, R.F.

Competing interests

The author has no conflicts of interest to declare.

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