Biochemical and Biophysical Research Communications 498 (2018) 1022–1027

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

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

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ARTICLE INFO

Article history:
Received 9 March 2018
Accepted 13 March 2018
Available online 16 March 2018

Keywords:
Dicer-2
Loquacious
siRNA
Inorganic phosphate
dsRNA
RNA silencing

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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https://doi.org/10.1016/j.bbrc.2018.03.108
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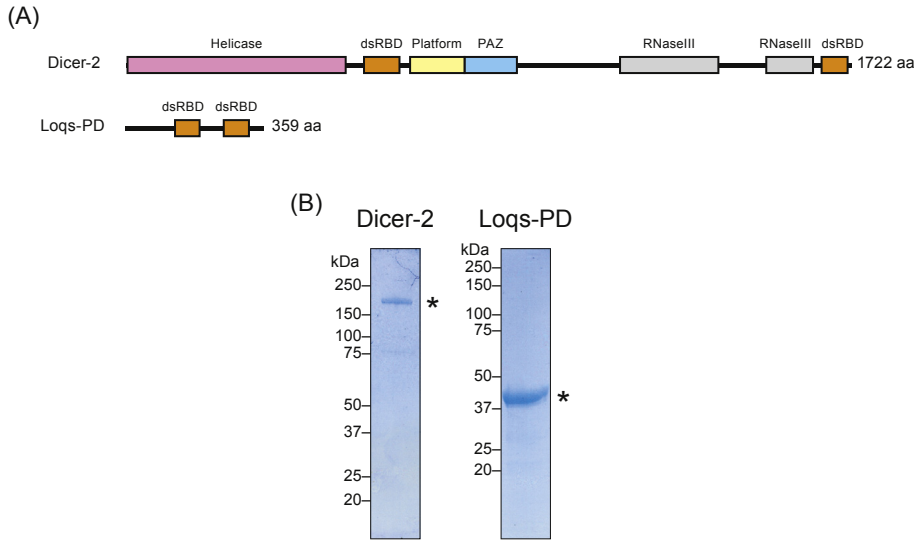

Fig. 1. Recombinant proteins of Drosophila Dicer-2 and Loqs-PD.
(A) Domain structures of *Drosophila* Dicer-2 and Loqs-PD.
(B) Coomassie-stained SDS-PAGE gels of purified recombinant Dicer-2 and Loqs-PD proteins.

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