Functional Anatomy of the *Drosophila* MicroRNA-generating Enzyme

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**Abstract**

In *Drosophila melanogaster*, the multidomain RNase III Dicer-1 (Dcr-1) functions in tandem with the double-stranded (ds)RNA-binding protein Loquacious (Loqs) to catalyze the maturation of microRNAs (miRNAs) from precursor (pre)-miRNAs. Here we dissect the molecular mechanism of pre-miRNA processing by the Dcr-1-Loqs complex. The tandem RNase III (RIII) domains of Dcr-1 form an intramolecular dimer such that one RIII domain cleaves the 3′ strand, whereas the other cuts the 5′ strand of pre-miRNA. We show that the functional core of Dcr-1 consists of a DUF283 domain, a PAZ domain, and two RIII domains. Dcr-1 preferentially associates with the Loqs-PB splice isoform. Loqs-PB uses the second dsRNA-binding domain to bind pre-miRNA and the third dsRNA-binding domain to interact with Dcr-1. Both domains of Loqs-PB are required for efficient miRNA production by enhancing the affinity of Dcr-1 for pre-miRNA. Thus, our results provide further insights into the functional anatomy of the *Drosophila* miRNA-generating enzyme.

RNA interference is a post-transcriptional gene silencing mechanism mediated by microRNAs (miRNAs) and small interfering RNAs (siRNAs). In the initiation step, siRNAs and miRNAs are generated respectively from mostly exogenous long double-stranded RNA (dsRNA) and endogenous short hairpin pre-miRNA. In the effector step, nascent siRNA and miRNA are assembled into similar RNA-induced silencing complexes termed siRISC and miRISC, respectively. In RISCs, a single-stranded siRNA or miRNA functions as the guide RNA to direct sequence-specific degradation and/or translational repression of cognate mRNA.

Biogenesis of miRNAs involves two RNome III enzymes: Drosha and Dicer. In the nucleus, the primary transcript (pri-miRNA) of a miRNA gene is processed by Drosha into ~60-nucleotide stem-loop pre-miRNA. Drosha requires the assistance of a dsRNA-binding protein, known as Pasha or DGCR8, to accurately process pri-miRNA to pre-miRNA. The pre-miRNA is then exported by Exportin 5 to the cytoplasm, where it is further cleaved by Dicer into miRNA.

Dicer is a conserved family of ~200-kDa multidomain RNase III (RIII) enzymes. The canonical prokaryotic RNase III contains a single RIII domain and functions as a homodimer. In contrast, a typical eukaryotic Dicer consists of a DExH helicase domain, a domain of unknown function (DUF)283, and a PAZ domain at the N terminus as well as two RIII domains and a dsRNA-binding domain (dsRBD) at the C terminus. Recent biochemical studies have shown that human Dicer operates as a monomer and that its tandem RIII domains form one processing center and cleave the opposite strand of dsRNA. This model is supported by the x-ray crystal structure of Dicer (Protein Data Base number 2FFL) from the human parasite *Giardia intestinalis*. The primitive *Giardia* Dicer only carries a PAZ domain followed by tandem RIII domains. As Dicer resembles the shape of a hatchet, the two RIII domains form the blade as an intramolecular dimer that is similar to the homodimeric structure of prokaryotic RNome III.

In *Drosophila melanogaster*, two Dicer enzymes, Dcr-1 and Dcr-2, are responsible for miRNA and siRNA production, respectively (8, 21, 24). Despite extensive sequence homology, Dcr-1 and Dcr-2 display distinct substrate specificities and ATP requirements (8). Dcr-1 prefers to process pre-miRNA to miRNA in an ATP-independent manner. Dcr-2 is much better at processing dsRNA and requires ATP hydrolysis for efficient siRNA production (8). By contrast, human and most other organisms contain a single Dicer that generates both miRNAs and siRNAs.

Typically, Dicer functions in tandem with a specific dsRNA-binding protein (5). For example, Dcr-2 and R2D2 constitute the *Drosophila* siRNA-generating enzyme (24). R2D2 contains two dsRBD domains and forms a heterodimeric complex with Dcr-2 (24). Although R2D2 does not regulate siRNA production, it cooperates with Dcr-2 to promote assembly of the effector siRISC complexes (24–26). Only the Dcr-2-R2D2 complex, neither Dcr-2 nor R2D2 alone, could efficiently interact with duplex siRNA. Both Dcr-2 and R2D2 are critical components of the RISC loading complex, the formation of which precedes and is required for RISC activation (27–29).
**Functional Anatomy of Dcr-1-Loqs Complex**

Loqacious (Loqs) functions as a co-factor for Dcr-1 in the *Drosophila* miRNA pathway (8, 30, 31). The *loqs* gene produces alternative spliced transcripts encoding three protein isoforms (PA-PC). All three isoforms are expressed in S2 cells, whereas only PA and PB are expressed in fly tissues (30). Both Loqs-PA and Loqs-PB carry three dsRBD domains (8, 30). Loqs-PB is slightly larger than Loqs-PA by 46 amino acids. As shown by biochemical fractionation of S2 extracts, Dcr-1 and Loqs-PB, but not Loqs-PA or Loqs-PC, correlate perfectly with the miRNA-generating activity (8). Although recombinant Dcr-1 is able to process pre-miRNA to miRNA, Loqs-PB greatly enhances miRNA production by increasing the affinity of Dcr-1 for pre-miRNA (8). Similar to Loqs, TRBP and PACT contain three dsRBD domains and have recently been identified as two dsRNA-binding proteins for human Dicer. In the current study, we performed detailed domain structure and functional analyses to dissect the molecular mechanism of pre-miRNA processing by the *Drosophila* miRNA-generating (Dcr-1-Loqs) enzyme.

**EXPERIMENTAL PROCEDURES**

**Cloning and Expression of Recombinant Protein—**All truncated constructs were generated by PCR and subcloning using full-length Dcr-1, Loqs-PA, and Loqs-PB cDNA as templates (8). The catalytic mutants of Dcr-1 were constructed by using “QuiChange” (Stratagene). Recombinant His- or FLAG-tagged full-length or truncated Dcr-1-Loqs proteins were produced in insect cells using the BAC-to-BAC baculovirus expression system (Invitrogen). Large scale productions of recombinant Dcr-1 proteins were conducted as described previously (8, 24). For interaction studies in insect cells, 200 μl of each virus was added to 10 ml of 10° cells/ml SF21 cells in a T25 flask. Cell extracts were prepared in the lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) 40 h after viral infection. For interaction studies in S2 cells, expression constructs for full-length or truncated Myc-Dcr-1 and GFP-Loqs were generated by the “Gateway” system (Invitrogen). Transient transfections of S2 cells were conducted using Cellfectin (Invitrogen). Cell lysates were prepared 40 h after transfection.

**Antibodies and Immunoprecipitation (IP)—**Mouse monoclonal anti-Dcr-1 and anti-Loqs antibodies were generated against purified recombinant Dcr-1-Loqs-PB complex. The polyclonal anti-GFP antibodies were purchased from Invitrogen, whereas monoclonal anti-FLAG (M2), anti-Myc (9E10), and anti-His (HIS-1) antibodies were from Sigma. For co-IPs, ~2 mg of cell extract was incubated with 5 μl of antibodies at 4 °C for 2 h followed by the addition of 10 μl pre-washed protein A beads (Santa Cruz Biotechnology). After rotating at 4 °C overnight, the beads were washed six times with the IP wash buffer (110 mM potassium acetate, 10 mM HEPES, pH 7.4, 100 mM NaCl, 2 mM Mg(OAc)2, 5 mM dithiothreitol, and 1% Nonidet P-40) and boiled in 2× SDS sample buffer for 5 min.

**Sedimentation Analysis—**In parallel, 300 μl (10 μg/μl) of S100 extract of S2 cells and 50 μM purified recombinant Dcr-1 or Dcr-1-Loqs-PB complex were applied onto a 3-ml 10–30% glycerol gradient. The contents were centrifuged at 55,000 rpm at 4 °C for 3.5 h using an SW60Ti rotor (Beckman). Fourteen fractions (250 μl/fraction) were taken from the top of the tube and subsequently used for Western blotting to detect the presence of Dcr-1.

**The Pre-miRNA Processing Assay—**Synthetic pre-let-7 and pre-bantam RNA (Dharmacon) were radiolabeled at the 5’ end by T4 polynucleotide kinase (New England Biochemicals) and [γ-32P] ATP (Valent Pharmaceuticals) or at the 3’ end by T4 RNA ligase (Ambion) and [α-32P] pCp (Amersham Biosciences) followed by PAGE purification. Typically, 5 × 104 cpm pre-miRNA was incubated with recombinant Dcr-1 enzymes at 30 °C for 30 min in 10-μl reactions (25). For the kinetic studies (see Fig. 3g), 2.5 × 105 cpm (~0.2 pmol) pre-miRNA was incubated with 0.2 pmol of recombinant Dcr-1 enzymes.

**The Pre-miRNA Gel-shift Assay—**Recombinant Dcr-1-Loqs-PB proteins were incubated with 5 × 105 cpm of 5’-radio-labeled pre-miRNA at 30 °C for 30 min in the same buffer as the processing assay. The reaction mixtures were resolved on a 6% native PAGE as described previously (8).

**The UV Cross-linking Assay—**The 20-μl pre-miRNA gel-shift reactions were performed as described above followed by exposure to UV light for 20 min in a 96-well dish. After adding 5 μl of 4× SDS sample buffer, the mixture was boiled for 5 min, resolved on a 4–20% SDS-PAGE, and transferred to cellulose membrane followed by autoradiography.

**RESULTS**

**Two RNase III Domains of Dcr-1 Form an Intramolecular Dimer—**Previous studies have revealed that the two RIII domains of human Dicer form an intramolecular dimer and cleave the opposite strands of dsRNA (22). To determine whether *Drosophila* Dcr-1 had a similar action mechanism, we generated a series of catalytic mutant Dcr-1 enzymes. Based on the sequence alignment, Asp-1749 and Glu-1908 of RIIIa and Asp-2036 and Glu-2139 of RIIIb were predicted to be the corresponding catalytic residues of Dcr-1 (Fig. 1a). We inactivated either or both of the RIII domains of Dcr-1 by changing these residues to alanine by site-directed mutagenesis. Wild type and mutant His-tagged Dcr-1 recombinant proteins were produced using an insect cell expression system and highly purified by Ni2+ affinity column followed by Q-Sepharose and SP-Sepharose chromatography (8).

We compared the cleavage patterns of wild type and various catalytic mutant Dcr-1 enzymes by *in vitro* pre-miRNA-processing assays using either a 5’-radio-labeled or a 3’-radio-labeled substrate. Mutations of both RIII domains (E1908A/E2139) completely abolished the ability of Dcr-1 to process pre-miRNA (Fig. 1b, compare lane 1 and lane 6). Interestingly, the RIIla (D1749A or E1908A) mutants could only cleave the 5’ (top) strand, but not the 3’ (bottom) strand, of pre-miRNA (Fig. 1b, compare lane 1 and lanes 2 and 3). Conversely, the RIIlb (D2036A or E2139A) mutants were able to cut the 3’ strand, but not the 5’ strand, of pre-miRNA (Fig. 1b, compare lane 1 and lanes 4 and 5). The mirroring cleavage patterns indicate that 5’-RIIla cleaves the 3’ strand, whereas 3’-RIIlb cuts the 5’ strand of pre-miRNA. Thus, like human Dicer, the tandem RIII domains of Dcr-1 form one processing center. The staggered pair of cuts made by RIIla and RIIlb excises miRNA from pre-
miRNA and creates a characteristic two-nucleotide 3' overhang terminus (Fig. 1c).

**Dcr-1 Exists as a Monomer**—We characterized the sizes of the endogenous and recombinant Dcr-1 proteins by sedimentation analysis. On a 10–30% glycerol gradient, the Dcr-1 protein in S2 extracts fractionated with a peak of ~400 kDa (Fig. 2). The data suggested that the bulk of endogenous Dcr-1 existed as a monomeric form and in complex with other cellular components, such as Loqs and Ago1 (8, 30). At an equivalent concentration, recombinant Dcr-1 and Dcr-1-Loqs-PB heterodimer migrated at positions that were consistent with a Dcr-1 monomer and a Dcr-1-Loqs-PB heterodimer (Fig. 2). Together, these results indicate that Dcr-1 functions as a monomer at physiological condition.

**Pre-miRNA Processing by Truncated Dcr-1**—Dcr-1 is a multidomain RNase III enzyme that contains a helicase domain, a DUF283 domain, a PAZ domain, two RIII domains, and a dsRBD domain. To determine which of these domains were critical for Dcr-1 function, we generated a series of truncated (T1–T6) Dcr-1 and compared their activities to that of wild type enzyme by pre-miRNA-processing assays (Fig. 3, a and b). As shown in Fig. 3, c and d, removal of the DUF283 domain (T2) and/or the PAZ domain (T3) completely abolished the ability of Dcr-1 to generate miRNAs. Similar results were obtained whether using a pre-let-7 (miRNA located at the 5' strand) (Fig. 3c) or pre-bantam (miRNA at the 3' strand) substrate (Fig. 3d). In contrast, Dcr-1 could efficiently process pre-miRNA to miRNA without the N-terminal helicase domain (T1) or the C-terminal dsRBD domain (T5). Intriguingly, deletion of both domains (T6) still resulted in a functional Dcr-1 enzyme (Fig. 3, e and f). Further kinetic studies revealed that T1, T5, and T6 retained ~38, 61, or 15% of the miRNA-generating activity of a wild type Dcr-1 enzyme (Fig. 3g).

**Dcr-1 Preferentially Associates with Loqs-PB**—As shown by coimmunoprecipitation (co-IP) experiments, FLAG-tagged Dcr-1 could form a complex with either His-tagged Loqs-PA or His-tagged Loqs-PB in insect cells following co-infection of baculoviruses (Fig. 4a). Intriguingly, when insect cells were co-infected with all three viruses, Dcr-1 associated Loqs-PB almost exclusively, although Loqs-PA and Loqs-PB were expressed at equivalent levels (Fig. 4a). This result suggests that the two Loqs isoforms compete for Dcr-1 binding and that Dcr-1 may have higher affinity for Loqs-PB than Loqs-PA.

We further examined the association between the endogenous Dcr-1 and Loqs proteins by co-IPs using anti-Dcr-1 and anti-Loqs monoclonal antibodies. Although anti-Loqs antibodies brought down all three Loqs (PA, PB, PC) proteins from S2 extracts, Loqs-PB was the predominant isoform detected in the IPs of anti-Dcr-1 antibodies (Fig. 4b). This was consistent with our previous observation that Dcr-1 and Loqs-PB, but not Loqs-PA or Loqs-PC, correlated perfectly with the miRNA-generating activity (8). Together, these biochemical results indicate that Dcr-1 and Loqs-PB constitute the miRNA-generating enzyme.

**Interaction Domains between Dcr-1 and Loqs**—To determine how Dcr-1 interacted with Loqs, we examined the interaction between FLAG-Loqs-PB and various His-tagged truncated...
Dcr-1 proteins in insect cells (Fig. 3 a). Following co-infection of the Dcr-1-Loqs baculoviruses, IPs were performed with anti-FLAG antibodies followed by Western blotting with anti-His antibodies. As shown in Fig. 5a, Loqs-PB was unable to interact with T1, T2, or T3 of Dcr-1, all of which lacked the N-terminal helicase domain. Conversely, Loqs-PB interacted strongly with the full-length, T4, or T5 of Dcr-1 that shared the helicase domain in common. These results indicate that Loqs interacts with the helicase domain of Dcr-1.

Next, we generated four truncated Loqs-PB constructs to map the region of Loqs that interacted with Dcr-1 (Fig. 5b). We performed co-IP experiments to examine the interaction between Myc-tagged helicase domain (Myc-helicase) of Dcr-1 and GFP-tagged truncated Loqs (L1–L4) in S2 cells after co-transfection of the Dcr-1-Loqs expression constructs. The anti-GFP antibodies specifically brought down Myc-helicase only in the presence of L3 (dsRBD2-dsRBD3) or L4 (dsRBD3), but not L1 (dsRBD1-dsRBD2) or L2 (dsRBD2) (Fig. 5c). Therefore, the Dcr-1-Loqs-PB association involves the N-terminal helicase domain of Dcr-1 and the C-terminal dsRBD (dsRBD3) of Loqs.

Loqs-PB is larger than Loqs-PA by 46 amino acids that immediately precede the C-terminal dsRBD domain. To determine whether these 46 residues were responsible for high affinity binding between Loqs-PB and Dcr-1, we generated the L5 construct by specifically removing the 46 residues from L4 (Fig. 5d). As shown in Fig. 5d, full-length Myc-Dcr-1 could interact individually with either GFP-L4 or GFP-L5, which represented the minimal Dcr-1 interaction domains of Loqs-PB and Loqs-PA, respectively. However, Myc-Dcr-1 preferentially associated with GFP-L4 over GFP-L5 when all three constructs were co-transfected into S2 cells. Thus, the miniature L4 and L5 constructs recapitulate the difference in Dcr-1 affinity between Loqs-PB and Loqs-PA.

Loqs-PB Requires Its Second and Third dsRBDs for Dcr-1 Regulation—Although Dcr-1 alone is able to process pre-miRNA to miRNA, Loqs-PB greatly enhances miRNA production by increasing the affinity of Dcr-1 for pre-miRNA (8). To determine which portions of Loqs-PB were critical for Dcr-1 regulation, we made recombinant full-length and various truncated Loqs-PB proteins (Fig. 6a) and compared their abilities to enhance Dcr-1 activity in the pre-miRNA-processing assay (Fig. 6b). As expected, L1 and L2 did not affect miRNA production because they lacked the third dsRBD domain that interacted with Dcr-1. Although L4 (dsRBD3) interacted with Dcr-1, it was unable to increase miRNA production. Only L3.
In parallel, native gel-shift assays were conducted to compare the premiRNA affinity of recombinant Dcr-1 in the absence or presence of full-length and truncated Loqs-PB (8). In these assays, we employed the catalytic mutant (E1908A/E2139) Dcr-1 to prevent the cleavage of radiolabeled pre-miRNA. As shown in Fig. 6c, neither L1 nor L2 could increase the binding of Dcr-1 to pre-miRNA. On the other hand, L4 modestly increased, whereas L3 greatly enhanced the binding of Dcr-1 to pre-miRNA.

We performed photocross-linking experiments to examine the physical interaction between pre-miRNA and recombinant Dcr-1-Loqs-PB proteins. As shown in Fig. 6, d and e, both Dcr-1 and Loqs-PB were efficiently cross-linked to radiolabeled pre-miRNA after exposure to ultraviolet light. In presence of Dcr-1, only L3 (dsRBD2-dsRBD3), but not L4 (dsRBD3), could be cross-linked to pre-miRNA.

Furthermore, we specifically inactivated the dsRBD2 of Loqs in L3 by mutating two conserved alanines (Ala-308, Ala-309) to lysine residues as described previously for R2D2 (24). These point mutations abolished the ability of L3 to either interact with pre-miRNA or enhance miRNA production (Fig. 6, d–f). Together, these results indicate that Loqs-PB uses its dsRBD2 to bind pre-miRNA and dsRBD3 to interact with Dcr-1. Both domains of Loqs-PB are required for its ability to regulate Dcr-1 activity.

DISCUSSION

The primitive Giardia Dicer carries only the PAZ domain and two RIII domains but displays robust dsRNA-processing activity (23). Here we show that Drosophila Dcr-1 can efficiently process pre-miRNA to miRNA without the N-terminal helicase and the C-terminal dsRBD domains. By contrast, removal of the PAZ and/or the DUF283 domains completely abolished the ability of Dcr-1 to generate miRNA. A similar phenomenon has been observed for human Dicer (32). Thus, we propose that the functional core of eukaryotic Dicer consists of a DUF283 domain, a PAZ domain, and two RIII domains.
Both Loqs-PA and Loqs-PB use the third dsRBD domain to interact with the helicase domain of Dcr-1. However, Dcr-1 preferentially associates with Loqs-PB rather than Loqs-PA when both isoforms are co-expressed. As shown by biochemical fractionation, Dcr-1 and Loqs-PB, but not Loqs-PA, correlate perfectly with the miRNA-generating activity. Furthermore, transgenic expression of Loqs-PB, but not Loqs-PA, can rescue both developmental and miRNA-processing defects of homozygous loqs knock-out flies (33). Thus, the Loqs-PB, but not Loqs-PA, isoform is necessary and sufficient for Drosophila development and the miRNA pathway (33). Taken together, our previous and current studies demonstrate that the Dcr-1-Loqs-PB and Dcr-2-R2D2 complexes catalyze Drosophila miRNA and siRNA biogenesis, respectively (8, 24).

Although R2D2 does not regulate the siRNA production of Dcr-2, Loqs-PB greatly enhances miRNA production by increasing the affinity of Dcr-1 for pre-miRNA (8). The current study suggests a simple model of pre-miRNA processing by the Dcr-1-Loqs complex. On its own, Dcr-1 is able to generate miRNA. The minimal requirements for Dcr-1 to process pre-miRNA include a DUF283 domain and a central PAZ domain followed by two RII domains. The PAZ domain is believed to interact with the terminus of pre-miRNA stem. The DUF283 domain is a dsRNA-binding domain (34) and may be critical for the binding or processing of pre-miRNA. The tandem RII domains form an intramolecular dimer such that RIIa cleaves the 3’ strand, whereas RIIb cleaves the 5’ strand of pre-miRNA. The pair of cuts creates a two-nucleotide 3’ overhang and excises miRNA from pre-miRNA.

On the other hand, Loqs-PB uses the C-terminal dsRBD3 to interact with the N-terminal helicase domain of Dcr-1. The middle dsRBD2 of Loqs-PB is responsible for direct binding to pre-miRNA. Interestingly, although L4 (dsRBD3) of Loqs-PB does not physically contact pre-miRNA, it modestly enhances the binding of Dcr-1 to pre-miRNA. One possible explanation is that the binding of dsRBD3 to Dcr-1 triggers a conformational change in Dcr-1, thereby increasing its affinity for pre-miRNA. These specific interactions greatly enhance the miRNA-generating activity of Dcr-1, possibly by strengthening the interaction between Dcr-1 and pre-miRNA. Additionally, they may organize the enzyme/substrate in a proper conformation for efficient processing. Our biochemical studies provide further insights into the functional anatomy of the Drosophila miRNA-generating enzyme. The results may help understand the process of miRNA biogenesis in other model organisms.

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