Dicer-1, but not Loquacious, is critical for assembly of miRNA-induced silencing complexes

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ABSTRACT

Double-stranded RNA-binding proteins (dsRBPs), such as R2D2 and Loquacious (Loqs), function in tandem with Dicer (Dcr) enzymes in RNA interference (RNAi). In *Drosophila*, Dcr-1/Loqs and Dcr-2/R2D2 complexes generate microRNAs (miRNAs) and small interfering RNAs (siRNAs), respectively. Although R2D2 does not regulate siRNA production, R2D2 and Dcr-2 coordinately bind siRNAs to promote assembly of the siRNA-induced silencing (siRISC) complexes. Conversely, Loqs enhances miRNA production. It is uncertain if Dcr-1 and Loqs facilitate miRNA loading onto the miRISC complexes. Here we used *loqs* knockout (KO) flies to characterize the physiological functions of Loqs in the miRNA pathway. Northern analysis revealed consistent accumulation of precursor (pre)-miRNAs in *loqs*^{KO} flies. However, the lack of Loqs had differential effects on mature miRNAs: some are diminished, whereas others maintain wild-type levels. Importantly, the data suggest that miRNA production is not the rate-limiting step of the miRNA pathway. We show that Dcr-1, but not Loqs, is critical for assembly of miRISCs by using *dcr-1* or *loqs* null egg extract. Consistent with this, recombinant Dcr-1 could efficiently interact with miRNA duplex in the absence of Loqs. Together, our results indicate that Loqs plays a prominent role in miRNA biogenesis, but is largely dispensable for miRISC assembly. Thus, Loqs and R2D2 represent two distinct functional modes for dsRBPs in the RNAi pathways.

Keywords: Dcr-1; Loqs; pre-miRNA; miRNA; miRISC

INTRODUCTION

RNA interference (RNAi) is a post-transcriptional gene silencing mechanism guided by 21- to 25-nucleotide(nt) small interfering RNAs (siRNAs) and microRNAs (miRNAs) (Fire et al. 1998; Hannon 2002; Tomari and Zamore 2005; Paroo et al. 2007). The catalytic engine of RNAi consists of two enzymes, Dicer and Argonaute (Ago) (Paroo et al. 2007). Dicer, a multidomain RNase III enzyme, processes long double-stranded (ds)RNA to siRNA or short stem-loop precursor (pre)-miRNA to miRNA (Bernstein et al. 2001; Hutvagner et al. 2001). Ago, in conjunction with a siRNA or miRNA, forms the catalytic core of the RNA-induced silencing complexes termed siRISC or miRISC, which directs sequence-specific cleavage and/or translational repression of complementary mRNA (Liu et al. 2004; Song et al. 2004; Rivas et al. 2005).

A typical Dicer is comprised of a DExH helicase domain, a domain of unknown function (DUF)283, a PAZ domain, two RNase III domains, and a dsRNA-binding (dsRBD) domain (Bernstein et al. 2001). Most organisms, including humans, contain a single Dicer that generates both miRNAs and siRNAs. In *Drosophila*, two Dicers, Dcr-1 and Dcr-2, catalyze miRNA and siRNA production, respectively (Liu et al. 2003; Lee et al. 2004). Biochemical studies establish that Dcr-1 and Dcr-2 enzymes have distinct substrate specificities and ATP requirements (Liu et al. 2003; Jiang et al. 2005). Dcr-1 prefers to process pre-miRNA to miRNA in an ATP-independent manner, whereas Dcr-2 is much more efficient at processing dsRNA and requires ATP hydrolysis for efficient siRNA production (Liu et al. 2003; Jiang et al. 2005).

The Ago family of proteins contains two signature domains, PAZ and PIWI. The PAZ domain of Ago has an oligonucleotide-binding (OB) fold and is believed to bind the 2-nt 3' overhang of duplex siRNA or miRNA (Lingel et al. 2003; Song et al. 2003; Yan et al. 2003; Ma et al. 2004). The PIWI domain resembles the structure of RNase H and functions as the slicer of RISC that cleaves the target mRNA (Song et al. 2003, 2004; Liu et al. 2004;

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Rivas et al. 2005). However, not all Ago proteins possess the slicer activity. Among the four human Ago proteins, the slicer activity has only been demonstrated for Ago2 (Liu et al. 2004; Meister et al. 2004). In *Drosophila*, Ago1 and Ago2 both possess slicer activity and serve, respectively, as the catalytic core of the miRISC and siRISC complexes (Miyoshi et al. 2005).

It is unclear how siRNA or miRNA is transferred from Dicer to Ago to form the effector RISC complexes. A novel component of RNAi has been revealed by purifying *Drosophila* siRNA-generating enzyme, which consists of Dcr-2 and R2D2 (Liu et al. 2003). R2D2 is named because of its two dsRBD domains (R2) and association with Dcr-2 (D2). However, R2D2 does not regulate siRNA production (Liu et al. 2003, 2006). Rather, R2D2 cooperates with Dcr-2 to bind siRNA and facilitate siRNA loading onto the effector siRISC complexes (Liu et al. 2003, 2006). Neither Dcr-2 nor R2D2 alone could efficiently interact with duplex siRNA (Liu et al. 2006). Both Dcr-2 and R2D2 are critical components of the RISC loading complex (RLC), the formation of which precedes and is required for RISC activation (Pham et al. 2004; Tomari et al. 2004a; Pham and Sontheimer 2005). R2D2 is a homolog of the previously discovered RDE-4, which also contains two dsRBDs, associates with Dicer and RDE-1 (Ago2 homolog), and is required for RNAi in *Caenorhabditis elegans* (Tabara et al. 2002). RDE-4 and R2D2 are two founding members of a growing family of dsRBPs that play important roles in the siRNA and miRNA pathways.

Another dsRBP, Loquacious (Loqs), functions in tandem with Dcr-1 in the *Drosophila* miRNA pathway (Forstemann et al. 2005; Jiang et al. 2005; Saito et al. 2005). The *loqs* gene encodes three protein isoforms (PA-PC) through alternative splicing (Forstemann et al. 2005). Only Loqs-PA and Loqs-PB are expressed in fly tissues, and both contain three dsRBD domains. Biochemical fractionation of S2 extract shows that Dcr-1 and Loqs-PB correlate perfectly with the miRNA-generating activity (Jiang et al. 2005). Recombinant Dcr-1 alone is able to process pre-miRNA to miRNA in vitro, whereas Loqs-PB greatly enhances miRNA production by increasing Dcr-1's affinity for pre-miRNA (Jiang et al. 2005). Consistent with this, partial deficiency of Loqs, as a result of RNAi or a hypomorphic mutation, causes accumulation of pre-miRNAs in S2 cells or flies (Forstemann et al. 2005; Jiang et al. 2005; Saito et al. 2005). Thus, Loqs plays a critical role in assisting Dcr-1 to process pre-miRNAs to miRNAs.

To investigate the physiological functions of Loqs, we generate *loqs* knockout (KO) flies by gene targeting through ends-out homologous recombination (Park et al. 2007). Homozygous *loqs*^{KO} flies display severe developmental defects, including embryonic lethality and loss of germline stem cells (GSCs) (Park et al. 2007). Furthermore, mosaic studies indicate that Loqs is intrinsically required for self-renewal of female GSCs. These phenotypes can be rescued

by transgenic expression of Loqs-PB, but not Loqs-PA, suggesting that the Loqs-PB isoform is necessary and sufficient for *Drosophila* development and the miRNA pathway. In the current study, we performed biochemical analysis of *loqs*^{KO} flies to further characterize the functions of Loqs in the miRNA pathway. Our results show that Loqs is required for the production of some miRNAs, but dispensable for others. Moreover, Dcr-1, but not Loqs, is critical for loading miRNA onto the miRISC complexes. Thus, Loqs primarily functions in *Drosophila* miRNA biogenesis rather than miRISC assembly.

RESULTS AND DISCUSSION

To determine if Loqs was required for all miRNA biogenesis, we performed Northern blots to systematically examine the levels of 33 out of 78 known miRNAs among wild-type and homozygous *loqs*^{KO} flies (Park et al. 2007). Although these miRNAs were chosen based on previous miRNA cloning data from adult flies (Aravin et al. 2003), only half of Northern blots produced detectable signals and clean background. In the majority of cases, we observed significant accumulation of the ~60 nt pre-miRNAs in *loqs*^{KO} flies, indicating a general role of Loqs in assisting Dcr-1 to process pre-miRNA (Fig. 1). By contrast, the lack of Loqs had differential effects on the levels of various mature miRNAs. For example, eight miRNAs (e.g., mir-7 and mir-277) were reduced or eliminated in *loqs*^{KO} flies (Fig. 1A). Conversely, eight other miRNAs (e.g., mir-1 and mir-14) showed equivalent levels between wild-type and *loqs*^{KO} flies (Fig. 1B). These results indicate that Loqs is required for the production of only a subset of *Drosophila* miRNAs.

This was a surprising finding because *loqs*^{KO} flies not only had a reduced level of Dcr-1 enzyme (data not shown), but also completely lacked Loqs, an enhancer of miRNA production. However, despite these deficiencies in *loqs*^{KO} flies, the remaining Dcr-1 could still produce miRNAs at a sufficient rate to allow almost half of miRNAs to accumulate to wild-type levels (Fig. 1B). Importantly, these data suggest that miRNA production is not the rate-limiting step of the miRNA pathway. It is possible that more miRNAs are produced than can be loaded onto the miRISC complexes, and excess free miRNAs will be rapidly degraded in *Drosophila* cells.

Northern blotting measures the steady-state levels of miRNAs. In theory, the lack of Loqs may differentially affect the rate of synthesis, miRISC loading, or degradation of different miRNAs in *loqs*^{KO} flies. For example, some pre-miRNAs may represent perfect substrates for Dcr-1, and their processing is less dependent or independent of Loqs. Conversely, other pre-miRNAs may represent less optimal substrates or have low affinity for Dcr-1, thereby requiring Loqs for efficient miRNA production. However, our bioinformatic analysis of pre-miRNA sequences has not yielded

FIGURE 1. Loqs is required for the production of only a subset of miRNAs. (A) Northern blots of eight miRNAs showing pre-miRNA accumulation and miRNA reduction in *loqs*^{KO} flies. (B) Northern blots of eight miRNAs showing pre-miRNA accumulation accompanied by wild-type levels of mature miRNAs in *loqs*^{KO} flies. For each Northern blot, size markers are shown on the left. Pre-miRNA and miRNA are marked by arrow and arrowhead, respectively. 2S rRNA is used as loading control. Question marks refer to the inability to determine ~60 nt pre-miRNA.

any obvious features that could distinguish the two miRNA classes. Alternatively, other dsRBPs, such as R2D2, may partially substitute for Loqs in miRNA processing. However, this notion is not supported by our analysis of *r2d2 loqs* double mutant (data not shown). Therefore, future studies are required to uncover the molecular basis of this interesting phenomenon.

Dcr-2 and R2D2 coordinately bind siRNAs to promote efficient assembly of the siRISC complexes (Liu et al. 2003, 2006; Lee et al. 2004; Pham et al. 2004; Tomari et al. 2004b). It will be important to determine if Dcr-1 and Loqs play a similar role in loading miRNAs onto the miRISC complexes. To examine this possibility, we first performed native gel-shift assays to compare the binding of recombinant Dcr-1 proteins to a radiolabeled (Loqs-independent) let7/let* duplex in the absence or presence of Loqs-PA and Loqs-PB. As shown in Figure 2, recombinant Dcr-1 alone

could efficiently interact with duplex miRNA. While Loqs-PA reduced the binding of Dcr-1 to let-7/let* duplex, Loqs-PB slightly enhanced it. A similar result was obtained by using a radiolabeled (Loqs-dependent) bantam/ban* duplex. By contrast, only Dcr-2/R2D2 complex, but neither Dcr-2 nor R2D2 alone, can efficiently interact with duplex siRNA (Liu et al. 2006).

To determine if Dcr-1 and Loqs were required for *Drosophila* miRISC assembly, we generated *dcr-1* or *loqs* null extract by using the FLP/ovo^D system (Chou et al. 1993). This mosaic germline system allowed us to bypass the lethality of homozygous *dcr-1*^{Q1147X} or *loqs*^{KO} animals and to produce only mutant eggs that lack maternal and zygotic Dcr-1 or Loqs proteins (see details in Materials and Methods). The *dcr-1*^{Q1147X} was considered a null allele because of a nonsense mutation before the catalytic RIII domains (Lee et al. 2004). Western blots confirmed the lack of Dcr-1 or Loqs in *dcr-1* or *loqs* null extract (Fig. 3A). The levels of Loqs proteins were reduced in *dcr-1* null extract and vice versa, suggesting that Dcr-1 and Loqs stabilized each other in vivo.

Next, we performed duplex siRNA and miRNA-initiated RISC (mRNA cleavage) assays using wild-type and *loqs* or *dcr-1* null extracts. Both siRISC and miRISC activity were greatly diminished in *dcr-1* null extract (Fig. 3B). The miRISC activity was likely Ago1 mediated since wild-type and *ago2* mutant extracts showed equivalent activities (Fig. 3C). The role of Dcr-1 in siRISC assembly was consistent with previous analysis of the *dcr-1*^{Q1147X} mutant (Lee et al. 2004). In contrast, we observed equivalent miRNA- and

FIGURE 2. Dcr-1 interacts with duplex miRNA in the absence of Loqs. Native gel-shift assays were performed as previously described (Liu et al. 2006) by incubating radiolabeled let-7/let* (A) or bantam/ban* (B) with 8 pmol recombinant Dcr-1 alone (lanes 1, 5) or in combination with 8 pmol Loqs-PA (lanes 2, 6), Loqs-PB (lanes 3, 7), or Loqs-PA+Loqs-PB (lanes 4, 8).

FIGURE 3. Dcr-1, but not Loqs, is critical for assembly of miRISC complexes. (A) Western blots were performed to detect Dcr-1, Ago1, Loqs, and R2D2 in wild-type (WT), *loqs*^{KO}, or *dcr-1* null extract. (B) Duplex siRNA- (lanes 1–4) and miRNA- (lanes 5–8) initiated RISC assays were performed in buffer or 25 μ g wild-type (WT), *loqs*^{KO}, or *dcr-1* null egg extract. (C) Duplex siRNA- (lanes 1–3) and miRNA- (lanes 4–6) initiated RISC assays were performed in buffer or 25 μ g wild-type (WT) or *ago2* ovary extract. (D) The pre-miRNA- and dsRNA-initiated RISC assays were performed in wild-type (odd lanes) and *loqs*^{KO} (even lanes) extracts with water (lanes 1,2), pre-let7 (lanes 3,4), or dsRNA (lanes 5,6) as triggers. (E) The pre-miRNA-processing assays were performed in buffer or 10 μ g of wild-type, *loqs*^{KO}, or *dcr-1* null extract (Jiang et al. 2005).

siRNA-initiated RISC activities between wild-type and *loqs* null extracts (Fig. 3B). These results indicate that Dcr-1, but not Loqs, is critical for *Drosophila* miRISC assembly.

Interestingly, the RISC assembly defect of *dcr-1* null extract could not be rescued by recombinant Dcr-1 or Dcr-1/Loqs complex (data not shown). It is possible that the lack of Dcr-1 causes reduction in other critical RNAi components. Alternatively, the *dcr-1* mutant may fail to produce a miRNA that normally represses an inhibitor of RNAi. Thus, it is currently unclear if Dcr-1 is directly or indirectly involved in miRISC or siRISC assembly.

Although *loqs* null extract was defective for pre-miRNA-initiated RISC activity, the magnitude of the effect (2–3-fold) was significantly less than the defect in pre-miRNA-processing activity (>10-fold) (Fig. 3D,E). Thus, the primary defect of *loqs* null extract is likely in pre-miRNA processing

rather than miRISC assembly. Moreover, this result supports the idea that miRNA production is not the rate-limiting step of *Drosophila* miRNA pathway.

We further examined *in vivo* miRNA loading by using a monoclonal anti-Ago1 antibody to immunoprecipitate (IP) the endogenous miRISC complexes followed by Northern blots to measure the abundance of miRNAs (Miyoshi et al. 2005). While the level of (Loqs-independent) mir-14 was equivalent in total RNAs of wild-type (WT) and *loqs*^{KO} eggs, the Ago1-associated mir-14 in *loqs*^{KO} extract was 83% of that from WT control (Fig. 4A). On the other hand, the level of (Loqs-dependent) bantam was reduced (60% of WT) in *loqs*^{KO} eggs, and a similar reduction was observed (43% of WT) for Ago1-associated bantam miRNA (Fig. 4B). These results suggest that Loqs is largely dispensable for miRISC assembly *in vivo*. It is likely that the small decrease in miRNA loading is due to the reduction of Dcr-1 protein in *loqs*^{KO} flies (Fig. 3A).

Recent studies have shown that Dcr-1 and Loqs are both required for embryonic viability and self-renewal of *Drosophila* ovarian GSCs (Lee et al. 2004; Jin and Xie 2007; Park et al. 2007). However, *dcr-1*^{−/−} GSCs, but not *loqs*^{−/−} GSCs, also display a cell cycle defect at G1/S transition (Hatfield et al. 2005; Park et al. 2007). Additionally, Dcr-1, but not Loqs, is required for somatic stem cell (SSC) maintenance (Jin and Xie 2007; Park et al. 2007). These results

suggest that the *dcr-1* mutant phenotype is more severe than that of *loqs*^{KO} flies. Consistent with this, we showed that only a subset of miRNAs was diminished, whereas others maintained wild-type levels in *loqs*^{KO} animals (Fig. 1). By contrast, we could not detect mir-14 or bantam in *dcr-1* null eggs by Northern blotting (Fig. 4), suggesting that Dcr-1 was likely responsible for all miRNA biogenesis. Furthermore, our studies showed that Dcr-1, but not Loqs, was critical for loading miRNA onto the miRISC complexes. Together, these results provide the biochemical basis for the phenotypic differences between *dcr-1* and *loqs* mutant flies.

Both R2D2 and Loqs belong to a growing family of dsRBPs that function in tandem with Dicer enzymes in the siRNA and/or miRNA pathways (Paroo et al. 2007). Previous and current studies present two different modes of

FIGURE 4. *Loqs* is largely dispensable for miRISC assembly in vivo. The mir-14 (A) and bantam (B) Northern blots of total RNA isolated from wild-type (WT), *loqs*^{KO}, and *dcr-1* null eggs (lanes 1–3), and miRNAs extracted from Ago1 IPs from wild-type and *loqs*^{KO} egg extracts (lanes 4,5). The bar graphs beneath indicate the quantification of the relative levels of mature miRNAs.

functions for dsRBPs: (1) R2D2 does not regulate siRNA production, but is required for loading siRNA onto the siRISC complexes (Liu et al. 2003, 2006; Tomari et al. 2004b). (2) *Loqs* plays a prominent role in miRNA biogenesis, but is largely dispensable for assembly of the miRISC complexes (Forstemann et al. 2005; Jiang et al. 2005; Saito et al. 2005). These studies will have important implications for understanding the functions of dsRBPs in other RNAi model systems.

MATERIALS AND METHODS

Northern blot

Total RNA was isolated from wild-type and homozygous *loqs*^{KO} unfertilized eggs or young adult flies by using TRIzol (Invitrogen), resolved by 12% denaturing (7 M urea) polyacrylamide gels, and transferred onto Zeta-probe membrane (Bio-Rad). While 30 µg total RNA from whole flies were loaded per lane (Fig. 1), 100 µg of total RNA were used for unfertilized eggs (Fig. 4). The Ago1 IPs were performed essentially as previously described (Miyoshi et al. 2005). In brief, 5 mg of wild-type and *loqs* null extract were

incubated with 10 µL of anti-Ago1 monoclonal antibody for 2 h at 4°C followed by addition of 10 µL of Protein A beads and rotating at 4°C for 4 h. After washing five times with the IP-wash buffer (see above), 200 µL of buffer A and 800 µL of TRIzol were added to extract the miRNA associated with Ago1. Northern hybridization was carried out at 40°C in 0.2 M sodium phosphate (pH 7.0) and 7% SDS for 16 h followed by two washes in 2× SSC and 0.1% SDS, once at room temperature for 5 min and again at 40°C for 20 min. All probes were synthetic 21-nt RNA oligos (IDT) that were complementary to miRNA sequences provided by miRBase (<http://microrna.sanger.ac.uk/>) and were 5'-end labeled with γ-³²P ATP by T4 polynucleotide kinase (NEB). A DNA oligo, 5'-TACAACCCTCAACCATATGTAGTCCAAGCA-3', was used for probing 2S rRNA as loading control.

Generation of *loqs* or *dcr-1* null egg extracts

To generate *loqs* null egg extract, we created mosaic germlines by crossing virgin female flies of yw [hsFLP]; *loqs*^{KO} [FRT]40A/CyO (Park et al. 2007) with male flies of ovo^{D1-18} [FRT]40A/MS(2)M¹ Sp/CyO (BL# 2121). The parents were flipped into fresh bottles every 3–4 d and the progenies heat shocked in a 38°C water bath for 1.5 h/day for two consecutive days. After eclosing, we sorted F1 virgin mosaic females with the genotype yw [hsFLP]/+; *loqs*^{KO} [FRT]40A/ovo^{D1-18} [FRT]40A. Similarly, we used virgin female flies of yw[hsFLP]; [FRT]40A/CyO to generate wild-type control eggs. For *dcr-1* null extract, we crossed virgin female flies of yw [hsFLP]; [FRT]82B *dcr-1*^{Q1147X}/TM3 (Lee et al. 2004) and male flies of w⁺; [FRT]82B ovo^{D1-18}/βTub85D^D/TM3 (BL# 2149). We sorted F1 virgin mosaic females with the genotype yw[hsFLP]/w⁺; [FRT]82B *dcr-1*^{Q1147X}/[FRT]82B ovo^{D1-18}.

Because ovo^{D1-18} is a dominant sterile marker, mature oocytes can only develop from GSCs that lack ovo^D and are homozygous for *loqs*^{KO} or *dcr-1*^{Q1147X} mutation, which are generated by the Flipase (FLP)/FRT-mediated mitotic recombination. Thus, the F1 virgin mosaic females could produce 100% of *loqs*^{KO} or *dcr-1*^{Q1147X} mutant eggs. Only a small time window allowed for mutant egg collection because Dcr-1 and *Loqs* were both required for self-renewal of GSCs (Jin and Xie 2007; Park et al. 2007). We optimized this procedure to generate thousands of F1 mosaic virgin females and collect *loqs* or *dcr-1* mutant eggs every 24 h on apple juice plates with wet yeast paste.

We treated unfertilized eggs with 50% bleach for 2 min followed by washing twice with DEPC-treated water and once with buffer A (10 mM KOAc, 10 mM HEPES at pH 7.4, 10 mM Mg(OAc)₂, and 5 mM DTT). Subsequently, we added to dechorionated eggs two volumes of buffer A that were freshly supplemented with protease inhibitors (Roche) and SuperRNase inhibitor (0.5 unit/µL) (Ambion). After sitting on ice for 15 min, the eggs were dounced 30 times followed by centrifugation at 13,000 rpm for 20 min at 4°C. We saved the supernatant as *dcr-1* or *loqs* null extracts in a –80°C freezer.

In vitro biochemical assays

The miRNA gel-shift assays were conducted as previously described (Liu et al. 2006). The let-7/let* miRNA (Dharmacon) were radiolabeled with γ-³²P at the 5' end by T4 polynucleotide kinase. All in vitro RISC assays were performed as described (Liu et al. 2003, 2006). The mRNA substrate was radiolabeled at the

5' G cap by guanylyl transferase (Ambion) followed by PAGE purification. In brief, 25,000 cpm radiolabeled mRNA was incubated with fly egg extracts for 1 h at 30°C in 1× buffer 12 (100 mM KOAc, 10 mM HEPES at pH 7.4, 2 mM Mg(OAc)₂, and 5 mM DTT) in the presence of an ATP-regenerating system (1 mM ATP, 30 mM creatine phosphate [Fluka], and 30 unit creatine phosphokinase [Sigma]). Both pre-let-7 and dsRNA (130 bp) triggers were used at 250 nM final concentration. Duplex let-7 siRNA and let-7/let* miRNA were used at 25 nM and 100 nM final concentration, respectively.

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