Article

Dicer's Helicase Domain Discriminates dsRNA Termini to Promote an Altered Reaction Mode

Noah C. Welker,[1] Tuhin S. Maity,[1,3] Xuecheng Ye,[2,3] P. Joseph Aruscavage,[1] Ammie A. Krauchuk,[1] Qinghua Liu,[2] and Brenda L. Bass[1,*]
[1]Department of Biochemistry, University of Utah, Salt Lake City, UT 84112, USA
[2]Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas, TX 75390, USA
[3]These authors contributed equally to this work
*Correspondence: bbass@biochem.utah.edu
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SUMMARY

The role of Dicer's helicase domain is enigmatic, but in vivo it is required for processing certain endogenous siRNA, but not miRNA. By using *Caenorhabditis elegans* extracts or purified *Drosophila* Dicer-2 we compared activities of wild-type enzymes and those containing mutations in the helicase domain. We found the helicase domain was essential for cleaving dsRNA with blunt or 5′-overhanging termini, but not those with 3′ overhangs, as found on miRNA precursors. Further, blunt termini, but not 3′ overhangs, led to increased siRNAs from internal regions of dsRNA; this activity required ATP and a functional helicase domain. Our data suggest that blunt or 5′-overhanging termini engage Dicer's helicase domain to facilitate accumulation of siRNAs from internal regions of a dsRNA, an activity suited for processing long siRNA precursors of low abundance, but not necessary for the single cleavage required for miRNA processing.

INTRODUCTION

Dicer endonucleases cleave long double-stranded RNA (dsRNA) and short hairpin RNA (pre-miRNA) into 20–27 nucleotide (nt) RNAs, called siRNAs and miRNAs, respectively (Carthew and Sontheimer, 2009). These short RNAs function as sequence-specific guides in targeting mRNAs for silencing. Most Dicer orthologs share a common domain architecture (Figure 1A). Biochemical and structural studies have provided detailed information about how the RNase III nuclease domains direct cleavage of dsRNA in the active site (MacRae et al., 2006; Zhang et al., 2004). In contrast, the role of the helicase domain is unclear, although it contains conserved motifs that in other Superfamily 2 helicases couple ATP hydrolysis to motor activities such as unwinding or translocation (Lohman et al., 2008; Pyle, 2008).

Several studies indicate the helicase domain is required for siRNA, but not miRNA, processing. *Caenorhabditis elegans* strains expressing Dicer (DCR-1) with point mutations in any of three different helicase motifs (Figure 1B, underlined) show normal levels of mature miRNAs, but are defective for the production of certain endogenous siRNAs (endo-siRNAs),

particularly a longer 26 nt species with a 5′ guanosine (26G RNAs; Gent et al., 2010; Pavelec et al., 2009; Welker et al., 2010). While *Homo sapiens* and *C. elegans* encode a single Dicer, *Drosophila melanogaster* encodes two enzymes (Lee et al., 2004), one for processing miRNA precursors (Dicer-1) and the other for processing siRNAs from exogenously introduced, or endogenous, dsRNA (Dicer-2; Chung et al., 2008; Czech et al., 2008; Ghildiyal et al., 2008; Kawamura et al., 2008; Okamura et al., 2008a, 2008b). Dicer-1 lacks a functional helicase domain (Figure 1A) and does not require ATP for activity, again indicating pre-miRNA processing does not require a helicase function. In contrast, Dicer-2 has a well-conserved helicase domain and requires ATP to efficiently cleave long dsRNA (Jiang et al., 2005; Liu et al., 2003). Consistent with the idea that the helicase domain of Dicer-2 is required for siRNA processing, a mutation in its Walker A motif (G31R, Figure 1B) reduces siRNA derived from dsRNA produced from a transgene in vivo (Lee et al., 2004). Similarly, *Schizosaccharomyces pombe* strains expressing Dicer with a point mutation in the same helicase motif (K38A, Figure 1B) are defective for centromeric silencing and generation of siRNAs (Colmenares et al., 2007).

While these studies point to the importance of the helicase domain in siRNA processing, how the domain contributes to this process is unclear. Previous in vitro studies have not provided an obvious mechanistic function (Colmenares et al., 2007; Ma et al., 2008; Zhang et al., 2002). In fact, deletion or mutation of the helicase domain of human Dicer leads to a more active enzyme in vitro (Ma et al., 2008). In continued pursuit of the mechanistic function of Dicer's helicase domain, we performed in vitro studies with cell-free extracts of *C. elegans*, as well as purified recombinant *Drosophila* Dicer-2. For both systems we compared activities of wild-type and helicase mutant forms of Dicer. The results of our studies indicate Dicer's helicase domain is required for recognizing certain dsRNA termini to promote a distinctly different reaction. Our studies offer insight into why the helicase domain is required in vivo for accumulation of certain endo-siRNA, but not miRNA.

RESULTS

The Helicase Domain of *C. elegans* Dicer Mediates Recognition of Duplex Termini

To gain mechanistic insight into the role of Dicer's helicase domain, we compared processing of dsRNA by cell-free embryo
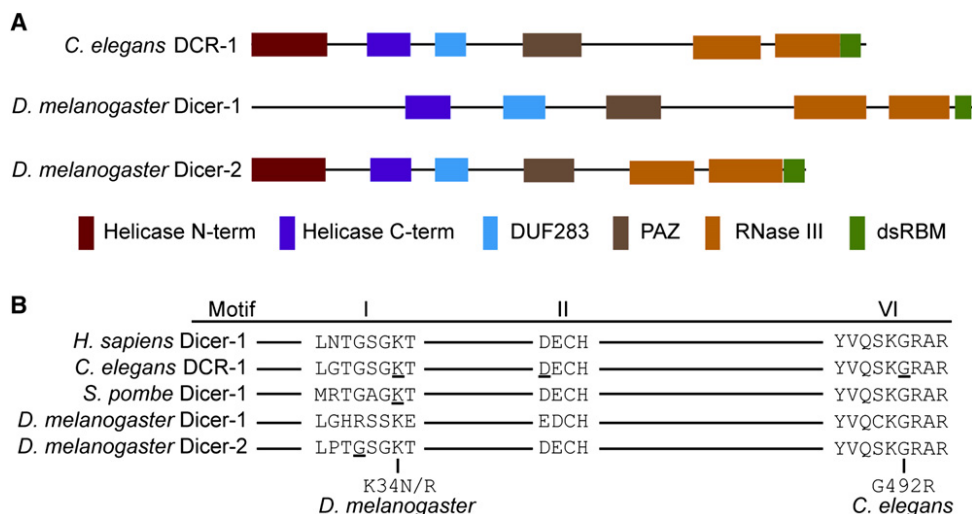
Figure 1. Dicer's Helicase Domain Has Well-Conserved Motifs
(A) Domain organization of *C. elegans* DCR-1 and *Drosophila* Dicer-1 and Dicer-2, color-coded to indicate: helicase domain, domain of unknown function 283 (DUF283), Piwi Argonaute Zwille domain (PAZ), RNase III domains, and the dsRNA binding motif (dsRBM).
(B) Amino acids within the highly conserved Motif I (Walker A), Motif II (Walker B), and Motif VI are shown, with mutants analyzed in this study indicated. Underlined amino acids show residues mutated in previous studies and discussed in the text.

extracts of wild-type *C. elegans* or strains harboring a point mutation in motif VI (G492R, Figure 1B) of the helicase domain [*dcr-1(mg375)*; see Experimental Procedures]. We first monitored the reaction of a ^{32}P-end-labeled pre-*let-7* RNA with the endogenous *C. elegans* sequence, including the characteristic 2 nt 3′ overhang. Consistent with previous analyses of endogenous RNA from these strains (Pavelec et al., 2009; Welker et al., 2010), both extracts processed pre-*let-7*, showing a similar accumulation of 22 nt mature *let-7* (Figures 2A and 2B).
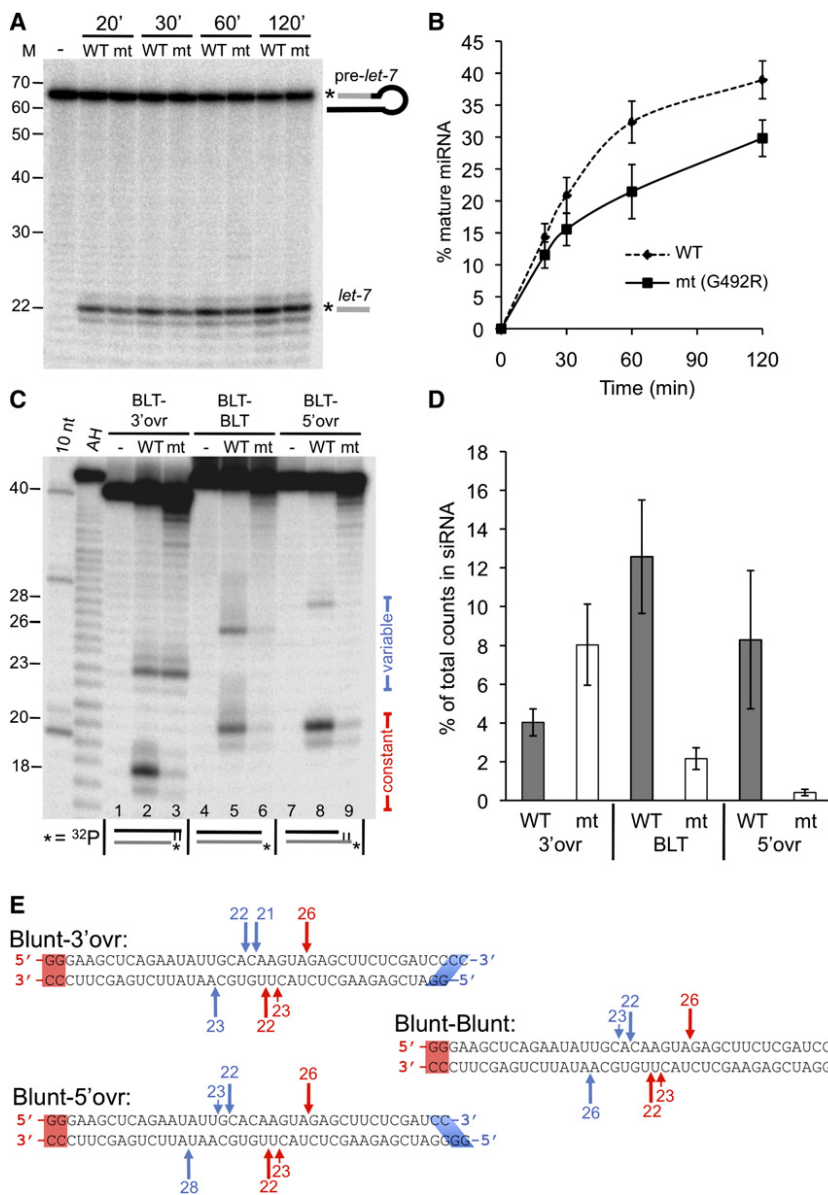
Precursors of endo-siRNA are ill-defined, but in *D. melanogaster* can arise from long, genomically encoded hairpins or overlapping genes that give rise to complementary transcripts (Marques et al., 2010). In addition, in vitro studies of human Dicer show that the length of an siRNA depends on the termini of its dsRNA precursor (Vermeulen et al., 2005; Zhang et al., 2004). Thus, as a first step in evaluating the requirement of Dicer's helicase domain in processing siRNA precursors, we hybridized 40 and 42 nt RNAs to create completely base-paired dsRNA with a variety of termini (Figures 2C and 2E). All dsRNAs had one blunt terminus and one terminus that varied to include a 2 nt 3′ overhang (BLT-3′ovr), a second blunt end (BLT-BLT), or a 2 nt 5′ overhang (BLT-5′ovr).

In choosing cleavage sites, Dicer measures from duplex termini (MacRae et al., 2007; Zhang et al., 2004) and the short length of the 40/42 dsRNAs allowed only one cleavage event, which could occur from either end. 40/42 dsRNAs were 5′ ^{32}P-end-labeled on one strand and designed so that cleavage resulting from measuring from the constant blunt terminus gave shorter products (∼18–20 nt, constant) than cleavage from the variable terminus (23–28 nt, variable; Figure 2C). Each substrate was incubated with wild-type or G492R mutant extract and reaction products resolved by electrophoresis on a denaturing gel. In wild-type extracts, cleavage products were observed from all three variable termini and the constant blunt terminus (Figures

2C, lanes 2, 5, and 8 and Figure 2D). In contrast, while cleavage from the variable 3′ 2 nt overhang was observed in the G492R mutant extract (Figure 2C, lane 3), these extracts were markedly deficient in processing from blunt termini or those with 5′ 2 nt overhangs (Figures 2C, lanes 3, 6, and 9 and Figure 2D). These data indicated that Dicer requires a functional helicase domain for efficient cleavage of dsRNA with blunt or 5′-overhanging termini, but not dsRNA with 3′-overhanging termini. The latter is consistent with the observation that Dicer's helicase domain is not required for processing pre-miRNA, which have 2 nt 3′ overhangs.

Dicer Activity in *C. elegans* Extracts Produces siRNA with 3–4 Nucleotide Overhangs and Lengths Dependent on dsRNA Termini

To more precisely define cleavages occurring in *C. elegans* extracts, we performed reactions in wild-type extracts by using 40/42 dsRNAs that were ^{32}P-end-labeled on the opposite strand (Figures 2C, black strand in cartoon and Figure S1). We compared migration of alkaline hydrolysis (AH) and T1 ribonuclease products with 10 nt RNA ladders on long sequencing gels to obtain accurate sizes of cleavage products (Figure S1). When combined, data for each strand allowed precise characterization of Dicer cleavage sites (Figure 2E). Each arrow in Figure 2E indicates a site of cleavage and the number of nucleotides from a constant (red) or variable (blue) terminus. While the distance from cleavage sites to 5′ termini varied between molecules (e.g., compare blue numbers for bottom strands), the distance to 3′ termini was similar (compare blue numbers for top strands), indicating that *C. elegans* Dicer measures from 3′ termini as observed in other organisms (MacRae et al., 2006, 2007). However, the distance to 3′ termini was 21–22 nt when measuring from 3′-overhanging termini, but 1 nt longer (22–23 nt) when measured from blunt or 5′-overhanging termini

Figure 2. The Helicase Domain of *C. elegans* Dicer Is Required for Cleavage of dsRNA with Blunt or 5′-Overhanging Termini, but Not 3′-Overhanging Termini

(A) PhosphorImage showing production of mature *let-7* miRNA (*let-7*) from 5′ ^{32}P-end-labeled (*) pre-*let-7,* incubated for indicated times without (−), or with wild-type (WT) or G492R mutant (mt) *C. elegans* extract in extract cleavage buffer (12 mM MgOAc; see Experimental Procedures). M, positions of RNA decade ladder and 22 nt miRNA.

(B) Data from multiple assays as in (A) were quantified to determine % miRNA (100 × [radioactivity in miRNA band/total radioactivity in lane]); miRNA band included radioactivity in major and two immediately adjacent bands, but plots did not differ significantly when only the predominant band was monitored. Data points show average values; error bars = standard error of the mean; n = 2.

(C) PhosphorImage of reaction products generated after 60 min incubation of 40/42 dsRNAs with indicated termini, without (−) or with WT or G492R mutant (mt) extract in extract cleavage buffer (10 mM MgOAc; see Experimental Procedures). As cartooned, 40/42 dsRNAs were 5′ ^{32}P-end-labeled on the bottom strand. Products generated by Dicer measuring from constant or variable termini are indicated. Marked lengths (left) were determined with reference to AH ladders, 10 nt RNA ladders (10 nt), and T1 ribonuclease ladders (Figure S1).

(D) Data from multiple assays as in (C) were quantified to compare cleavage from three types of termini. Percent siRNA = 100 × (radioactivity in siRNA band/total radioactivity in lane); radioactivity in siRNA band included predominant and immediately adjacent bands if present. Percent siRNA values for BLT end processing included constant ends of 3′ovr and 5′ovr dsRNAs as well as both termini of BLT-BLT dsRNAs. Error bars = standard error of the mean (n ≥ 6).

(E) Schematic of observed cleavage sites in 40/42 dsRNAs reacted as in (C) with extracts of wild-type *C. elegans*. Sites were determined by using data for 40/42 dsRNAs labeled on the top (Figure S1) or bottom (Figure 2C) strand. Length in nt is shown above arrows marking major (long arrows) and minor (short arrows) cleavage with respect to Dicer measurement from constant blunt end (red) or variable end (blue).

(Figure 2E). Given the necessity of a functional helicase domain for cleavage of dsRNA with blunt and 5′-overhanging termini, but not dsRNA with 3′-overhanging termini (Figure 2C), these data suggest Dicer uses an alternate mode of cleavage when the helicase domain is engaged. We also found that product lengths were consistent with a staggered cleavage of each strand to yield 3 and 4 nt overhangs, rather than the canonical 2 nt overhang associated with other RNase III family members (MacRae and Doudna, 2007). Deep sequencing of *C. elegans* small RNAs reveals 26 nt endo-siRNAs that pair with their sense partner strands in a manner that predicts a 3 nt 3′ overhang (Ruby et al., 2006). Our data raise the possibility that 26 nt endo-siRNAs arise from a helicase-dependent cleavage of a blunt terminus (Figure 2E). Most *C. elegans* 26 nt endo-siRNAs
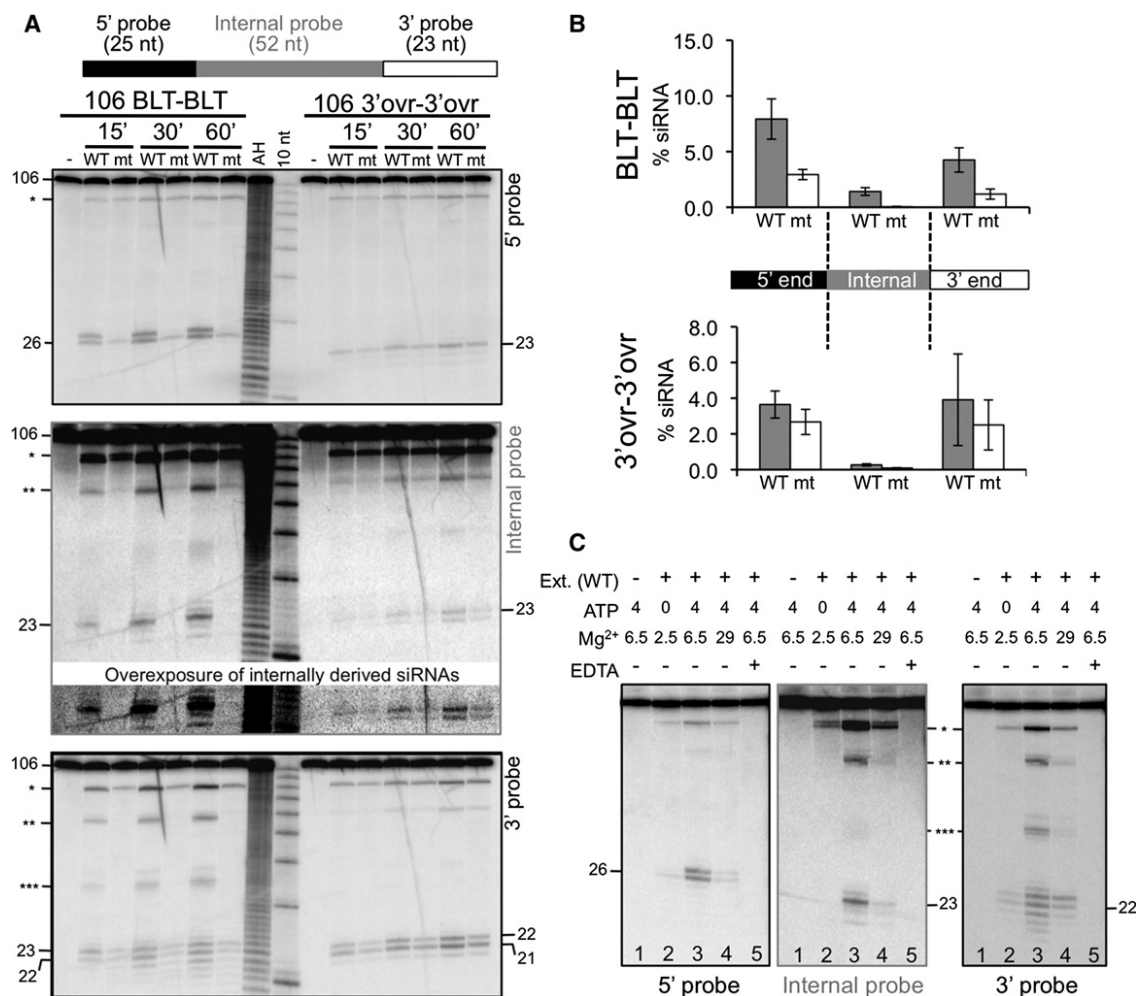
have a guanosine at their 5′ terminus. We found that duplexes with different 5′ nt were cleaved similarly by *C. elegans* extracts (Figure S1, available online), suggesting this feature does not derive from Dicer.

Dicer's Helicase Domain Facilitates Production of siRNAs from Internal Regions of Long dsRNA with Blunt but Not 3′-Overhanging Termini

Processing of pre-miRNAs by Dicer requires only a single cleavage step that results in a staggered cleavage of both strands. While the precursors of endo-siRNAs are ill-defined, endo-siRNAs map to the genome in clusters, suggesting they are produced by multiple cleavage steps along the length of a dsRNA precursor. We considered the possibility that recognition of blunt

Figure 3. dsRNA with Blunt Termini Give Rise to More Internally Derived siRNAs Than Those with 3′ Overhangs

(A) PhosphorImages of northern blots comparing the reactions of 106 BLT-BLT and 3′ovr-3′ovr dsRNA incubated with wild-type (WT) and G492R mutant (mt) extracts for various times, in extract cleavage buffer (10 mM MgOAc; see Experimental Procedures). Cartoon shows the relative position of probes designed to detect siRNAs generated from termini (top and bottom panels) or middle (middle panel) of dsRNAs. Asterisks denote intermediates, with each representing one cleavage event (*∼80 nt, **∼57 nt, ***∼34 nt). Marked lengths (nt) were determined from the data of Figure S1.

(B) Data from multiple analyses as in (A) were quantified to determine the average % siRNA (see Figure 2D) for a 60 min incubation with dsRNAs and extracts indicated. Conditions were as in (A) except some reactions contained 6.5 mM MgOAc (control experiments showed no difference in reactions with 6.5 or 10 mM MgOAc). When siRNA bands were heterogeneous, siRNA radioactivity included all proximal bands. Error bars = standard error of the mean (n ≥ 3).

(C) Northern blots as in (A) show reaction of 106 BLT-BLT dsRNA incubated for 60 min with wild-type extract (Ext.) in extract cleavage buffer modified to contain varying amounts of ATP (in mM), MgOAc (in mM) and EDTA (10 mM). ATP stimulates cleavage and is required for accumulation for internally derived siRNAs (see Figure S2 for overexposure of middle panel). Asterisks denote cleavage intermediates as in (A). Marked lengths (nt) were determined from data in Figure S1.

and 5′-overhanging termini by Dicer's helicase domain triggered an altered mode of reaction uniquely suited for the production of endo-siRNAs from a long dsRNA precursor. To test for this, we performed cleavage reactions using longer, nonradioactive dsRNA (formed by hybridizing 106 nt RNAs) and analyzed reaction products by northern blot with probes that hybridized to siRNAs from duplex termini, or alternatively, to those from internal regions (Figure 3).

Representative blots for three time points of a reaction with a BLT-BLT or a 3′ovr-3′ovr dsRNA incubated in wild-type or G492R mutant extract are shown (Figure 3A) and average values from multiple analyses plotted (Figure 3B). When probed for

siRNAs resulting from the first cleavage event at either end (top and bottom panels, 5′ probe and 3′ probe respectively), the sizes of predominant siRNA bands were reminiscent of those observed with 40/42 dsRNA. For example, the predominant 3′ cleavage products (3′ probe) from the 106 BLT-BLT dsRNA (22/23 nt) were 1 nt longer than those from the 106 3′ovr-3′ovr dsRNA (21/22). Similarly, a 5′ terminal siRNA (5′ probe) of 26 nt was observed for the 106 BLT-BLT dsRNA, and 1 of 23 nt for the 106 3′ovr-3′ovr dsRNA, as observed for 40/42 dsRNAs with similar termini (compare to Figures 2C and 2E). However, in addition to the 26 nt siRNA from the 5′ terminus of the 106 BLT-BLT dsRNA, a 27 nt band was also observed after

incubation in wild-type, but not G492R mutant extracts. Because this helicase-dependent siRNA was observed with 106 dsRNAs, but not 40/42 dsRNAs, possibly it relates to processing of longer dsRNA. Analysis of blots with internal probes allowed visualization of subsequent cleavages on the longer molecules, and interestingly, siRNAs from subsequent cleavages were predominantly 23 nt in length, regardless of which strand was probed (Figure 3A, middle panel; data not shown).

The 40/42 dsRNAs only allow a single cleavage event, measured from either end, and this was most analogous to the first cleavage from the termini of the 106 dsRNAs, detected by the 5′ and 3′ probes. As observed for 40/42 dsRNAs (Figures 2C and 2D), wild-type and G492R mutant extracts produced similar levels of terminal siRNAs from the 3′ovr-3′ovr dsRNA, but the G492R mutant extracts were much less efficient in processing the termini of BLT-BLT dsRNA compared to the wild-type extracts (Figure 3A, 5′ and 3′ probes) and this was validated in multiple analyses (Figure 3B). These data reiterate the importance of the helicase domain for cleavage from blunt termini. In addition, we observed a striking difference between the reactions of the two dsRNAs with the probe that detected internal siRNAs. Only very low levels of internal siRNAs were detected with the 3′ovr-3′ovr dsRNA and these low levels were detected in both wild-type and G492R mutant extracts. In contrast, accumulation of internal siRNAs was much greater for the reaction of the 106 BLT-BLT dsRNA and in this case the detection of internal siRNAs was completely dependent on a functional helicase domain; internal siRNAs were undetectable after incubation in the G492R mutant extract, even after overexposure (Figures 3A and 3B).

Thus, in comparing reactions of dsRNA with blunt or 3′-overhanging termini, we observed that accumulation of siRNAs from internal regions was most efficient with dsRNA containing blunt termini reacted with Dicer containing a functional helicase domain. Consistent with this, higher molecular weight reaction intermediates corresponding to one, two, or three cleavages along the length of the dsRNA (asterisks; Figure 3A) were observed predominantly with the BLT-BLT molecule. Such intermediates were observed with the internal probes as well as with the 5′ and 3′ probes because the latter could detect siRNAs from terminal regions and intermediates corresponding to cleavage from the opposite end.

In *C. elegans* Extracts the Accumulation of siRNAs from Internal Regions of dsRNA Is ATP Dependent

The activity of DEXH helicases is often coupled to ATP binding and/or hydrolysis and we reasoned that helicase-dependent cleavage events might also be dependent on ATP. Indeed, similar to what was observed with the G492R mutant extract, without the addition of ATP, reaction of 106 BLT-BLT dsRNA showed a small amount of siRNA from termini but none from internal regions (Figure 3C, lane 2 in all panels), even after overexposure (Figure S2). Similarly, the helicase-dependent 27 nt siRNA observed with the 5′ probe (Figure 3A) was also ATP dependent (Figure 3C, compare lanes 2 and 3, 5′ probe). Further, addition of ATP enhanced siRNA accumulation from internal regions of the 106 BLT-BLT dsRNA (compare lanes 2 and 3, internal probe) and gave rise to intermediates (lane 3, internal

and 3′ probe). As expected from Dicer's known dependence on divalent metals (MacRae and Doudna, 2007), magnesium was necessary for cleavage (lane 5, all panels), albeit at high concentrations (∼25 mM free Mg^{2+}) it inhibited the reaction (lane 4, all panels).

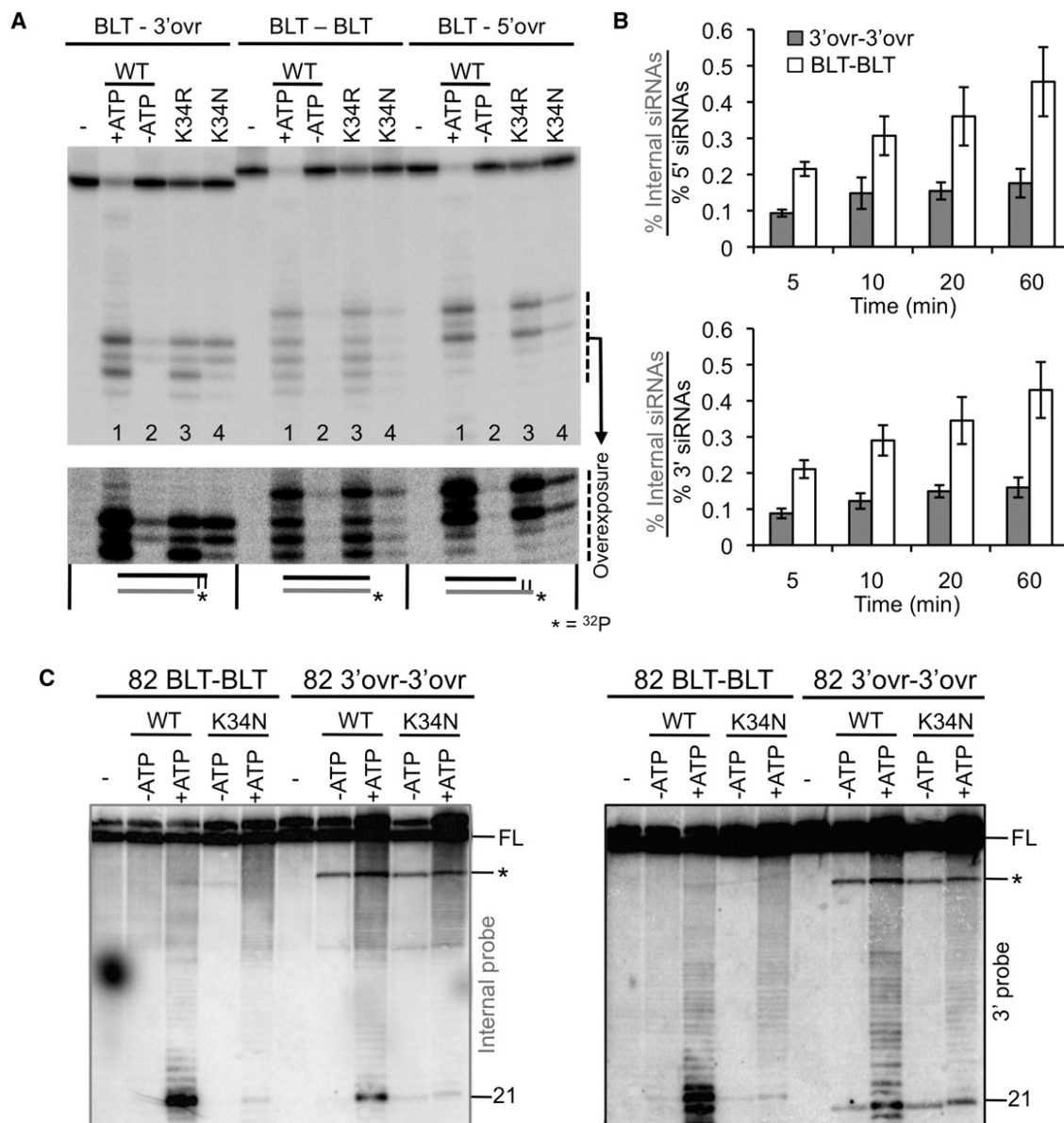### Purified *Drosophila* Dicer-2 Also Discriminates Duplex Termini

Our comparisons of cleavage in wild-type and G492R mutant *C. elegans* extracts indicated that the helicase domain was involved in the recognition of duplex termini and the efficient accumulation of siRNAs from internal regions of dsRNA. However, because these studies used extracts containing many proteins, they did not reveal whether helicase-dependent activities were intrinsic to Dicer or mediated in concert with other proteins. Attempts to purify active recombinant *C. elegans* Dicer have been unsuccessful and thus, to address this question, we initiated studies of *Drosophila* Dicer-2, which has a well-conserved helicase domain (Figure 1A) and, like *C. elegans* DCR-1, is implicated in endo-siRNA processing (Chung et al., 2008; Czech et al., 2008; Ghildiyal et al., 2008; Kawamura et al., 2008; Okamura et al., 2008a, 2008b).

We overexpressed and purified recombinant wild-type Dicer-2 and variants with mutations in the Walker A motif of the helicase domain (Figure 1B and Figure S3) and first monitored cleavage of the 40/42 dsRNAs. As observed previously (Liu et al., 2003), cleavage by *Drosophila* Dicer-2 was very dependent on ATP (Figure 4A, compare lanes 1 and 2 for all dsRNAs). However, in the absence of ATP, cleavage was virtually undetectable for the BLT-BLT and BLT-5′ovr 40/42 dsRNAs, but a small but reproducibly detectable amount of cleavage was observed for the BLT-3′ovr dsRNA (Figure 4A, compare lane 2 for all dsRNAs). Thus, purified *Drosophila* Dicer-2 reacted differently on dsRNA with 3′-overhanging termini compared to those with blunt and 5′-overhanging termini, providing the first hint that the properties observed in *C. elegans* extracts were intrinsic to Dicer.

The residue we mutated in the *Drosophila* Dicer-2 helicase domain (K34) is well studied in other helicases and in some cases eliminates helicase function (Kim et al., 1997; Pause and Sonenberg, 1992). However, we chose relatively conservative substitutions and found that when reacted with 40/42 dsRNAs the K34R mutant showed nearly wild-type levels of cleavage (Figure 4A, compare lanes 1 and 3 for all dsRNAs). In contrast, the K34N mutant, like the G492R helicase mutant of *C. elegans*, was able to process 3′-overhanging termini but was very inefficient in processing blunt and 5′-overhanging termini (Figure 4A, lane 4 for all dsRNAs).

We next tested whether Dicer-2 behaved similarly to *C. elegans* DCR-1 when processing long dsRNA. As in our analyses of *C. elegans* extracts (Figures 3A and 3B), we performed multiple time course experiments by using wild-type Dicer-2 and the 106 BLT-BLT and 3′ovr-3′ovr dsRNAs and analyzed reaction products with northern blots probed for internal or terminal siRNAs (Figure 4B). Again, processing of 106 BLT-BLT dsRNA gave rise to many more internal siRNAs relative to terminal siRNAs than processing of 106 3′ovr-3′ovr dsRNA.

To rule out sequence-specific effects, we also monitored the reaction of 82 nt dsRNAs (82 BLT-BLT; 82 3′ovr-3′ovr) that had

**Figure 4. Purified *Drosophila* Dicer-2 Discriminates Duplex Termini**

(A) PhosphorImage of products separated by 17% denaturing PAGE after a 30 min incubation of 0.8 nM ^{32}P-end-labeled (bottom strand) 40/42 dsRNAs (as in Figure 2C) in cleavage buffer without Dicer (−) or with 30 nM wild-type (WT) Dicer-2 or helicase mutants as indicated. All reactions contained 8 mM ATP except those lacking ATP (-ATP).

(B) Data from multiple northern blot analyses (probes as in Figure 3A) from cleavage reactions of 106 3′ovr-3′ovr and BLT-BLT with WT Dicer-2 with incubation times as indicated were quantified to determine the ratio of siRNAs derived from internal cleavage to those from 5′ and 3′ termini. Percent siRNA, as in Figure 2D; error bars = standard error of the mean (n ≥ 3).

(C) PhosphorImage of northern blot showing products electrophoresed as in (A), produced by incubation of 1 nM 82 BLT-BLT or 3′ovr-3′ovr dsRNAs in cleavage buffer (30 min), without (−) or with 10 nM wild-type or K34N Dicer-2, without ATP (−ATP) or with 5 mM ATP (+ATP). Asterisks denote cleavage intermediates. Left blot, internal probe; right blot, 3′ end probe; FL, full-length 82 dsRNA. siRNA sizes were determined by comparing to AH ladder. See also Figure S3.

a different sequence from the 106 dsRNA (see Supplemental Experimental Procedures). We reacted 82 dsRNAs with wild-type and K34N *Drosophila* Dicer-2 in the presence or absence of ATP and performed northern blot assays with probes for terminal and internal siRNAs (Figure 4C). Our results for Dicer-2 (wild-type and K34N) with 82 dsRNAs were similar to those ob-

tained with *C. elegans* wild-type and G492R mutant extracts with 106 dsRNAs. Wild-type and K34N Dicer-2 produced similar levels of terminal siRNAs (3′ probe) from 3′ovr-3′ovr dsRNAs, but the K34N Dicer-2 was much less efficient in processing terminal siRNAs from the BLT-BLT dsRNA compared to wild-type Dicer-2; in all cases the production of the terminal siRNA
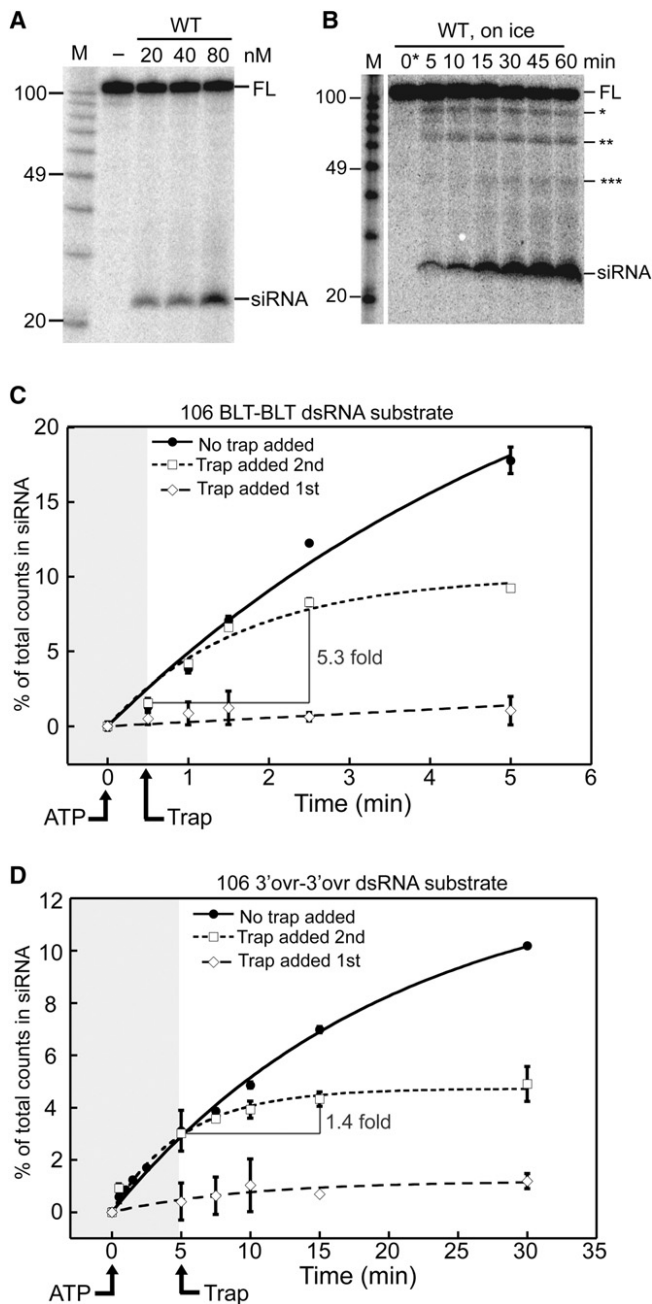
Figure 5. Dicer-2 Exhibits Two Modes of Cleavage, Depending on dsRNA Termini

(A) PhosphorImage of products resolved by 12% denaturing PAGE after incubation of 0.5 nM ^{32}P internally labeled 104 bp dsRNA in cleavage buffer (30 min; 24°C) with varying wild-type (WT) Dicer-2 and 5mM ATP. M, RNA decade markers, labeled for length (nt) by comparison to AH and P1 nuclease products of end-labeled ^{32}P-104 bp dsRNA. Mobility of full-length dsRNA (FL) and siRNA are marked.

(B) As in (A) except wild-type Dicer-2 was constant (80 nM), reactions were on ice, and reaction time was varied. Asterisks denote intermediates with one (*), two (**), or three (***) cleavages. 0*, aliquots taken immediately (≤10 s) after starting reaction with ATP.

(C and D) Quantification of multiple trap experiments using 80 nM Dicer-2 and 0.5 nM ^{32}P internally labeled 106 BLT-BLT (C) or 106 3'ovr-3'ovr (D) dsRNA. All reactions were initiated by adding ATP (5 mM) followed shortly

was stimulated by ATP. Importantly, as observed with the *C. elegans* extracts, a functional helicase domain and ATP were required for efficient production of internal siRNAs (internal probe) with a BLT-BLT dsRNA; however, the K34N mutant differed from the G492R mutant in that a small amount of internal siRNAs was observed after incubation of the BLT-BLT dsRNA in the presence of ATP. In contrast to experiments with the *C. elegans* extracts, in the purified system we were able to use defined levels of Dicer. Under the conditions of this experiment (10-fold more Dicer than dsRNA), reaction of the 3'ovr-3'ovr, but not BLT-BLT dsRNA, gave rise to cleavage intermediates (Figure 4C, asterisks).

Multiple siRNAs Are Produced from dsRNA Containing Blunt Termini without Dicer-2 Dissociation

Our studies of DCR-1 in *C. elegans* extracts, and purified *Drosophila* Dicer-2, indicated the helicase domain engages blunt termini, but not 3' overhangs, in a way that facilitates the production of siRNAs from internal regions of dsRNA. Dicer cleaves from termini, and one explanation for the inefficient production of internal siRNAs from dsRNAs with 3' overhangs is that access to internal siRNAs requires multiple reactions, each involving Dicer binding, cleavage, and dissociation. By contrast, if production of multiple siRNAs from dsRNA with blunt termini could occur without Dicer dissociation, this would explain the greater accumulation of internal siRNAs with these dsRNAs.

This idea was consistent with the fact that with purified *Drosophila* Dicer-2 it was difficult to observe intermediate cleavage products in reactions of dsRNA with blunt termini (Figure 4C). In addition, by using a 104 bp dsRNA with a 1 nt 5' overhang that was internally labeled with ^{32}P-104 dsRNA (FL, Figure 5A), we verified that under single-turnover conditions (excess Dicer-2), intermediates were not observed. This was as expected if Dicer-2 was rapidly cleaving dsRNA to completion without dissociation. However, we reasoned we should be able to detect cleavage intermediates if we could slow the reaction and trap RNA-Dicer-2 complexes prior to complete cleavage of the dsRNA. To this end, we performed Dicer-2 reactions with ^{32}P-104 dsRNA on ice (Figure 5B). Indeed, under these conditions we observed intermediates (asterisks, Figure 5B). Similarly, a slow cleavage rate may explain intermediates detected with *C. elegans* extracts when analyzing a long dsRNA with blunt termini (Figure 3A).

To provide further evidence that blunt termini triggered a reaction whereby multiple siRNAs along the length of the dsRNA were produced without Dicer-2 dissociation, we used a pulse-chase approach. Dicer-2 was allowed to cleave ^{32}P internally labeled dsRNA for a short time to allow Dicer-2-dsRNA complex formation, followed by the addition of a vast excess of

nonradiolabeled (cold) dsRNA trap. If Dicer-2 cleaved distributively, dissociating and rebinding after each cleavage event, addition of trap should quench the reaction and siRNAs should fail to accumulate after trap addition. In contrast, siRNAs should continue to accumulate even after the addition of trap if multiple siRNAs could be produced along the length of a dsRNA without Dicer-2 dissociation.

We first monitored the reaction of internally labeled ^{32}P-106 BLT-BLT or ^{32}P-106 3′ovr-3′ovr dsRNA in the absence of all trap (Figures 5C and 5D, filled circles, solid lines) and also confirmed that 2000-fold excess of cold trap (82 BLT-BLT) over ^{32}P-labeled dsRNA was sufficient to quench the reaction if added at the beginning of the reaction (diamonds, dashed lines). We then reacted each ^{32}P-dsRNA for a short time (Figures 5C and 5D, shaded), followed by the addition of trap dsRNA. Quantification of multiple experiments revealed that even after the addition of trap, siRNAs continued to accumulate from ^{32}P-106 BLT-BLT dsRNA (Figure 5C), increasing 5.3 fold in the 2 min after trap addition. In contrast, siRNAs from 3′ovr-3′ovr dsRNA increased only 1.4 fold in the 10 min after trap addition; this small increase may be due to the final cleavage event for Dicer-2 productively bound to dsRNA immediately prior to the trap addition. While multiple siRNAs were clearly produced without Dicer-2 dissociation from the ^{32}P-106 BLT-BLT dsRNA, siRNAs did not reach the same maximum after trap addition as in the complete absence of trap, suggesting a small amount of dissociation occurred from all reacting complexes or, alternatively, that a small subset of complexes was not resistant to trap.

DISCUSSION

We compared the activity of wild-type Dicer and Dicer containing point mutations in its helicase domain by using *C. elegans* extracts or purified recombinant *Drosophila* Dicer-2. In both systems we found that the helicase domain allows Dicer to discriminate between dsRNA with different termini. Our studies indicate the helicase domain is required for efficient cleavage of dsRNA with blunt or 5′-overhanging termini, but not of dsRNA with 3′-overhanging termini. The latter agrees with previous in vivo studies that indicate the helicase domain is required for processing certain endo-siRNAs, but not miRNAs, which have a 3′ overhanging terminus (Pavelec et al., 2009; Welker et al., 2010).

By using long dsRNAs that can accommodate multiple cleavages, we observed that the helicase domain also increases production of siRNAs from internal regions of dsRNA for molecules with blunt, but not 3′ overhanging, termini. Further, for Dicer-2 we found that siRNAs continue to accumulate from dsRNA with blunt, but not 3′ overhanging, termini after the addition of a vast excess of trap dsRNA. Taken together, our data suggest a model whereby dsRNA with blunt or 5′-overhanging termini engage the helicase domain in a way that leads to the production of multiple siRNAs along the length of a dsRNA without Dicer dissociation; such an activity is suited for the production of endo-siRNAs from a long dsRNA precursor, but unnecessary for the single cleavage event required for miRNA processing.

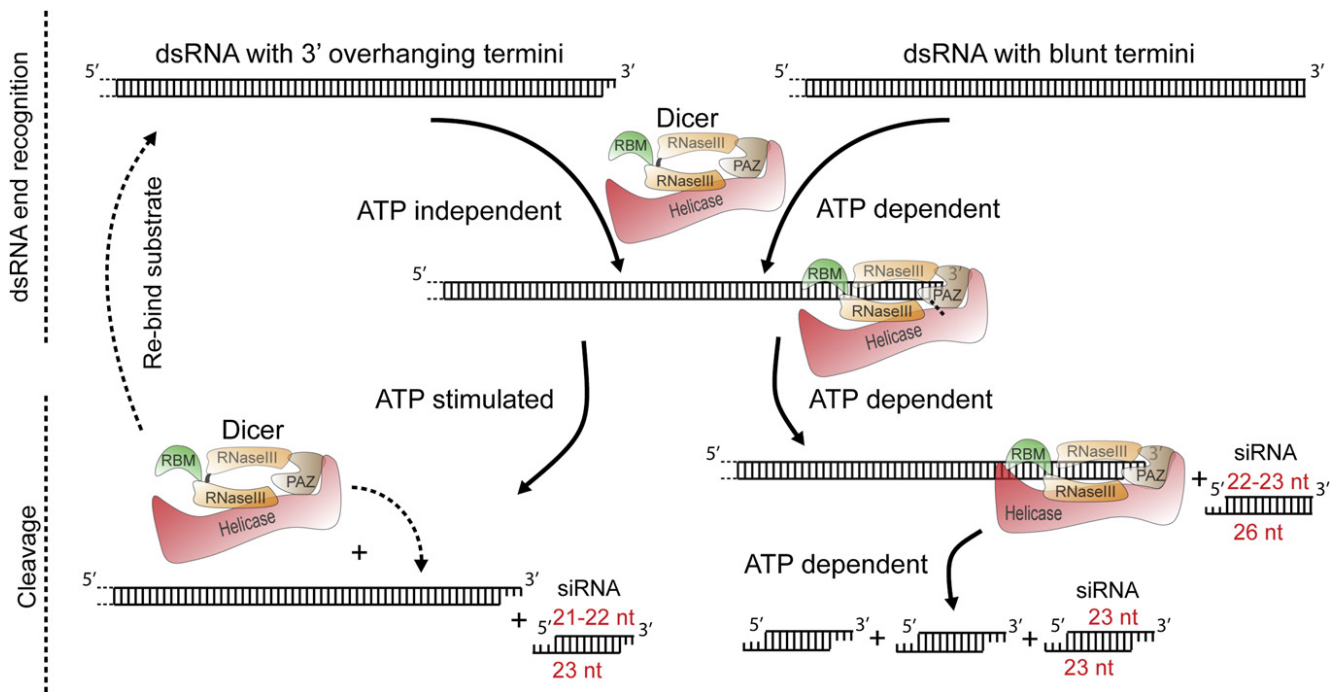## Model for Helicase-Dependent Cleavage of dsRNA by Dicer

Figure 6 illustrates a model for the function of Dicer's helicase domain based on data we present here and on other data existing in the literature. Given the properties of the helicase mutants and the observed ATP requirements, we describe the reaction in two steps: dsRNA end recognition and cleavage. However, because our assays only monitored cleavage, the first step is inferred. Binding of the Piwi Argonaute Zwille (PAZ) domain to the 3′ terminus of dsRNA is crucial for orienting the RNase III domains for cleavage (MacRae et al., 2007), and our model shows this as the first step, regardless of the terminal structure of dsRNA. However, as illustrated, this would be an ATP-independent step for dsRNA with 3′ overhangs, but for blunt termini would require ATP, possibly to enable the helicase domain to unwind termini, allowing the PAZ domain access to the 3′ end. Assuming ATP dependence equates to a dependence on the helicase domain, this aspect of the model is consistent with our observation that *C. elegans* G492R mutant extracts, or the *Drosophila* K34N mutant, are defective for cleavage of dsRNA with blunt or 5′ overhangs but show wild-type levels of terminal siRNA from dsRNA with 3′ overhangs (Figures 2C and 2D, 3A and 3B, 4A and 4C).

Depending on duplex termini, end recognition can be ATP independent or ATP dependent, but in our model the consequence is the same: interaction of the PAZ domain with the 3′ terminus. However, in our model, subsequent steps differ depending on duplex termini. With duplexes containing 3′-overhanging termini, Dicer acts distributively, dissociating from the dsRNA after each cleavage event, followed by rebinding and another cleavage. This aspect of the model is supported by the observation that production of siRNAs from internal regions of a dsRNA with 3′ overhangs is inefficient (Figures 3B and 4B) and in trap experiments siRNAs ceased to be produced from 3′ovr-3′ovr dsRNA after trap was added (Figure 5D).

While we observed a small amount of cleavage from 3′-overhanging termini in the absence of ATP (Figure 4A, lane 2), we show this cleavage step as ATP stimulated (Figure 6), because addition of ATP increased siRNA production by wild-type *Drosophila* Dicer-2 (Figure 4A, lane 1). As yet we do not know the role of ATP in this step. ATP binding or hydrolysis could be important for turnover, or as observed in other helicases (Hopfner and Michaelis, 2007), for a protein conformational change, in this case to stabilize Dicer binding to duplex termini.

The model proposes that multiple siRNAs are produced along the length of dsRNA with blunt or 5′-overhanging termini, without Dicer dissociation. This aspect of the model is supported by our observation that the helicase domain is required for (Figures 3A and 3B) or greatly stimulates (Figures 4B and 4C) the accumulation of siRNAs from internal regions of dsRNA with blunt termini, which is completely dependent on ATP (Figures 3C and 4C). Further, with purified *Drosophila* Dicer-2, siRNAs accumulate without the appearance of intermediates (Figure 5A). Finally, during trap experiments siRNAs continue to accumulate from BLT-BLT dsRNAs even after the addition of 2000-fold excess trap, indicating that multiple siRNAs are produced without Dicer-2 dissociation (Figure 5C). Many DEXH helicases act as translocases that couple ATP hydrolysis to movement along

Figure 6. Model for Termini-Dependent Cleavage of dsRNA by Dicer

Two steps, end recognition and cleavage, are shown for dsRNA with 3'-overhanging termini (top left) or blunt termini (top right) reacting with Dicer (shown with color-coded domains). A common intermediate is shown for end recognition (dots, nt of blunt dsRNA); this step is ATP independent for dsRNA with 3'-overhanging termini, but for blunt termini requires ATP. Subsequently, cleavage of either dsRNA produces siRNA (red font, lengths based on *C. elegans* DCR-1). For dsRNA with 3' overhangs, Dicer dissociates after cleavage and subsequent cleavage events require rebinding. Blunt or 5' overhangs trigger a conformational change that allows multiple siRNAs to be produced along the length of the dsRNA without dissociation. We show siRNAs with 3 nt overhangs, but 4 nt overhangs were also observed in *C. elegans* extracts.

a nucleic acid (Lohman et al., 2008; Singleton et al., 2007). In fact, the ATP dependence of *Drosophila* Dicer-2 is proposed to reflect a role of the helicase domain in translocation along dsRNA (Bernstein et al., 2001; Hutvágner and Zamore, 2002; Nykänen et al., 2001). Our data are consistent with the idea that Dicer-2 acts processively, with a single enzyme translocating along the length of a dsRNA to catalyze multiple cleavage events without dissociation. However, at present we cannot rule out other mechanisms in which Dicer would not be strictly processive. For example, our data are also consistent with a model whereby blunt and 5'-overhanging dsRNAs trigger a cooperative oligomerization of Dicer along the length of the dsRNA. While multiple siRNAs would be produced along the length of dsRNA without Dicer dissociation, each Dicer would produce only a single siRNA prior to dissociation, and thus would not be considered processive.

Dicer's PAZ domain acts as part of a ruler that measures from the 3' end of dsRNA to specify the cleavage site (MacRae et al., 2006, 2007). By using *C. elegans* extracts, we observed that duplexes with 3'-overhanging termini were cleaved at 21–22 nt from the 3' terminus, while dsRNA with blunt termini were cleaved at 22–23 nt from the 3' terminus. Unexpectedly, *C. elegans* cleavage products from both blunt and 3'-overhanging dsRNAs showed noncanonical 3 and 4 nt 3' overhangs. Here it is important to note that after cleavage to remove the first siRNA from the end of a dsRNA, all dsRNAs have 3' overhangs

and subsequent cleavage events yield 23 nt siRNAs for all dsRNAs. Despite this, subsequent cleavage of dsRNA with blunt and 3' overhangs is very different, emphasizing that the former is cleaved along its length without dissociation.

Previous studies in *C. elegans* indicate that miRNAs represent about 80%–90% of all small RNAs, with primary endo-siRNAs accounting for only a small fraction, ∼1% (Ruby et al., 2006; Welker et al., 2010). Endo-siRNA precursors are likely of low abundance, and multiple cleavage events along the dsRNA length without Dicer-2 dissociation would be an efficient way to maximize endo-siRNA levels.

Implications for the Role of Dicer's Helicase Domain in Processing Small RNAs of Other Organisms

Precursors of miRNAs have been analyzed in many organisms, and all are assumed to have 2 nt 3' overhangs. Our data suggest processing of these small RNAs will not require Dicer's helicase domain. This is supported by the existence of a second Dicer in *D. melanogaster* (Dicer-1), which is dedicated to miRNA processing and lacks a functional helicase domain. Further, *C. elegans* with mutations in the helicase domain of DCR-1 are viable and have wild-type miRNA levels (Pavelec et al., 2009; Welker et al., 2010).

While endo-siRNAs have been identified in several organisms (Golden et al., 2008) their precursors are ill-defined. *D. melanogaster* endo-siRNAs arise from loci predicted to form long,

intramolecular hairpins, as well as sense and antisense transcripts of overlapping genes. In plants, endo-siRNAs arise from dsRNA synthesized by an RNA-dependent RNA polymerase (RdRP) from a noncoding template RNA (*trans*-acting small interfering RNAs) or from natural antisense transcripts (natural antisense small interfering siRNAs; Chen, 2009). While a requirement of Dicer's helicase domain for processing these precursors has not been tested, our studies predict this highly conserved domain will be required for cleavage of dsRNA with blunt or 5′ overhangs in all organisms. That said, in vitro experiments with human Dicer have yet to reveal a requirement for ATP or the helicase domain in dsRNA processing; rather, this domain is inhibitory (Ma et al., 2008; Zhang et al., 2002). Given the propensity of dsRNA to trigger the interferon response in mammalian cells, cleavage of such molecules may be under tight regulation and require additional proteins.

Long dsRNA precursors of endo-siRNAs may not accumulate when Dicer cleavage is coupled to dsRNA synthesis by an RdRP, as in *Tetrahymena thermophila* and *S. pombe* (Colmenares et al., 2007; Lee and Collins, 2007). For example, accumulation of dsRNA in *S. pombe* is only observed when Dicer is mutated to disrupt its RNase III activity, leading to a model whereby Dicer cleaves dsRNA as soon as it is synthesized by the RdRP. It seems likely that at least some *C. elegans* endo-siRNAs are produced by an RdRP-coupled mechanism, because DCR-1 is found in a complex that includes the RdRP RRF-3, and a 3′ to 5′ exonuclease, ERI-1 (Duchaine et al., 2006; Kennedy et al., 2004). RRF-3 might synthesize dsRNA with heterogenous termini that are subsequently polished by ERI-1 to create blunt termini that require DCR-1's helicase domain for processing. Cleavage from the blunt terminus would yield a 26 nt endo-siRNA, while subsequent cleavages would produce shorter siRNAs. Interestingly, RRF-3, ERI-1, and DCR-1's helicase domain are all necessary for the production of 26G endo-siRNAs (Gent et al., 2010; Han et al., 2009; Vasale et al., 2010; Welker et al., 2010), which are crucial for endo-siRNA-mediated gene silencing in *C. elegans* (Gent et al., 2010; Vasale et al., 2010).

EXPERIMENTAL PROCEDURES

C. elegans Extract Cleavage Assay

Fifty microliter reactions containing extract cleavage buffer (30 mM HEPES [pH 7.4], 100 mM KOAc, 10% glycerol, 1 mM DTT, 80 units RNasin, 0.4 nM dsRNA, 4 mM ATP, and 6.5–12 mM MgOAc as indicated) were incubated at 20°C with 40 μg *C. elegans* embryo extract [wild-type, N2; G492R, *dcr-1(mg375)*], prepared as described (Parker et al., 2006). dsRNA sequences and preparation are described in Supplemental Experimental Procedures. Experiments used *dcr-1(mg375)* strain YY011, reported to contain a secondary mutation, *mut-16(mg461)*(Gent et al., 2010); subsequent experiments with strain YY470, which lacks the mutation, gave identical results. Reactions were stopped with an equal volume of phenol/CHCl₃/isoamyl alcohol (25:24:1) followed by organic extraction and ethanol precipitation after adding 15 μg glycogen. RNAs were resolved by 17% denaturing PAGE and either exposed wet at −20°C overnight on a PhosphorImager screen (^{32}P-end-labeled dsRNAs), or subjected to northern blot analysis (cold dsRNAs). For northern blots, nucleic acid was transferred from gels to Hybond-NX membrane (Amersham) in a wet transfer cell (80 V, 1 hr; 0.5 × TBE). Blots were crosslinked with EDC (30 min, 60°C; Pall et al., 2007) and incubated with 3 pmol of ^{32}P-end-labeled DNA probe (for sequences, see Supplemental Experimental Procedures) at 42°C in ULTRAhyb-Oligo buffer (Ambion). Membranes were washed three to four times at 42°C in 2× to 4× SSC plus 0.1–0.2% SDS and exposed

on a PhosphorImager screen (Molecular Dynamics). Between probings blots were stripped by rotating at 80°C with 20 mM Tris (pH 7.5), 1 mM EDTA, 1% SDS, three to four times over 1.5 hr and exposed to ensure all radioactivity was removed. Data were quantified by using ImageQuant software. AH ladder was prepared by incubating 20 fmol of ^{32}P-end-labeled dsRNA and 10 μg of nonspecific (torula) RNA in 10 μl 50 mM sodium carbonate (pH 9.0, NaHCO₃/Na₂CO₃; 85°C, 10 min).

Dicer-2 Cleavage Assay

Wild-type and mutant Dicer-2 were cloned, overexpressed, and purified as described (Ye and Liu, 2008; see Supplemental Experimental Procedures). Dicer-2 cleavage assays were at 24°C for times indicated, in cleavage buffer (30 mM HEPES [pH 7.4], 100 mM KOAc, 10 mM MgOAc, 1 mM DTT, 50 units RNasin) with Dicer-2 and dsRNA as specified. Protein and RNA were preincubated (15 min, 24°C) and reactions were started by adding ATP-MgOAc (5 mM final). Reactions of 40/42 dsRNAs were in cleavage buffer except with 8 mM final ATP-MgOAc and reactions were initiated by adding protein.

Dicer-2 cleavage reactions were stopped by adding an equal volume of 2× formamide loading buffer (85% formamide, 0.5× TBE, 50 mM EDTA [pH 8.0], 0.05% bromophenol blue and 0.05% xylene cyanol). Products were separated by 12% denaturing PAGE unless indicated, visualized on a PhosphorImager screen (Molecular Dynamics), and quantified by using ImageQuant software. Northern blots were performed as for *C. elegans* in vitro cleavage assays.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.molcel.2011.02.005.

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