Short Interfering RNA Strand Selection Is Independent of dsRNA Processing Polarity during RNAi in Drosophila

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Summary

Short interfering RNAs (siRNAs) guide mRNA cleavage during RNA interference (RNAi) [1–3]. Only one siRNA strand assembles into the RNA-induced silencing complex (RISC) [4], with preference given to the strand whose 5′ terminus has lower base-pairing stability [5, 6]. In Drosophila, Dcr-2/R2D2 processes siRNAs from longer double-stranded RNAs (dsRNAs) [7, 8] and also nucleates RISC assembly [7, 9, 10], suggesting that nascent siRNAs could remain bound to Dcr-2/R2D2. In vitro, Dcr-2/R2D2 senses base-pairing asymmetry of synthetic siRNAs and dictates strand selection by asymmetric binding to the duplex ends [11]. During dsRNA processing, Dicer (Dcr) liberates siRNAs from dsRNA ends [1, 12] in a manner dictated by asymmetric enzyme-substrate interactions [13]. Because Dcr-2/R2D2 is unlikely to sense base-pairing asymmetry of an siRNA that is embedded within a precursor, it is not clear whether processed siRNAs strictly follow the thermodynamic asymmetry rules [5, 6] or whether processing polarity can affect strand selection [1]. We use a Drosophila in vitro system in which defined siRNAs with known asymmetry can be generated from longer dsRNA precursors. These dsRNAs permit processing from specifically either the 5′ or the 3′ end of the thermodynamically favored strand of the incipient siRNA. Combined dsRNA-processing/mRNA-cleavage assays indicate that siRNA strand selection is independent of dsRNA processing polarity during Drosophila RISC assembly in vitro.

Results and Discussion

Independent Variation of siRNA Base-Pairing Asymmetry and Dcr Polarity In Vitro

If the Dcr-2/R2D2 heterodimer establishes asymmetric contacts with an incipient siRNA during dsRNA processing [13] before it can sense the relative base-pairing stabilities of the siRNA ends, then the polarity of Dcr processing could modulate strand selection when siRNAs are generated from longer precursors [14]. To test this possibility, we sought a biochemical system in which the relevant variables (the polarity of Dcr processing and the thermodynamic asymmetry of the processed siRNAs) could be independently controlled. Tuschl and coworkers previously showed that RNA duplexes as short as 39 nt (but not 29 nt) are efficiently diced in Drosophila embryo lysates and that a ~20 nt 3′ overhang blocks Dcr processing at the extended end [1]. On the basis of these observations, we designed two pairs of short dsRNA triggers, each forcing Dcr to initiate cleavage from opposite ends to generate a single, common 21 nt siRNA (see the Supplemental Experimental Procedures in the Supplemental Data online for specific sequences). One of these pairs forces unidirectional processing to generate a common asymmetric siRNA derived from the human Cyclophilin B (cyc) gene, and the other pair generates a common symmetric siRNA derived from the human Cu, Zn-superoxide dismutase (sod) gene [5] (Figure 1A). The triggers that force Dcr initiation from the 2 nt 3′-overhanging end of the sense or antisense strand are referred to as “Dcr-R” or “Dcr-L,” respectively (Figure 1A).

For the cyc dsRNAs, Dcr-R forces Dcr to enter from the more weakly base-paired end of the cyc siRNA (blue in Figure 1A, top), and Dcr-L forces Dcr to enter from the more stable end (red in Figure 1A, top). In the case of cyc Dcr-R, selection of the siRNA strand with the exposed 3′ end is disfavored on the basis of thermodynamic asymmetry [5, 6]. Elbashir et al. [1] previously reported preliminary evidence that the processed siRNA strand with the 3′ terminus exposed in the precursor is preferentially selected during RISC assembly. Therefore, the cyc Dcr-R dsRNA places the two reported strand-selection parameters in conflict (Figure 1B). We included the sod triggers to test whether dsRNA processing could impart asymmetric strand selection on a processed siRNA that is thermodynamically symmetric [5]. In each case, the sequence immediately outside of the predicted dsRNA processing site was designed to preserve the relative thermodynamic end stabilities in the event of Dcr-2 cleavage heterogeneity.

To confirm that the Dcr-R and Dcr-L dsRNAs gave rise to the expected siRNA products, we 5′-radiolabeled the longer (64 nt) strand, allowing us to track the progress of the dsRNA processing step by electrophoresis in a 15% denaturing polyacrylamide gel. All of the dsRNAs were efficiently and specifically processed into the predicted 21 and 22 nt siRNAs in vitro (Figure 2). We confirmed that the shorter strand of the dsRNA was also accurately diced by labeling this strand and observing the appearance of the expected dsRNA processing products (Figure S1).

Guide Strand Selection Is Independent of Dicer Processing

To test whether Dcr processing polarity influences siRNA strand selection, we performed a combined dsRNA-processing/target-cleavage assay. To examine target mRNA cleavage, we added 5′-cap-radiolabeled sense or antisense mRNA target (to assess relative siRNA strand selection) to the dsRNA processing reactions shown in Figure 2B and Figure S2. The targets were

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added after dsRNA processing and RISC assembly had proceeded for 15 min (for the sod triggers) or 30 min (for the cyc triggers). Following further incubation, we analyzed the reactions in a 6% denaturing polyacrylamide gel to detect the cleaved mRNA target. Because the concentration of dsRNA in each reaction was below saturation for target cleavage [15], we normalized the fraction of target cleaved to the extent of Dcr processing in each reaction as determined by the fraction of 21–22 nt siRNA produced relative to the 64 nt precursor (Figure 2A and Figure S2).

The cyc and sod siRNAs directed cleavage of sense and antisense targets with the expected levels of asymmetry [5] (Figures 3A and 3C, left panels, and Figures 3B and 3D, black circles). cyc siRNA cleaved ~40% of cyc sense target but only ~5% of antisense target in 5 hr, whereas sod siRNA cleaved ~60% of both sense and antisense targets in 2 hr. In all cases, the sod and cyc Dcr-R triggers (Figures 3B and 3D, orange triangles) and Dcr-L triggers (green squares) were equally efficient at cleaving their corresponding targets and generally followed the expected thermodynamic asymmetry pattern. Unexpectedly, both of the sod 3' -extended dsRNAs directed less target cleavage than the synthetic siRNA (Figure 3D, upper panel). Although we do not know the reason for the reduced potency of the processed dsRNAs with this target, it cannot be explained as a result of differential strand selection induced by the polarity of Dcr processing, given that the reduction affected both sod Dcr-R and sod Dcr-L dsRNAs equally. Because both pairs of Dcr-R/Dcr-L triggers showed identical potency in guiding cleavage of their respective sense and antisense targets, we conclude that the direction of Dcr processing is not likely to influence selection of the guide strand in Drosophila embryo lysates.

To directly test whether a processed siRNA proceeds into RISC without dissociating from the dsRNA processing machinery, we performed a chase experiment with sod Dcr-L dsRNA that was prebound to Dcr in vitro (Figure 4A). We first confirmed that Drosophila Dcr-2 is capable of binding but not cleaving dsRNA when incubated at 4°C in embryo lysates (Figure S3), as is the case for human Dcr (hDcr) [12]. To chase newly diced siRNA into RISC, we first preincubated sod Dcr-L dsRNA in embryo lysate for 20 min on ice at a concentration close to saturation for RISC assembly (50 nM) [15]. An unrelated competitor siRNA was then added to the reaction mix on ice at various saturating concentrations, up to 250-fold over that of sod Dcr-L dsRNA. The reactions were shifted to 25°C, and radiolabeled...
sense and antisense mRNA targets were added for 1 hr to measure RISC assembly. If the newly processed siRNA was directly loaded into RISC without first being released, then the unrelated siRNA would fail to compete during RISC assembly. Regardless of whether the competitor siRNA was added during or after the preincubation with sod Dcr-L, we observed strong and comparable inhibition of both sense and antisense sod target cleavage, consistent with the idea that a freshly diced siRNA is released from Dcr before it can initiate RISC assembly in Drosophila embryo lysate (Figures 4B and 4C).

Synthetic siRNAs Are More Potent than Dicer-Substrate dsRNAs in Drosophila Eggs

In mammalian cells, Dcr substrates (such as short hairpin RNAs or 27 nt duplexes) exhibit greater silencing potency than 21 nt siRNAs [16–18], suggesting that Dcr processing might facilitate entry into the RISC assembly pathway. To test whether the same is true in the Drosophila RNAi system, we injected 0–1 hr fertilized eggs with siRNA or 36 bp Dcr-substrate dsRNA (with a 2 nt 3' overhang) directed against bicoid (bcd) mRNA. The 36 bp dsRNA was designed such that Dicer would produce an siRNA identical to the synthetic bcd siRNA when processing was initiated from the 3' end of the guide strand. We confirmed that this 36 bp dsRNA (like the 36 bp sod dsRNAs used in Figure 2C) was a competent dsRNA processing substrate in vitro (data not shown). At every concentration tested, the Dcr-substrate dsRNA was approximately half as potent at reducing bcd mRNA levels as the corresponding “pre-diced” siRNA, as measured by RT-PCR analysis (Figures 4D and 4E). The reduced activity of Dcr-substrate dsRNAs in vivo suggests that RISC assembly and Dicer processing are not coupled in Drosophila eggs, contrary to findings in mammalian cells [16–18].

Implications for siRNA Strand Selection during RNAi In Drosophila, Dcr enzymes are required for RISC assembly as well as dsRNA processing [8, 9], suggesting that the two phases of RNAi might be functionally coupled in a manner that affects siRNA strand selection [1, 14]. However, our experiments indicate that Drosophila RISC assembly and siRNA strand selection are not significantly influenced by the dsRNA processing step and that the thermodynamic asymmetry rules [5, 6] apply equally well with processed and unprocessed siRNAs in this system. This suggests that Drosophila Dcr enzymes do not channel newly generated siRNAs directly into RISC, but rather release the siRNAs into solution (or onto another factor) before they enter the RISC assembly pathway.

Several observations have suggested that thermodynamic asymmetry governs strand selection for processed RNAi triggers. MicroRNAs (miRNAs) are diced from stem-loop precursors, and in most instances only one strand of the processed miRNA duplex is stably incorporated into RISC [19]. The mature strand can be present at either the 5’ or the 3’ end of the stem-loop, but either way, the selected strand is generally compatible with the thermodynamic asymmetry guidelines [5]. In addition, artificial dsRNAs introduced into plant cells give rise to a stable set of siRNAs [20] that adhere to the asymmetry rules [6]. Similar results have been reported with natural dsRNAs in plants [21]. However, interpretation of these results is difficult because the Dcr processing polarities were not defined, and it is also not clear whether small RNA stability is always a suitable surrogate measure of RISC assembly. Furthermore, plant cells (unlike insect and mammalian cells) export...
siRNAs into the vasculature to enable systemic RNAi, and therefore the plant dsRNA processing machinery may have specifically evolved the propensity to release newly processed siRNAs. Thus the applicability of the plant analyses to insects and mammals has not been clear.

While this work was in progress, Rose et al. [18] characterized modified 27 nt duplexes that force a defined Dcr processing polarity and give rise to specific, predictable 21 nt siRNAs. Experiments with these Dcr substrate RNAs revealed that hDcr processing polarity can in fact influence siRNA strand selection in transfected human cells, although it does not completely supercede thermodynamic asymmetry [18]. The reasons for the discrepancy between our results and those of Rose et al. [18] are not clear, although one possibility is that different Dcr enzymes may vary in their tendencies to remain associated with newly generated siRNAs. It is noteworthy that Drosophila Dcr-2 (which is primarily devoted to the siRNA pathway [7, 8]) appears to lack the canonical PAZ domain that normally provides Dcr with a binding pocket for 2 nt 3' overhangs [22, 23]. A PAZ domain is present in hDcr, and mutational analyses indicate that the hDcr PAZ domain assists with dsRNA binding and processing when a 2 nt 3' overhang is present [13]. The apparent lack of a PAZ domain in Dcr-2 may compromise its ability to remain bound to newly cleaved siRNA. It is curious that Drosophila Dcrs are required for RISC assembly [8] but do not appear to couple dsRNA processing to siRNA strand selection, whereas mammalian Dcrs are not required for RISC assembly [4, 24, 25] but apparently do couple dsRNA processing to siRNA strand selection [16–18, 26].

Finally, it remains to be determined whether Dcr enzymes associate with long dsRNA processing substrates and siRNA RISC-assembly substrates in the same way. This issue is undoubtedly important for understanding the functional relationship between Dcr’s roles in the initiator and effector phases of RNAi. Crystal structures of E. coli RNase III [27, 28], an ancestor of eukaryotic Dcrs, are likely to be informative. The structural data, and models derived from them, depict a protein that can engage dsRNAs in a dynamic fashion. A single dsRBD on each subunit of the RNase III homodimer is tethered to the endonuclease domain by a flexible linker that can rotate roughly 90º around the catalytic core [28]. Thus, there are likely to be at least two binding modes for dsRNA in complex with an RNase III enzyme: one in which the dsRBD braces the RNA helix from either side as it is channeled into the catalytic cleft [13, 29], and another where the dsRBD holds the dsRNA above and orthogonal to the active site [28]. It is possible that Dcr enzymes also exhibit alternate dsRNA binding modes depending on whether they are actively processing dsRNA or channeling siRNA into RISC. Interconversion between these two conformations may require at
least transient release of the siRNA product. Additional
dynamic dsRNA/protein interactions during dsRNA pro-
cessing and RISC assembly presumably involve the
dsRNA binding proteins Loquacious/R3D1 [30–32],
R2D2 [7], and TRBP [33, 34] , which associate with
Dcr-1, Dcr-2, and hDcr, respectively. Further functional
analysis of Dcr’s PAZ, RNase III, and dsRNA binding do-
mains, aided by recent advances in the structural biol-
ogy of Dcr [35], will be necessary to understand Dcr’s
roles in the transition between the initiation and effector
phases of RNAi.

Supplemental Data
Supplemental Data include Supplemental Experimental Procedures
and four figures and are available with this article online at:http://
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