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siRNA Repositioning for Guide Strand Selection by Human Dicer Complexes

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SUMMARY

The human ribonuclease Dicer and its double-stranded RNA (dsRNA) binding protein (dsRBP) partners TRBP and PACT play important roles in the biogenesis of regulatory RNAs. Following dicing, one dsRNA product strand is preferentially assembled into an RNA-Induced Silencing Complex (RISC). The mechanism of strand selection in humans and the possible role of Dicer in this process remains unclear. Here we demonstrate that dsRNAs undergo significant repositioning within Dicer complexes following dicing. This repositioning enables directional binding of RNA duplexes, thereby biasing their orientation for guide strand selection according to the thermodynamic properties of the helix. Our findings indicate that Dicer is itself capable of sensing siRNA thermodynamic asymmetry regardless of the dsRBP to which it is bound. These results support a model in which Dicer employs two distinct RNA binding sites – one for dsRNA processing and the other for sensing of siRNA thermodynamic asymmetry – during RISC loading in humans.

INTRODUCTION

RNA interference (RNAi) and related pathways are conserved modes of post-transcriptional gene silencing in which single-stranded guide RNAs bind to cognate mRNAs and direct their endonucleolytic cleavage or translational repression by RNA-induced silencing complexes (RISCs) (Jinek and Doudna, 2009; Siomi and Siomi, 2009). Silencing is initiated by long dsRNAs or hairpin pre-microRNAs, which are processed by the endonuclease Dicer to yield 21-23 nt short interfering RNAs (siRNAs) or microRNAs (miRNAs), respectively (Bernstein et al., 2001; Hutvagner et al., 2001; Zamore et al., 2000). These small dsRNAs are then loaded onto Argonaute2 (Ago2), the endonuclease component of RISC (Liu et al., 2004; Rivas et al., 2005). In humans, this process of RISC-loading involves both Dicer and TRBP (Chendrimada et al., 2005; Rivas et al., 2005). Together these proteins, along with Ago2, comprise the core of a complex termed the RISC-Loading Complex (RLC) (Gregory et al., 2005; MacRae et al., 2008). Another dsRBP called PACT also binds to Dicer, but its specific function is as yet poorly understood (Kok et al., 2007; Lee et al., 2006).

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RISC association with either an siRNA or a miRNA involves the selection of one strand of the dsRNA to be used as a guide for downstream targeting events. The other strand, termed the passenger (or miRNA* in the case of miRNAs), is cleaved and/or removed (Matranga et al., 2005; Rivas et al., 2005; Wang et al., 2009a). Based on crystallographic studies of a bacterial Argonaute homolog, Dicer-generated dsRNAs are thought to bind Argonaute such that the 3′ end of the guide strand fits into a hydrophobic pocket of the Ago PAZ domain (Wang et al., 2008b), and the 5′ end of the guide sits within a conserved basic pocket of the Mid domain (Frank et al., 2010; Ma et al., 2005). When bound in this way, the passenger strand likely makes few protein contacts and is primed for cleavage and/or dissociation (Parker et al., 2005; Wang et al., 2008a). This specific loading of the guide strand is essential to overall siRNA efficacy, and the parameters for designing siRNAs that maximize the loading of this strand in human cells have been the subject of significant scientific inquiry (Czech et al., 2009; Khvorova et al., 2003; Krol et al., 2004; Okamura et al., 2009; Sano et al., 2008; Schwarz et al., 2003).

How Dicer and its protein partners orient dsRNAs for proper guide strand loading has been unclear in humans, but recent structural work, together with studies of a homologous complex in *Drosophila*, have provided some clues. In thermodynamically asymmetric siRNA duplexes, the strand whose 5′-end lies at the less thermodynamically stable end of the helix is preferentially loaded onto Ago2 as the guide strand (Czech et al., 2009; Khvorova et al., 2003; Schwarz et al., 2003). In flies, this process is assisted by the Dicer-2/R2D2 heterodimer, which coordinately binds siRNAs in a directional manner such that the dsRBP R2D2 interacts with the more thermodynamically stable siRNA end and Dicer-2 interacts with the less stable end (Liu et al., 2006; Tomari et al., 2004b). It remains unclear which protein in the heterodimer represents the actual asymmetry sensor, and in humans the fact that both TRBP and PACT can bind to Dicer makes it difficult to draw a direct analogy to this fly sensor.

In the present study, we sought to use a structurally informed approach to determine how the Dicer/TRBP heterodimer orients nascent siRNA duplexes for strand-selective RISC loading. Recent structural studies using electron microscopy demonstrated that human Dicer has an L-shaped architecture. The long arm of Dicer contains the catalytic center and binds to Ago2, and the short arm contains the N-terminal helicase domain and binds to TRBP (Kok et al., 2007; Lau et al., 2009; Tahbaz et al., 2004; Wang et al., 2009b). Using reconstituted human Dicer/TRBP/dsRNA complexes, we show here that dsRNAs are released and repositioned along the helicase domain of Dicer following cleavage in order to enable sensing of siRNA thermodynamic asymmetry. We demonstrate that Dicer itself is capable of sensing this asymmetry, and that this functionality is activated upon association with either TRBP or PACT. Taken together, these findings support a model for human siRNA biogenesis in which two distinct RNA binding sites on Dicer enable the re-positioning of dsRNAs, allowing for sensing of siRNA thermodynamic asymmetry and strand-selective RISC loading.

RESULTS

The Dicer/TRBP heterodimer binds differently to substrate versus product RNAs

Negative-stain electron microscopy demonstrates that Dicer has an L-shaped structure, and that TRBP localizes to the distal end of Dicer adjacent to the N-terminal helicase domain, consistent with previous biochemical data (Lau et al., 2009; Lee et al., 2006; Wang et al., 2009b). Additionally, TRBP is required for high affinity binding of product, but not substrate dsRNAs (Chakravarthy et al., 2010; Chendrimada et al., 2005). These studies suggest the possibility that substrate and product dsRNAs might bind to different regions of Dicer. For dsRNA cleavage to occur, a substrate must be bound by the catalytic arm of

Dicer. However, the similarity between the length of the helicase domain and the length of an siRNA duplex in EM reconstructions hinted that following dicing, products might re-localize to Dicer's helicase domain.

To test this hypothesis, we reconstituted the human Dicer enzyme by co-expression of an N-terminal ("Helicase") fragment containing the N-terminus to the PAZ domain and a separate C-terminal ("Catalytic") fragment containing the RNaseIII domains and C-terminal dsRBD (Figure 1A; Ma and Doudna, unpublished data). This bipartite Dicer lacks a small region of polypeptide corresponding to a flexible loop and co-elutes with full length Dicer on a sizing column (data not shown), thus its structure is likely to be identical to that of the intact protein. Dicing assays confirmed that this two-piece Dicer retains catalytic activity that is highly similar to that of the wild type protein (Supplemental Figure S1). Following purification, this bipartite Dicer was combined with an equimolar amount of TRBP to reconstitute a Dicer/TRBP complex.

To determine how the Dicer/TRBP complex positions Dicer substrates relative to products, we used a set of 5-iodo-uracil-modified substrate and product dsRNAs that could be photo-cross-linked to the protein complex by exposing binding reactions to 302 nm light ((Tomari et al., 2004a; Tomari et al., 2004b); Figure 1B, Supplemental Table S1). Substrate mimics consisted of a 37 bp chimeric dsRNA with one blunt end and a 2 nt 3′-overhang at the other end to promote unidirectional binding of the overhang by Dicer's PAZ domain (Rose et al., 2005). Deoxyribonucleotides present at the cleavage sites in each strand prevented the dicing reaction from occurring (data not shown). A single 5-iodo-uracil modification was incorporated at different locations along each dsRNA. Given that the 3′-overhangs of Dicer substrates are proposed to bind the PAZ domain (present in the helicase branch of the bipartite Dicer), care was taken to place substrate modifications at sites distant from this overhang. Product mimics consisted of 21 nt thermodynamically symmetric siRNA duplexes with a single 5-iodo-uracil modification. Modified strands were radiolabeled at their 5′-ends so that photo-cross-linked proteins could be identified by mobility using SDS-PAGE.

These experiments showed that regardless of sequence, Dicer substrates preferentially photo-cross-link to the C-terminal "catalytic" Dicer fragment, whereas Dicer products preferentially cross-link to the N-terminal "helicase" fragment (Figure 1C, D, Supplemental Figure S1). These differential photo-cross-linking propensities for substrates versus products are consistent with siRNA repositioning within the Dicer/TRBP complex following substrate cleavage.

The Dicer/TRBP heterodimer releases nascent siRNAs for re-binding

The product repositioning that we detected could occur by two potential mechanisms. Either siRNAs are released from the Dicer/TRBP complex and rebound in a new orientation, or they are reorganized without ever dissociating from the complex (Figure 2A). Studies using *Drosophila* embryo lysates or recombinant human Dicer and human cell extracts have led to conflicting views of siRNA fate immediately following dicing. In *Drosophila*, competition experiments indicated that siRNA products are released from Dicer prior to RISC-loading (Preall et al., 2006). Rose and colleagues, on the other hand, presented a model in which siRNAs remain bound to human Dicer and enter directly into RISC based on the observation that in some cases Dicer's processing polarity is predictive of silencing efficacy (Rose et al., 2005).

To clarify the immediate fate of nascent siRNAs within the Dicer/TRBP complex before Ago2 loading, we developed a native PAGE product-binding assay using a reconstituted human dicing system. A 40 bp dsRNA substrate was used (dsRNA 40a/b; see Supplemental Table S1) that had one blunt end and a 2 nt 3′-overhang at the other end. The strand

containing the 2 nt 3′-overhang also had a 5′-biotin modification to further promote unidirectional dicing. As anticipated, this dsRNA yields a single cleavage product upon incubation with Dicer (data not shown). The amount of product bound could be readily tracked by mobility shift on a 6% native gel (Supplemental Figure S2). Briefly, substrates were pre-incubated with the Dicer/TRBP heterodimer on ice to allow for binding but not cleavage (Supplemental Figure S2, Lane 3). Reactions were then transferred to tubes containing increasing concentrations of unlabeled siRNA 12a/b competitor (Supplemental Table S1) at 37°C to initiate the dicing reaction. If products remain bound to the Dicer/TRBP complex as they are moved to the helicase branch, increasing amounts of cold siRNA would compete with product binding to a similar extent as when the complex was instead pre-incubated with a labeled siRNA product. However, if products were first released and then re-bound by the helicase domain, one would expect a higher level of competition than in the case of a pre-bound product.

Product binding and dicing assays showed that excess unlabeled siRNA effectively competed with the 40a/b dicing product for binding to the Dicer/TRBP heterodimer without significantly affecting dicing efficiency (Figure 2B). We reproducibly observed a ~67% decrease in product binding when 100-fold excess competitor was present. By contrast, binding decreased by only ~11% when Dicer was pre-incubated with a 21 nt siRNA instead of the 40a/b substrate, indicating that we are seeing *bona fide* product release (Figure 2C). To control for non-specific effects, we repeated the competition experiments with an unrelated, unlabeled 116 nt structured human Alu RNA and observed only a ~16% decrease in product binding (Figure 2C). None of these decreases were accompanied by a commensurate decrease in overall dicing activity (Figure 2D). To address the possibility that these data simply reflect an ability of Dicer to bind both substrates and products simultaneously, we performed biotin affinity purification assays using the biotinylated dsRNA 40a/b substrate. Dicer/TRBP was pre-bound to this substrate, and increasing concentrations of radiolabeled siRNA 12a/b were then added to the reactions. Despite the fact that Dicer/TRBP complex was purified by this method, radiolabeled product RNA was not precipitated at any of the concentrations tested (Supplemental Figure S2 B, C). Our data are consistent with a model in which the Dicer/TRBP heterodimer releases nascent siRNAs into the bulk solvent and then rebinds them along Dicer's helicase domain rather than reorienting them within the confines of the complex.

The Dicer/TRBP heterodimer senses thermodynamic asymmetry of siRNA ends

A previous report demonstrated that the Dicer-2/R2D2 heterodimer functions as a sensor for siRNA asymmetry to direct strand-specific RISC-loading in *Drosophila* (Tomari et al., 2004b). We sought to determine the extent to which the Dicer/TRBP heterodimer functions analogously in humans. To test for sensing of siRNA asymmetry by the human complex, we used a set of synthetic siRNAs identical to that used previously ((Tomari et al., 2004b); Figure 3A; Supplemental Table S1). Briefly, these RNAs were each based on the thermodynamically symmetric sequence of siRNA 1 (this siRNA is identical to siRNA D in Supplemental Figure S1, see Supplemental Table S1), in which one strand contains a 5-iodo-uracil modification at the 20th base counting from the 5′ end. siRNAs 2-4 contained a single mismatch at one end to introduce thermodynamic asymmetry. The 5-iodo-uracil modification lies on either the 3′-overhang of the presumptive guide strand, passenger strand, or both. siRNAs 5-7 instead had a mismatch introduced at the opposite end of the duplex, flipping the asymmetry and thus the predicted guide strands of the duplexes.

The modified siRNAs were incubated with the Dicer/TRBP complex, followed by irradiation with 302 nm light and separation on an SDS-PAGE gel. If the Dicer/TRBP heterodimer behaves analogously to the Dicer-2/R2D2 heterodimer, Dicer would photo-cross-link preferentially to siRNAs that have a 5-iodo-uracil modification located at the less

stable end of the duplex. TRBP, on the other hand, would be expected to photo-cross-link preferentially to siRNAs containing the photo-cross-linkable base at the more stable end. We found that the Dicer/TRBP heterodimer did exhibit this cross-linking pattern (Figure 3B, C), indicating that the human complex functions as a sensor for siRNA asymmetry similar to that reported in flies. Based on additional cross-linking data, this sensor is also functional in the larger context of a complex containing Ago2 (Figure 3B, C).

Heterodimer formation is required for asymmetry sensing in humans

To test the extent to which Dicer or TRBP alone are capable of sensing siRNA thermodynamic asymmetry, we subjected each protein separately to photo-cross-linking assays. Although Dicer photo-cross-linked to siRNAs in control experiments using the Dicer/TRBP heterodimer, Dicer alone was unable to photo-cross-link to siRNAs (Figure 4A, B). This observation is consistent with the low affinity this protein has for siRNAs in the absence of a dsRBP (Chakravarthy et al., 2010). We also found that although TRBP photo-cross-linked to each siRNA, it did so with no pattern related to siRNA thermodynamic asymmetry (Figure 4A, B).

In light of the fact that neither Dicer nor TRBP alone could recapitulate asymmetric photo-cross-linking to siRNA ends, we wondered whether or not heterodimer formation is necessary for the human asymmetry sensor to function. Wild type TRBP consists of three dsRBDs connected by flexible linker regions (Figure 4C). The two N-terminal dsRBDs have a high affinity for dsRNA (Parker et al., 2008), whereas the C-terminal dsRBD is responsible for binding to Dicer (Kok et al., 2007). We therefore expressed a TRBP truncation mutant that lacks the C-terminal, Dicer-binding dsRBD. When combined with purified Dicer, no evidence of complex formation was detected by size exclusion chromatography (Supplemental Figure S3). This truncated TRBP was preincubated with equimolar amounts of human Dicer and subjected to photo-cross-linking assays. We found that under these conditions, Dicer again did not detectably photo-cross-link to any of the siRNAs. By contrast, the truncated TRBP did photo-cross-link, but again with no pattern related to the thermodynamic asymmetry of the siRNA ends (Figure 4D). These findings indicate that heterodimer formation is required for sensing of siRNA thermodynamic asymmetry, and that neither Dicer nor TRBP is competent for this function in their free states.

dsRBPs activate Dicer's asymmetry sensing functionality

In light of the finding that Dicer cannot sense siRNA asymmetry outside of the context of the Dicer/TRBP complex, we asked whether or not siRNA asymmetry sensing could be rescued by substituting PACT for TRBP since PACT also binds to Dicer's helicase domain (Lee et al., 2006). We reconstituted a Dicer/PACT heterodimer *in vitro* (Supplemental Figure S4) and subjected this complex to the same photo-cross-linking assays as for the Dicer/TRBP heterodimer. Likely due to the specific geometry of the photo-cross-linking reaction, we saw no cross-linking to PACT in this experiment (Figure 5A). Interestingly, however, we did observe photo-cross-linking to Dicer in the context of this heterodimer. When we compared the total fraction of Dicer photo-cross-linked for each siRNA, we saw a similar pattern of cross-linking to that of Dicer in the context of the Dicer/TRBP heterodimer (Figure 5A, B). Dicer reproducibly photo-cross-linked more readily when the 5-iodo-uracil modification was located at the less stable end of the duplex. This pattern indicates not only that PACT allows Dicer to bind its products (as does TRBP), but also that the Dicer/PACT heterodimer is capable of sensing the thermodynamic asymmetry of siRNA ends.

Based on this finding, we hypothesized that asymmetry sensing requires Dicer to be in complex with a dsRBP, but not necessarily TRBP or PACT. To investigate this prediction, we created a chimeric dsRBP in which we fused the two dsRBDs from the unrelated spermatid perinuclear RNA binding protein (SPNR) to the C-terminal Dicer binding domain of TRBP ((Schumacher et al., 1998; Schumacher et al., 1995); Figure 5C). Wild type SPNR does not bind to Dicer (Supplemental Figure S4). We reconstituted a heterodimer consisting of this chimeric dsRBP and Dicer (Supplemental Figure S4) and subjected the complex to photo-cross-linking assays to test for a functional asymmetry sensor. This tethering of SPNR dsRBDs, which are not implicated in RNAi, to TRBP's Dicer binding dsRBD was sufficient to impart asymmetry sensing capability to the heterodimer (Figure 5D, E).

The above results strongly suggest that Dicer is the protein sensor for siRNA thermodynamic asymmetry in humans. One clear question, however, was whether or not the third dsRBD of TRBP may itself retain some residual asymmetry sensing capabilities in a complex with Dicer. To rule out this possibility, we constructed a second fusion protein in which the SPNR dsRBDs were fused directly to the N-terminus of Dicer via an SPNR-derived linker containing a TEV cleavage site (Figure 5C, Supplemental Figure S4), giving a physically linked heterodimer containing no sequence from TRBP or PACT. We performed photo-cross-linking assays with this protein with an added TEV cleavage step following exposure to 302 nm light. Although the background of SPNR cross-linking was higher in this case (likely due to residual cross-linking that occurred following TEV cleavage), Dicer clearly retained its preference for cross-linking to the less stable end of siRNA duplexes in the absence of TRBP (Figure 5D, E). Taken together, these data demonstrate that Dicer itself is the functional sensor of siRNA thermodynamic asymmetry in humans, and that Dicer-associated dsRBPs play an essential positioning role to allow this sensing to occur.

Previous characterizations of mouse embryonic stem cells in which portions of Dicer's RNaseIII domains are excised demonstrated that although these cells cannot process Dicer substrates, exogenous siRNAs are still able to knock down gene expression (although strand selection was not specifically examined in either case (Kanellopoulou et al., 2005; Murchison et al., 2005)). One interpretation of these results is that Dicer is not necessary for RISC-loading in mammals. However, in both cases it was possible that a truncated form of Dicer containing the DExH/D helicase domain was still expressed. Such a Dicer fragment could retain its TRBP- and siRNA-binding capacity and may therefore sense siRNA thermodynamic asymmetry through the interactions proposed here. To investigate the possibility that such a Dicer fragment is expressed in these knockout cells, we performed a western blot analysis of cell lysates using an antibody that recognizes Dicer's N-terminus (Kanellopoulou et al., 2005; Murchison et al., 2005). Interestingly, in the mouse knockout cells, but not the conditional (un-floxed) control cells, we detected a band corresponding to ~100 kDa (Supplemental Figure S4). To rule out the possibility that this signal represented a non-specific antibody interaction with a protein expressed at higher levels in the mouse knockout cells than in the control cells, we used the same antibody to immunoprecipitate this fragment from cell lysates and ran the eluate on an SDS-PAGE gel. We then cut a band from the gel corresponding to the size of the fragment and submitted the sample for analysis by mass spectrometry. We were able to detect three tryptic fragments of mouse dicer each corresponding to the N-terminal portion of the protein using a cross-correlation cutoff that yielded results with greater than 95% confidence (Supplemental Table S2). A large, N-terminal fragment of Dicer is therefore expressed in these mouse Dicer “knockout” cells, indicating that although Dicer's main catalytic function is absent in these cells, it is possible that other Dicer functions such as asymmetry sensing are still carried out.

DISCUSSION

Considerable efforts have shed light on the mechanisms of both dicing and slicing in RNAi, but the process by which an siRNA is strand-selectively transferred to Ago2 from Dicer in humans is poorly understood. In this study, we used biochemical approaches to investigate the post-processing fates of siRNAs within the Dicer/TRBP complex as they are positioned and pre-oriented for strand-selective RISC-loading. The findings presented here allow us to propose a model for siRNA positioning and RISC-loading by the Dicer/TRBP complex that begins to establish the molecular basis of this process in humans (Figure 6).

Our data provide evidence that the Dicer/TRBP heterodimer binds Dicer substrates and products by distinct mechanisms. Dicer substrates are engaged by the catalytic region of the enzyme, as would be necessary for productive dicing. TRBP seems to stabilize this interaction, as human Dicer did not photo-cross-link to substrates in the absence of TRBP in our assays (data not shown). Following cleavage, dicing products are released into the bulk solvent, similar to the *Drosophila* system and in contrast to previous models of the human enzyme (Preall et al., 2006; Rose et al., 2005). Rose and colleagues approached this question by using modified pairs of synthetic dsRNAs that restricted Dicer binding to one or the other end of the duplex, but that would nonetheless each produce the same siRNA products. They reported that the processing polarity of Dicer affects the extent of gene silencing in human cells, and concluded based on this finding that human Dicer may not release its products before RISC-loading. This report, however, did not examine the potential effects that differing dicing efficiencies across different substrates would have on overall silencing. Additionally, the effects of heterogeneous product formation (21- versus 22 nt siRNAs) on silencing were not examined for all cases in which it occurred. While it is possible that the product release observed here is not obligate or that additional factors prevent siRNA dissociation from Dicer complexes *in vivo*, our *in vitro* data strongly favor a model of product release prior to repositioning.

We show that the Dicer/TRBP heterodimer rebinds its siRNA products (or exogenously administered siRNAs) at Dicer's helicase domain. Based on recently published EM structures, this repositioning represents an almost 90° shift in binding orientation, although it is possible that RNA binding to the helicase domain induces a conformational shift in Dicer that alters this angle (Lau et al., 2009; Wang et al., 2009b). This staging step allows an siRNA to be bound coordinately between Dicer's PAZ domain and TRBP (which binds at the distal end of Dicer's helicase branch, see Figure 6). Bound in this way, an siRNA can be spatially oriented by the heterodimer according to the thermodynamic asymmetry of its ends in a way that would not be possible if the products were bound along Dicer's catalytic domain, far from TRBP.

Our studies have demonstrated that the human core RLC, as well as a heterodimer containing Dicer and either TRBP or PACT, is capable of sensing the thermodynamic asymmetry of siRNA ends *in vitro*. These complexes bind siRNAs directionally such that Dicer interacts with the less stable end of the duplex and its dsRBP binding partners interact with the more stable end. This finding alone, however, does not directly address the question of which component represents the functional asymmetry sensing machinery. A previous report concluded that TRBP alone preferentially photo-cross-links to the more stable end of siRNA duplexes, which led to the conclusion that TRBP functions to sense siRNA thermodynamic asymmetry in humans, whereas Dicer acts as more of a passive element in the asymmetry sensing process (Gredell et al., 2010). These findings, however, were inconsistent across all siRNAs tested. Perhaps more importantly, given that in the same report the very siRNA strands that were found to photo-cross-link to TRBP preferentially in the context of an asymmetric duplex were also cross-linked preferentially to TRBP when

incubated as separate ssRNAs, one cannot rule out the possibility that these findings reflect a photo-cross-linking artifact related to the tendencies of individual ssRNAs to cross-link preferentially rather than to duplex stability.

In our study, the fact that TRBP alone photo-cross-links with no pattern related to thermodynamic asymmetry argues that its function is not to bind the more stable end of an siRNA and then directionally recruit that RNA to Dicer, as was previously proposed. Rather, our experiments using various heterodimers and chimeric proteins demonstrate that asymmetry sensing is only activated upon heterodimer formation, and that Dicer itself is the *bona fide* sensor of siRNA thermodynamic asymmetry. In this light, Dicer-associated dsRBPs likely act as positioning elements that hold siRNA products in place while Dicer senses the thermodynamic stabilities of siRNA ends, binding preferentially to the less-stable end.

Given that our data show siRNAs to be bound by Dicer's DExH/D helicase domain, it is tempting to implicate this domain in Dicer's asymmetry sensing functionality. Although in our assays asymmetry sensing does not require ATP, recent reports have shown that certain helicases are capable of unwinding short stretches of RNA in an ATP-independent manner (Liu et al., 2008). Partial unwinding of an unstable siRNA end could potentially allow Dicer to bind that siRNA end more favorably, imparting the observed directionality of product binding by the heterodimer. Unfortunately, point mutations in Dicer's helicase domain render the protein highly unstable, precluding a direct analysis of this hypothesis. Clearly, further structural and biochemical analyses of the precise nature of siRNA-binding by Dicer/dsRBP complexes are necessary to firmly establish the mechanistic basis of this asymmetry sensor.

In terms of strand-selective RISC-loading by the core human RLC, it is of mechanistic significance that asymmetry sensing by the heterodimers investigated here leads TRBP or PACT to preferentially bind the 3′ end of the guide strand (which lies at the more stable end of the duplex). In our previously reported EM structure of a minimal reconstituted RLC, we modeled the crystal structure of a guide strand-containing *Thermus thermophilus* Argonaute into the EM density (Wang et al., 2009b). Intriguingly, this docking placed the Ago PAZ domain near TRBP and the outer edge of Dicer's helicase domain. In binding the 3′ end of the guide strand, TRBP thus likely holds that end of the siRNA in close proximity to the Ago2 PAZ domain and in the proper orientation for guide-strand binding by the apposed domain. Given TRBP's apparently high level of flexibility, it is possible that this protein or PACT could directly hand off the 3′ end of the guide strand to Ago2 in a RISC-priming step that leads to a previously proposed state in which an siRNA is transiently coordinately bound between the Dicer and Ago2 PAZ domains (Wang et al., 2009b). Further studies will be needed to definitively establish whether or not this RISC-loading intermediate exists.

Our findings do not exclude the possibility that the asymmetry sensing observed here represents only one part of a more complex system of redundant guide strand selection checkpoints *in vivo*. For example, archaeal Argonaute structures indicate that the two 5′ bases of the guide strand splay apart and do not base pair with the passenger strand (Wang et al., 2009c), indicating that Ago2 itself may also bind siRNAs in a preferential orientation with respect to thermodynamic asymmetry that serves as a secondary proofreading mechanism for proper guide strand loading. Additionally, human Ago2-loading may be affected by internal elements of dsRNA structure, as is the case in *Drosophila* (Okamura et al., 2009). Nonetheless, the results of this study show that human Dicer/dsRBP heterodimers are able to position siRNAs distinctly from dsRNA substrates and orient them with respect to the relative thermodynamic stabilities of the ends of the duplex, strongly suggesting a role for these heterodimers in guide-strand selection during the RISC-loading process in humans.

EXPERIMENTAL PROCEDURES

Recombinant Protein Purification and Complex Reconstitution

Proteins were purified and complexes were reconstituted according to established protocols (MacRae et al., 2008), with the exceptions that all dsRBPs were expressed and purified from *E. coli* BL21 DE3 cells and that PACT was purified using two Superdex 75 16/60 columns connected in tandem. The chimeric Dicer protein was purified using a Superose 6 10/300 column.

UV Photo-cross-linking Assays

10 μL photo-cross-linking reactions composed of 100 nM protein, 10,000 cpm RNA (~50 nM), and 1x reaction buffer (20 mM HEPES, pH 7.5; 25 mM KCl; 1.5 mM $MgCl_2$; 1% glycerol; 0.01% Igepal 630-CA; 0.1 mg/mL BSA; 0.1 mg/mL tRNA; 2.5 mM TCEP) were pre-incubated at room temperature under foil for one hour. Reactions were then moved to a 96-well plate and exposed to 302 nm light for 15 minutes by placing a handheld UV lamp over the plate. In the case of the chimeric Dicer protein, reactions were then subjected to TEV-cleavage for 30 minutes under foil at room temperature. Reactions were run on a 4-20% SDS-PAGE gel. Gels were dried for 2 hours and observed by phosphorimaging following an overnight exposure. Data was quantified using ImageQuant TL software.

Competition Assays

18 μL competition assays containing 2,000 cpm dsRNA (~15 nM), 10 nM protein, and 1x reaction buffer were preincubated for one hour on ice to allow for substrate binding but not cleavage. Reactions were then transferred to pre-warmed tubes containing 2 μL cold siRNA of the appropriate concentration (from 0-fold to 100-fold over the concentration of substrate dsRNA) in 1x reaction buffer and incubated at 37°C for an additional 60 minutes. Reactions were split and 10 μL aliquots were run on either a 15% denaturing PAGE gel to analyze dicing efficiency or a 6% native PAGE gel to analyze product binding. Gels were dried overnight and exposed for ~8 hours prior to analysis by phosphorimaging. Data quantification was achieved using ImageQuant TL.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- Dicer/TRBP repositions products along Dicer's helicase domain.
- Dicer/TRBP releases nascent product RNAs prior to repositioning.
- dsRBPs position siRNAs for sensing of thermodynamic asymmetry by Dicer.

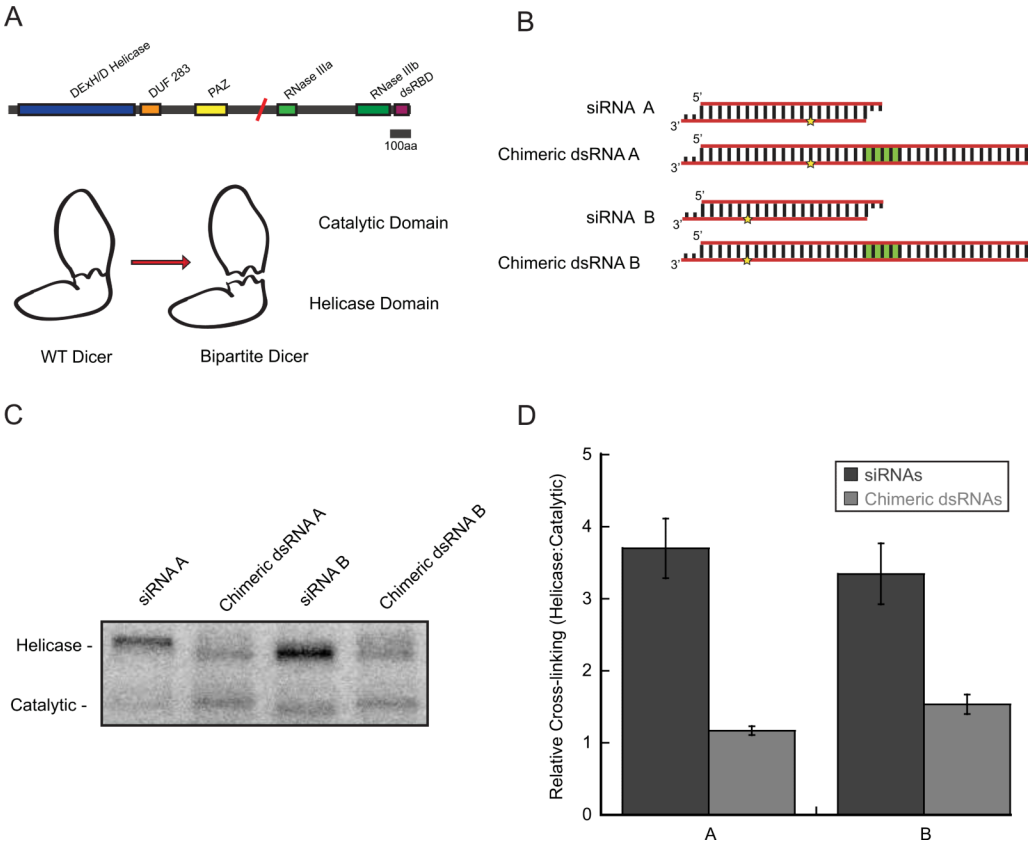

Figure 1. Human Dicer Binds dsRNA Substrates and siRNA Products along Distinct Axes

(A) Upper: Diagram of Dicer domain structure. Red slash indicates the approximate location of the interface between the two parts of the bipartite Dicer. Lower: Cartoon of recombinant bipartite Dicer indicating the approximate location of the interface between the two fragments, as estimated using previously reported EM models (Lau et al., 2009; Wang et al., 2009b). See also Figure S1.

(B) Diagram of synthetic, modified RNAs used with bipartite Dicer. Stars indicate the locations of 5-iodo-uracil modifications. Green areas indicate the presence of deoxyribonucleotides at the cleavage site. Overhangs are 2 nt 3′-overhangs. siRNAs are thermodynamically symmetric. Strands containing 5-iodo-uracil modifications were 5′-end radiolabeled and all unmodified strands had a 5′-phosphate.

(C) Photo-cross-linking assay using bipartite Dicer enzyme. Substrate mimics or siRNAs were incubated with the bipartite Dicer/TRBP complex at room temperature followed by irradiation with 302 nm light. Photo-cross-linked proteins were detected based on size by SDS-PAGE.

(D) Quantification of three experimental replicates (mean +/- SD) showing the ratio of photo-cross-linking to the bipartite Dicer's helicase domain versus the catalytic domain.

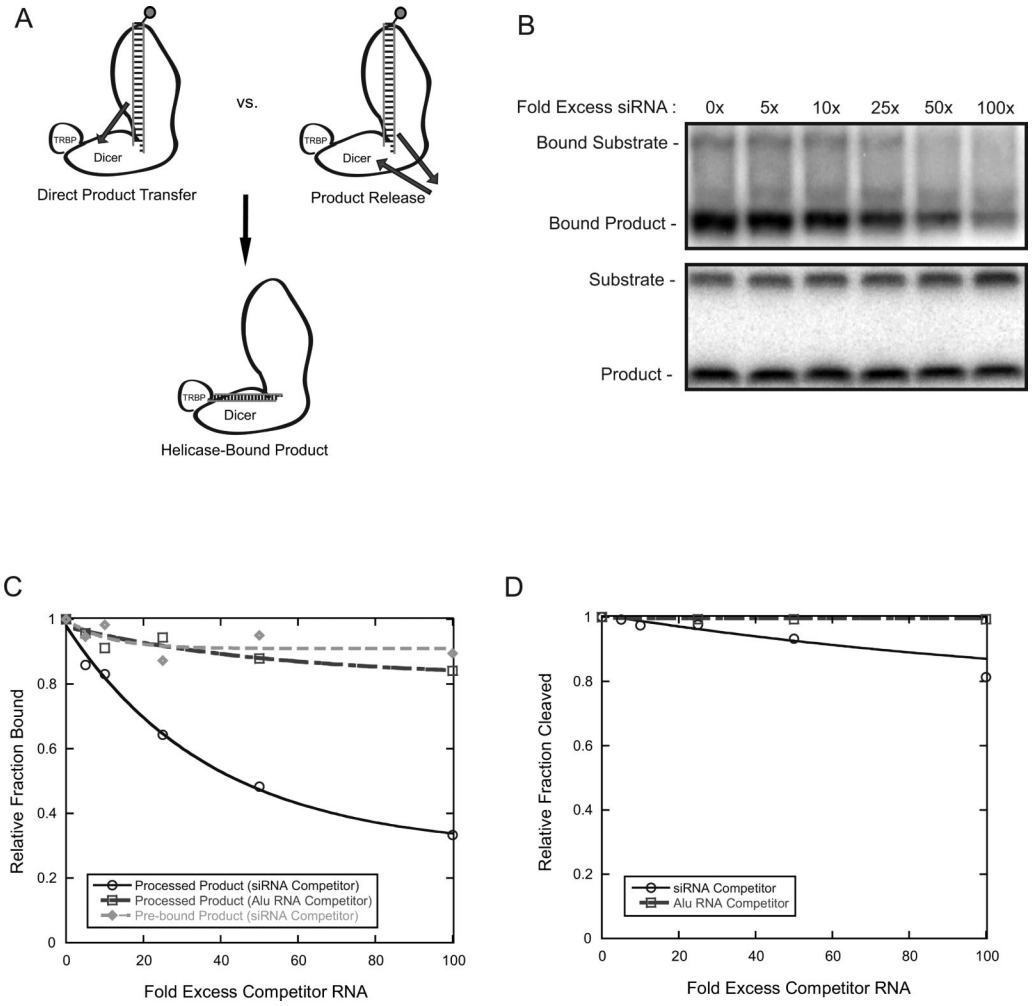

Figure 2. Human Dicer/TRBP Releases siRNA Products Prior to Repositioning

(A) Schematic of potential post-processing modes of siRNA repositioning. Products are either reorganized within the complex (upper left) or released and re-bound (upper right) prior to repositioning along Dicer's helicase domain (lower). Grey circle represents biotinylated dsRNA.

(B) Product binding competition assay. Dicer/TRBP complex was pre-incubated with radiolabeled 40a/b substrate at 4°C. To initiate cleavage, reactions were transferred to tubes at 37°C containing increasing concentrations of cold competitor siRNA 12a/b. Upper: 6% native PAGE analysis shows a decrease in bound product as the concentration of competitor siRNA is increased. Lower: 15% denaturing PAGE analysis demonstrates that competitor siRNA 12a/b has a negligible effect on dicing activity at the concentrations used in this study. See also Figure S2.

(C) Quantification of the fraction of product bound by Dicer/TRBP (Bound siRNA/Total siRNA) using data from three experimental replicates (means). Black circles: As the competitor concentration is increased, the fraction of bound product decreases substantially. Dark grey squares: Increasing concentrations of a structured Alu RNA did not compete away bound product. Light grey diamonds: When siRNA 12a/b is pre-bound, increasing concentrations of cold siRNA 12a/b do not lead to a significant decrease in the fraction of bound product. Values for each condition were normalized to the fraction product bound when no competitor was present.

(D) Quantification of product formation for dicing reactions (Fraction cleaved/Total) using data from three experimental replicates (means). Black circles: Increasing concentrations of cold siRNA 12a/b have a limited effect on dicing activity. Dark grey squares: Cold Alu RNA also fails to compete with dicing activity. Values for each condition were normalized to the fraction substrate cleaved when no competitor was present.

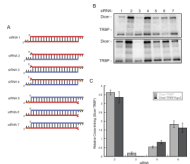

Figure 3. The Human Dicer/TRBP Heterodimer Senses siRNA Thermodynamic Asymmetry

(A) Diagram of synthetic, modified siRNAs used for asymmetry sensing assays. Stars indicate the location of 5-iodo-uracil modifications. Strands containing 5-iodo-uracil modifications were 5′-end labeled and all unmodified strands were 5′-phosphorylated. Overhangs are 2 nt 3′-overhangs. Curved ends indicate the presence of a mismatched base pair. Predicted guide strands are colored red and passenger strands are colored blue. See text for further details.

(B) Photo-cross-linking assay using reconstituted wild type Dicer/TRBP (upper panel) and Dicer/TRBP/Ago2 (lower panel) complexes. Modified siRNAs were incubated with Dicer/TRBP complex at room temperature followed by irradiation with 302 nm light. Photo-cross-linked proteins were detected based on size by SDS-PAGE.

(C) Quantification of the relative photo-cross-linking of siRNAs 2, 3, 5, and 6 to Dicer versus TRBP in asymmetry sensing assays using data from three experimental replicates (mean +/- SD). In the context of both the Dicer/TRBP heterodimer and the core RLC, Dicer was preferentially photo-cross-linked when the 5-iodo-uracil modification was introduced at the less stable end of the duplex, whereas TRBP preferentially photo-cross-linked to the more stable end.

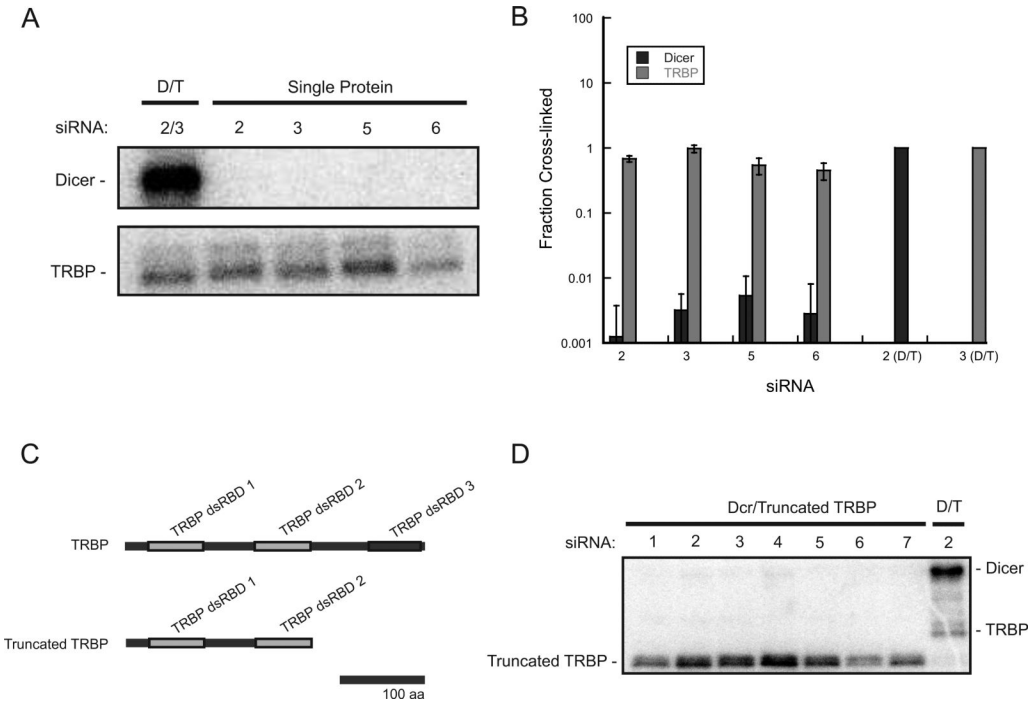

Figure 4. Heterodimer Formation is Required to Sense siRNA Thermodynamic Asymmetry

(A) Photo-cross-linking assay using free Dicer and TRBP. Dicer alone or TRBP alone was incubated with siRNA 2, 3, 5, or 6 prior to irradiation with 302 nm light. Photo-cross-linked proteins were detected based on size by SDS-PAGE. Upper: Dicer alone does not detectably photo-cross-link to any of the modified siRNAs used in this study. For comparison, the left lane shows the level of Dicer that photo-cross-links to siRNA 2 in the context of the Dicer/TRBP heterodimer (D/T). Lower: TRBP alone photo-cross-linked to each siRNA, but not with any pattern related to the thermodynamic asymmetry of siRNA ends. For comparison, the left lane shows the level of TRBP that photo-cross-links to siRNA 3 in the context of the Dicer/TRBP heterodimer (D/T).

(B) Quantification of the relative photo-cross-linking of siRNAs 2, 3, 5, and 6 to Dicer or TRBP alone (Fraction cross-linked/Total) in asymmetry sensing assays using data from three experimental replicates (means +/- SD). Data were normalized to the levels of Dicer or TRBP that photo-cross-linked to siRNA 2 or 3, respectively, in the context of the Dicer/TRBP heterodimer. Note that the y-axis is plotted on a logarithmic scale.

(C) Diagram of wild type and truncated TRBP domain structures. The dsRBD responsible for binding to Dicer is shown in dark grey.

(D) Dicer/TRBP heterodimer formation is necessary for sensing siRNA thermodynamic asymmetry. Dicer and truncated TRBP were pre-incubated on ice prior to incubation with siRNAs 1-7 at room temperature. Reactions were then irradiated with 302 nm light and analyzed by SDS-PAGE. siRNAs were only photo-cross-linked to the truncated TRBP protein, and with no pattern related to siRNA thermodynamic asymmetry. For reference, the far right reaction contained wild type Dicer/TRBP complex photo-cross-linked to siRNA 2. See also Figure S3.

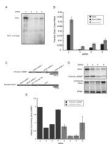

Figure 5. Dicer's Physical Interaction with a dsRBP Reconstitutes a Functional Asymmetry Sensor

(A) The Dicer/PACT heterodimer senses siRNA thermodynamic asymmetry. siRNAs 2, 3, 5, and 6 were incubated with Dicer/PACT complex at room temperature followed by irradiation with 302 nm light. Photo-cross-linked proteins were detected based on size by SDS-PAGE. No detectable cross-linking to PACT was observed. See also Figure S4.

(B) Quantification of the fraction Dicer photo-cross-linked (Fraction cross-linked/Total) to siRNAs 2, 3, 5, and 6 in asymmetry sensing assays using data from 3 experimental replicates (mean +/- SD) using either Dicer, the Dicer/PACT heterodimer, or the Dicer/TRBP heterodimer.

(C) *Upper*: Diagram of chimeric dsRBP domain structure. Sequence N-terminal to the vertical black line derives from SPNR. Light grey boxes indicate dsRBDs from SPNR. Sequence C-terminal of the vertical black line derives from TRBP. Dark grey box indicates TRBP's Dicer binding dsRBD. *Lower*: Diagram of chimeric Dicer domain structure. Light grey boxes indicate SPNR dsRBDs. Small black box indicates TEV cleavage site. Dark box represents full length Dicer. Diagonal break indicates a break from scale.

(D) Human Dicer is a protein sensor of siRNA asymmetry. Dicer/chimeric dsRBP complex (upper panel) or chimeric Dicer (lower panel) was incubated with siRNAs 2, 3, 5, or 6 at room temperature prior to irradiation with 302 nm light. Photo-cross-linked proteins were detected based on size by SDS-PAGE (chimeric Dicer was first TEV cleaved).

(E) Quantification of the relative photo-cross-linking of siRNAs 2, 3, 5, and 6 to Dicer versus the dsRBP in asymmetry sensing assays using data from 3 experimental replicates (mean +/- SD). Care was taken to ensure that in the case of the chimeric Dicer the small residual signal corresponding to uncleaved protein was not entered into the analysis. In both cases, Dicer was preferentially photo-cross-linked when the 5-iodo-uracil modification was introduced at the less stable end of the duplex, whereas the dsRBP preferentially photo-cross-linked to the more stable end. See also Figure S4.

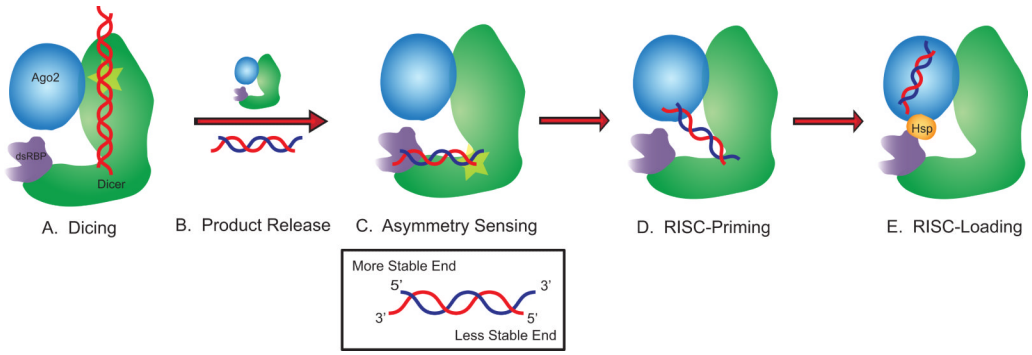

Figure 6. Model for RNA Positioning by the Human RISC-Loading Complex

(A) Human Dicer recognizes and orients dsRNA substrates along its catalytic domain, positioning them in the active site for cleavage.

(B) Following cleavage, Dicer releases nascent siRNA products for repositioning.

(C) Dicer and a dsRBP binding partner (TRBP or PACT) coordinately bind siRNAs, positioning them along Dicer's helicase domain for asymmetry sensing. Dicer preferentially binds the less stable end of the duplex while the dsRBP binds the more stable end. This likely positions the 3′ end of the guide strand in close proximity to the Ago2 PAZ domain prior to RISC-loading (Wang et al., 2009b). The guide strand is shown in red, and the passenger strand is shown in blue.

(D) In a hypothetical RISC-priming step, TRBP or PACT hands off the more stable end of the siRNA to the Ago2 PAZ domain, leading to a transient intermediate in which the siRNA is coordinately bound by Dicer and Ago2.

(E) Finally, pre-oriented siRNAs are loaded onto Ago2 such that the 3′ end of the guide strand is bound by the Ago2 PAZ domain and the 5′ end is bound by the Ago2 Mid domain. This final loading step may be facilitated by one or more RLC accessory proteins such as Hsc70 and Hsp90 (Iwasaki et al., 2010; Miyoshi et al., 2010).