Human RISC Couples MicroRNA Biogenesis and Posttranscriptional Gene Silencing

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Summary

RNA interference is implemented through the action of the RNA-induced silencing complex (RISC). Although Argonaute2 has been identified as the catalytic center of RISC, the RISC polypeptide composition and assembly using short interfering RNA (siRNA) duplexes has remained elusive. Here we show that RISC is composed of Dicer, the double-stranded RNA binding protein TRBP, and Argonaute2. We demonstrate that this complex can cleave target RNA using precursor microRNA (pre-miRNA) hairpin as the source of siRNA. Although RISC can also utilize duplex siRNA, it displays a nearly 10-fold greater activity using the pre-miRNA Dicer substrate. RISC distinguishes the guide strand of the siRNA from the passenger strand and specifically incorporates the guide strand. Importantly, ATP is not required for miRNA processing, RISC assembly, or multiple rounds of target-RNA cleavage. These results define the composition of RISC and demonstrate that miRNA processing and target-RNA cleavage are coupled.

Introduction

RNA interference (RNAi) is a term used to describe the phenomenon whereby double-stranded RNAs (dsRNAs) lead to silencing of genes of homologous sequence (Fire et al., 1998). RNAi occurs in a variety of evolutionarily diverse organisms (Novina and Sharp, 2004). Long dsRNAs are converted by the RNase III endonuclease Dicer to ~22 nucleotide (nt) small interfering RNAs (siRNAs) containing 2 nt 3′ overhangs (Bernstein et al., 2001). MicroRNAs (miRNAs) are a large family of endogenous, small regulatory RNAs that are generated by a two-step process from long primary miRNAs (pri-miRNAs) that are processed in the nucleus by the Microprocessor complex, comprised of the RNase III enzyme Drosha and the double-stranded RNA binding domain (dsRBD) protein DGCR8, to ~60–70 nt precursor miRNA (pre-miRNA) intermediates (Gregory et al., 2004, Denli et al., 2004, Han et al., 2004). These hairpin-shaped pre-miRNAs are transported to the cytoplasm, where they are subsequently cleaved by Dicer to generate an ~22 nt miRNA (Hutvagner et al., 2001, Ketting et al., 2001, Kim 2005). A single strand of the siRNA or miRNA duplex is incorporated into a ribonucleoprotein effector complex, known as the RNA-induced silencing complex (RISC) (Hammond et al., 2000, Martinez et al., 2002). RISC identifies target messages based on complementarity between the guide RNA and the mRNA and results in either endonucleolytic cleavage of targeted mRNA or translational repression (Bartel 2004, Lim et al., 2005). Although RISC has been purified from fly and human cells, its precise polypeptide composition and its assembly pathway remain unknown (Sontheimer 2005, Filipowicz, 2005). Indeed, siRNA-directed target mRNA cleavage activity has been reported in complexes that range in size from ~160 kDa to ~80S (Martinez et al., 2002, Nykanen et al., 2001, Hammond et al., 2001, Mourelatos et al., 2000, Hutvagner and Zamore, 2002, Pham et al., 2004). However, Argonaute2 (Ago2) was recently found to be the catalytic endonuclease of human RISC, and, accordingly, structural studies identified the PIWI domain as the catalytic center that resembles that of RNase H (Liu et al., 2004, Meister et al., 2004, Song et al., 2004; Ma et al., 2005). Target-RNA cleavage activity has recently been reconstituted using single-stranded (ss) siRNA to guide RNA cleavage by recombinant Ago2 protein (Rivas et al., 2005). However, this minimal RISC could not be loaded with the physiologically relevant duplex siRNA and, unlike RISC activity measured in cell lysates, did not display any ATP requirements (Hutvagner and Zamore, 2002, Haley and Zamore 2004). These data imply that the holo-RISC complex contains additional protein cofactors. Emerging data support Dicer as a potential RISC component, and a number of studies have reported a physical association of Ago proteins with Dicer both in vitro and in purifications from different organisms, including *Drosophila* (Hammond et al., 2001, Okamura et al., 2004, Pham et al., 2004, Jiang et al., 2005, Saito et al., 2005), *C. elegans* (Tabara et al., 2002), and humans (Tahbaz et al., 2004, Chendrimada et al., 2005). Furthermore, there is increasing support not only for Dicer's functioning in the initiation phase of generating siRNA/miRNA but also for its playing a downstream role in the effector phase of modulating posttranscriptional gene silencing (Doi et al., 2003, Lee et al., 2004, Pham et al., 2004, Chendrimada et al., 2005). Additional genetic and biochemical evidence for a mechanistic coupling between the two phases of RNAi was provided by the identification of the Dicer-interacting proteins R2D2 in *Drosophila* (Liu et al., 2003), RDE-4 in *C. elegans* (Grishok et al., 2000, 2001, Tabara et al., 2002), and TRBP in humans (Chendrimada et al., 2005), which are required for posttranscriptional gene silencing. Although *Drosophila* Dicer-2 (one of the two Dicer paralogs in this organism) can efficiently process long dsRNAs to siRNAs, the loading of the siRNAs into the RISC requires R2D2 (Liu et al., 2003, Tomari et al., 2004). Similarly, in humans, while Dicer alone can process dsRNAs and pre-miRNAs efficiently (Zhang et al., 2002, 2004, Provost et al., 2002, Chendrimada et al., 2005), the recruitment of the siRNA/miRNAs to Ago2 requires TRBP (Chendrimada et al., 2005).

In this study, we explore the functional significance of the physical association of Dicer with Ago2. Using a biochemical approach to isolate Dicer-containing protein complexes, we isolated an ~500 kDa protein complex containing Dicer-TRBP-Ago2. We show that this

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affinity-purified complex can be programmed with double-stranded siRNAs, or pre-miRNA hairpin RNAs, to direct Ago2-mediated target-RNA cleavage. Furthermore, we show that pre-miRNA processing and RISC assembly are functionally coupled and do not require ATP. Moreover, ATP is not required for this complex to catalyze multiple rounds of target-RNA cleavage. We propose that this Dicer-containing complex constitutes the human RISC.

Results

Dicer-TRBP-Ago2 Displays RISC Activity Using a MicroRNA Hairpin

Recently we isolated a trimeric protein complex of ~500 kDa that contains Dicer, TRBP, and Ago2. We demonstrated that this complex is required for miRNA biogenesis and links miRNA processing with the assembly of an active RISC (Chendrimada et al., 2005). The physical association of Dicer-TRBP and Ago2 prompted us to test the hypothesis that the two enzymes may be functionally linked. FLAG-Dicer was isolated from HEK293-derived cell lines using affinity chromatography, and the affinity eluate was subjected to Superose 6 gel filtration.

Analysis of the Dicer affinity eluate by silver staining and Western blot analysis confirmed the association of Dicer with TRBP and Ago2 (Figure 1A). Moreover, multiple mass spectrometric analyses have identified the 70 and 50 kDa bands as SKB1 and MEP50, common contaminants of FLAG-affinity purification (see Figure 1A mock, shown by asterisks). Other visible polypeptides were deemed to be Dicer breakdown products since they could be visualized using Dicer antibodies (Figure 1A).

Consistent with our previous report (Chendrimada et al., 2005), analysis of Superose 6 gel filtration fractions by Western blot demonstrated the coelution of a fraction of Dicer, with TRBP and Ago2 as components of an approximately 500 kDa complex (Figure 1B). Moreover, a fraction of FLAG-Dicer elutes at a smaller molecular weight corresponding to uncomplexed FLAG-Dicer (Figure 1B, fraction 32). To assess the activity of Dicer-TRBP-Ago2 complex, we utilized a synthetic hairpin corresponding to *Drosophila* let-7 pre-miRNA. We chose this *Drosophila* pre-let-7 for our functional assays because this pre-miRNA has been well characterized in other systems (Hutvagner and Zamore, 2002, Forstemann et al., 2005); is absolutely defined at the RNA sequence level owing to the fact that it is chemically synthesized; and, although the human genome contains orthologous let-7 miRNAs (most significantly, human let-7a), we wished to reduce the possibility that our purified complexes possessed significant activity from endogenous mature miRNA. In contrast to HeLa cells, HEK293 cells (or stable lines derived from such cells) contain very low levels of let-7a, and, unlike other miRNAs we have analyzed, let-7a is barely detectable by Northern blot (data not shown). To confirm that *Drosophila* pre-let-7 (herein referred to as pre-let-7) is a substrate for our affinity-purified Dicer-containing complexes and to examine pre-miRNA processing activity of Dicer-containing complexes, we assayed alternate fractions of the Superose 6 gel filtration column. 5′ end-labeled pre-miRNA was gel purified and used as a substrate for pre-miRNA processing assays. Column fractions were incubated with pre-let-7 for 1 hr at 37°C. After deproteinization using Proteinase K, the reaction products were separated on a 15% denaturing polyacrylamide gel (Figure 1C). Dicer-mediated cleavage of the 60 nt pre-let-7 hairpin resulted in an ~21 nt product representing mature let-7 miRNA. Furthermore, a broad peak of pre-miRNA processing activity was observed spanning fractions 24–34 of the column that corresponds with the presence of Dicer detected by Western blot (Figure 1B).

We next investigated whether this pre-let-7 could be used as a source of siRNA to direct Ago2-mediated cleavage of a complimentary target RNA. Column fractions were incubated with 5′-phosphorylated pre-let-7 for 30 min at 37°C prior to the addition of a 5′ end-labeled 21 nt target RNA. This analysis revealed the precise coelution (fractions 28–30) of Dicer-TRBP-Ago2 complex with target-RNA cleavage directed by the siRNA generated through the action of Dicer (Figure 1D). Importantly, no target-RNA cleavage was detected without pre-let-7 addition, demonstrating that there was no detectable activity from endogenous let-7a (see below and data not shown). To further examine the RISC complex, we subjected the RISC-containing fractions from the Superose 6 column to a second gel filtration through a Superdex 200 (S200). Superdex 200 is described by the manufacturer to display a greater resolution in the range of 700 to 200 kDa. Analysis of S200 column fractions using silver staining, Western blot analysis, and RISC activity demonstrated the precise coelution of siRNA-targeted cleavage and Dicer-TRBP-Ago2 complex (Figure 2A). These experiments demonstrate a role for Dicer-TRBP-Ago2 complex in mediating siRNA-directed cleavage of target mRNA and suggest a functional link between Dicer and Ago2.

Dicer Catalytic Activity Is Coupled with Ago2 Cleavage Activity

To directly assess the role of Dicer catalytic activity in siRNA-mediated target cleavage by Ago2, we compared the RISC activity of Dicer-TRBP-Ago2 complex using either a pre-miRNA let-7 hairpin or a mature 21 nt let-7 miRNA duplex which can no longer be cleaved by Dicer (Figure 2B). Analysis of RISC activity using increasing concentrations of let-7 miRNA hairpin or a duplex miRNA revealed an increased RISC activity when a hairpin pre-let-7 miRNA was used to trigger RISC activity, suggesting that programming RISC using a Dicer substrate is ~10-fold more efficient (Figure 2C). These experiments substantiate a role for Dicer catalytic activity in facilitating mRNA cleavage by RISC.

RISC Containing Dicer-TRBP-Ago2 Preferentially Loads the Guide Strand of the siRNA

Previous experiments have indicated that although double-stranded RNAs are cleaved on both strands by Dicer, only the strand with complementarity to the target mRNA (guide strand) is incorporated into RISC, while the passenger strand is excluded. Indeed, recent evidence indicates that the dsRBD protein R2D2 functions in choosing the guide strand of the duplex siRNA

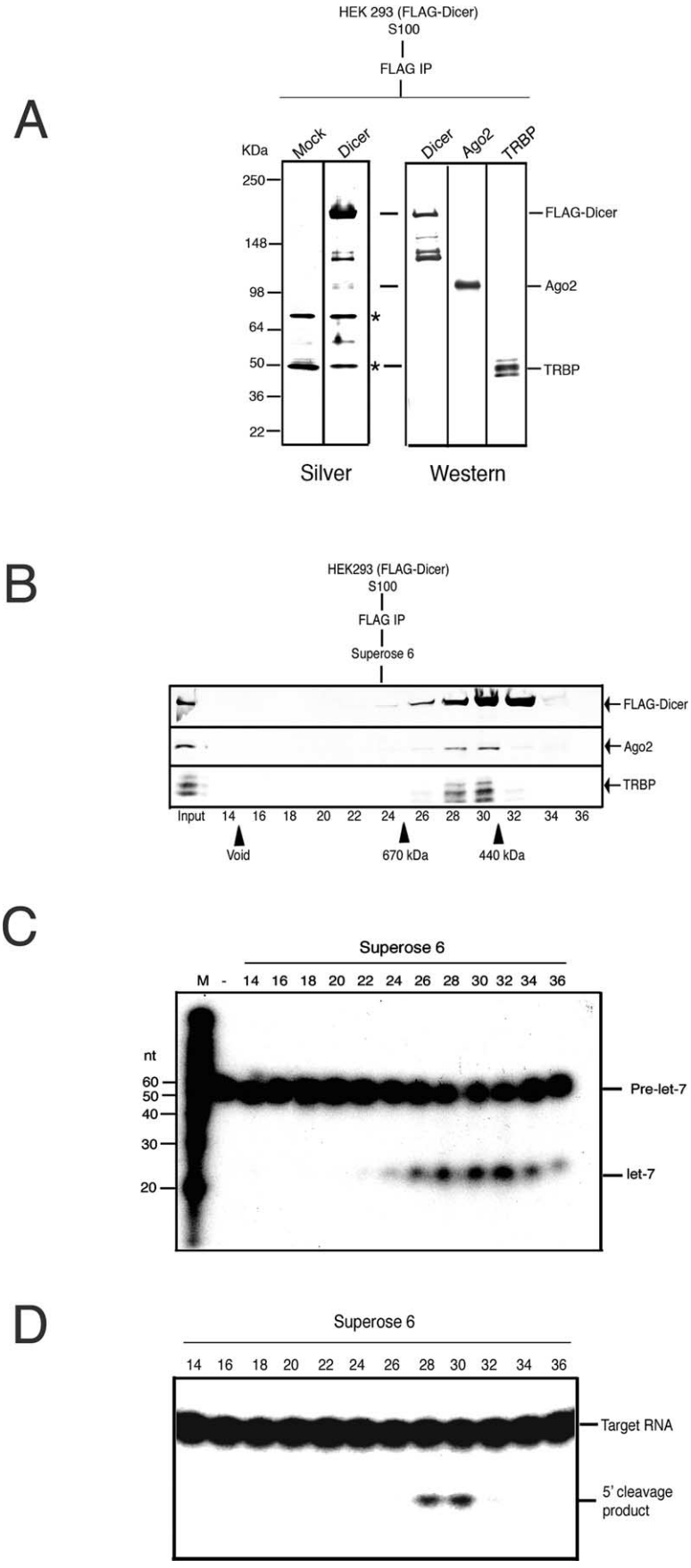


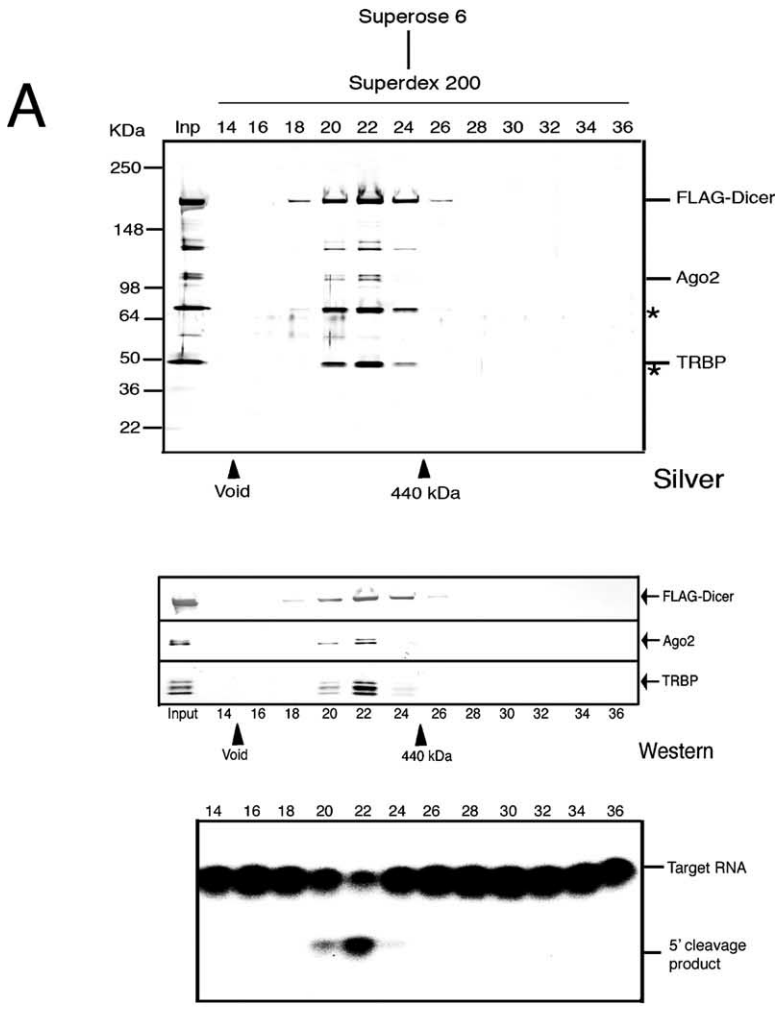
Figure 1. Isolation of the Dicer-TRBP-Ago2-Containing Human RISC

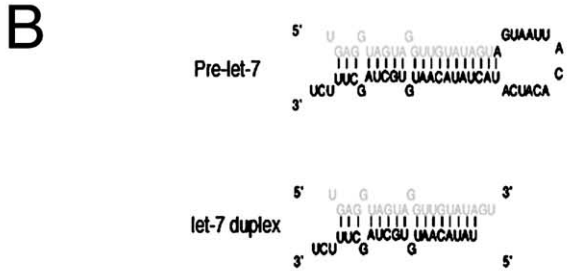
(A) FLAG-Dicer was isolated from a HEK293-derived S100 extract prepared from a cell line stably expressing FLAG-Dicer. An untagged HEK293 cell line was used as the mock. Following elution of the immunoaffinity eluate from M2 anti-FLAG beads, the fractions were resolved on an SDS-polyacrylamide (4%–12%) gel, and proteins were visualized by silver staining and Western blot analysis using anti-Dicer (N-terminal), anti-Ago2, and anti-TRBP polyclonal antibodies. Molecular masses (kDa) of marker proteins are indicated (left). Asterisks denote contaminating polypeptides. Multiple sequencing analyses have determined SKB1 and MEP50 to be common contaminants of FLAG-affinity purification.

(B) FLAG-Dicer affinity eluate purified from a HEK293-derived cytoplasmic extract (S100) subjected to Superose 6 size-exclusion chromatography and complexes resolved by SDS-PAGE and detected by Western blot analysis using anti-FLAG, anti-Argonaute2 (anti-Ago2), and anti-TRBP antibodies. Fractions of the column and molecular mass markers (kilodaltons) are indicated at the bottom. Arrows indicate the bands representing Dicer, TRBP, and Ago2 proteins.

(C) Pre-miRNA processing assay performed using 5′-labeled pre-let-7 RNA. Fractions of the column (10 μl) were incubated with 10 nM pre-let-7 RNA at 37°C for 90 min. Reaction products were resolved by 15% denaturing PAGE. The position of the 60 nt pre-let-7 substrate and ~21 nt let-7 Dicer cleavage product are indicated. A peak of pre-let-7 processing activity was detected in fractions 24–34 that corresponds with fractions containing Dicer protein detected by Western blot in (B).

(D) RISC assay performed on column fractions. Reactions were performed using 10 μl of each fraction incubated with pre-let-7 hairpin RNA (5 nM) for 30 min prior to the addition of target RNA (10 nM) as described in Experimental Procedures. Reaction products were resolved by 15% denaturing PAGE, and the band representing the specific 5′ cleavage product of the target RNA is indicated. The 3′ fragment is unlabeled and therefore not visible. The RISC activity detected in fractions 28–30 precisely coelutes with the presence of Ago2 and TRBP in (A).





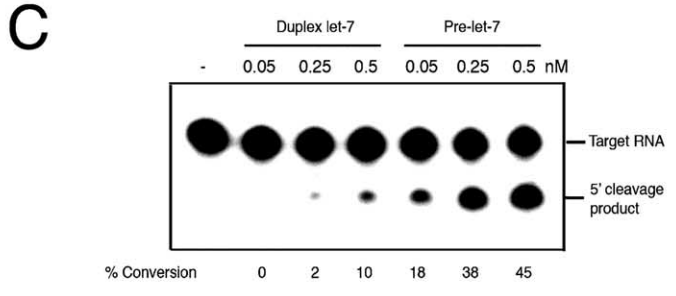


Figure 2. Dicer-Mediated Pre-miRNA Processing Is Coupled with Ago2-Mediated Target-RNA Cleavage Activity

(A) RISC-containing fractions from the Superose 6 column were subjected to a second step of purification by Superdex 200 (S200) gel filtration chromatography. Alternate fractions of the S200 column were analyzed by silver staining (top panel) and Western blot using anti-FLAG, anti-Ago2, and anti-TRBP antibodies (middle panel). Fractions of the column and molecular mass markers (kilodaltons) are indicated. Asterisks denote contaminating polypeptides. RISC assays were performed on S200 column fractions (lower panel). Reactions were performed using 10 μl of each fraction incubated with pre-let-7 hairpin RNA (0.5 nM) for 30 min prior to the addition of target RNA (10 nM). Reaction products were resolved by 15% denaturing PAGE. The RISC activity detected in fractions 20–22 precisely coelutes with the presence of Ago2 and TRBP.

(B) Sequences the pre-let-7 and 21 nt duplex let-7 RNA used to program RISC.

(C) Comparison of target-RNA cleavage activity of RISC programmed with 21 nt double-stranded RNA (dsRNA) (duplex let-7) or pre-let-7 hairpin RNA. Reactions were performed using samples from fraction 22 of the S200 gel filtration column that were preincubated with the indicated RNA prior to the addition of target RNA (10 nM). Reaction products were resolved by 15% denaturing PAGE, and the band representing the specific 5′ cleavage product is indicated.

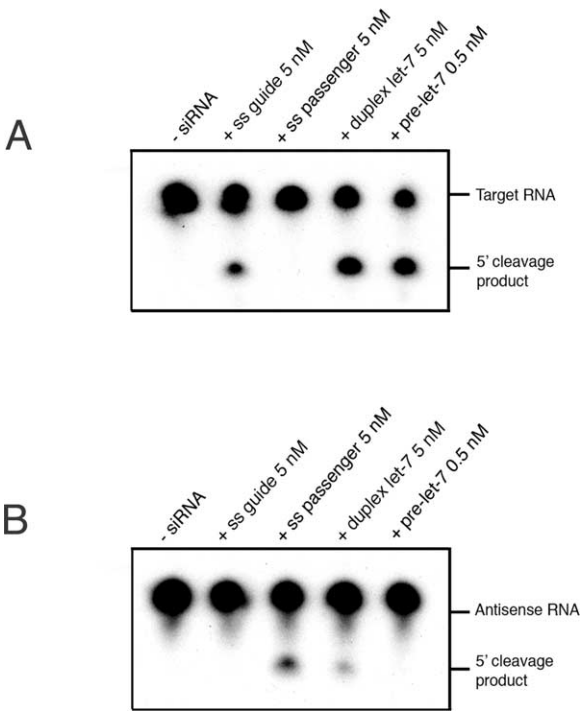


Figure 3. RISC Preferentially Loads the Guide Strand of the siRNA

(A) RISC assays performed using fraction 22 of the S200 column. RISC was preloaded using either 5 nM single-stranded 21 nt guide-strand let-7 RNA (+ss guide), 5 nM single-stranded 21 nt passenger strand let-7 RNA (+ss passenger), 5 nM 21 nt double-stranded let-7 RNA (+ds let-7), or 0.5 nM 60 nt hairpin RNA (+pre-let-7) prior to the target RNA (10 nM) cleavage reaction. When RISC was loaded with ss guide, pre-let-7, or ds let-7, but not with ss passenger, target-RNA cleavage activity was observed.

(B) RISC assays performed as in (A), except that the target RNA added after RISC loading corresponds to the antisense RNA sequence. Although RNA cleavage activity was detected from RISC loaded with complimentary sequence (+ss passenger), very little RISC activity was detected using ss guide, pre-let-7, or ds let-7.

by binding to the siRNA end with the greatest double-stranded character (Tomari et al., 2004). This thermodynamic asymmetry seems to be a general feature of miRNAs (Khvorova et al., 2003, Schwarz et al., 2003). Therefore, we tested whether the Dicer-TRBP-Ago2 complex could preferentially cleave the RNA corresponding to the target mRNA. Significantly, the complex could cleave both the authentic target mRNA as well as its complementary strand if presented by a single-stranded siRNA complementary to each RNA (Figures 3A and 3B). In contrast to single-stranded siRNAs, inclusion of the pre-miRNA corresponding to let-7 or 21 nt miRNA duplex as the source of siRNA in the reaction resulted in the preferential cleavage of the target mRNA (Figures 3A and 3B). These results attest to the ability of the Dicer-TRBP-Ago2 complex in specifically loading the guide strand of the miRNA precursor into an active RISC.

RISC Activity Does Not Require ATP

We next asked whether ATP is required for RISC assembly and function. Experiments were performed with

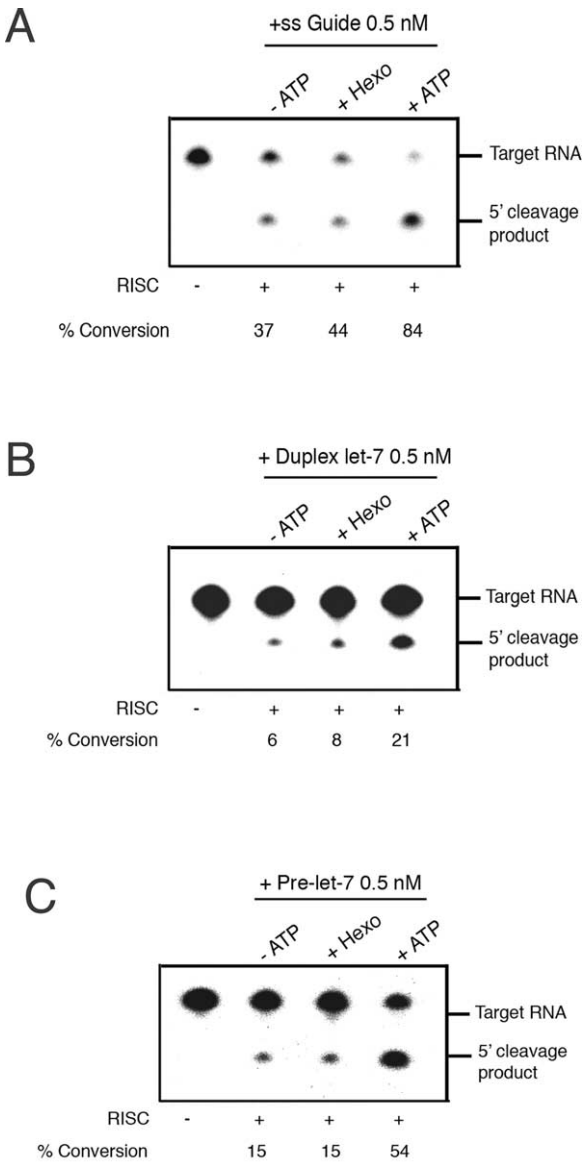


Figure 4. RISC Activity Does Not Require ATP

(A) RISC assays performed using fraction 22 of the S200 column. RISC was programmed using 0.5 nM single-stranded (ss) 21 nt RNA corresponding in sequence to the guide strand of let-7. After preincubation of the samples with the ss siRNA, complementary target was added to the reaction to a final concentration of 10 nM. Reactions were performed in the absence of adenosine triphosphate (–ATP), with ATP depletion using 2 mM glucose and 1 unit hexokinase (+Hexo), or with 1 mM ATP (+ATP).

(B and C) Reactions performed as in (A) except that 0.5 nM duplex let-7 RNA or pre-let-7 hairpin RNA, respectively, were used in place of the single-stranded siRNA.

and without ATP. Significantly, RISC assays performed using affinity-purified Dicer-TRBP-Ago2 complex displayed a modest stimulation (2- to 3-fold) by ATP (Figures 4A–4C). Furthermore, ATP could stimulate RISC activity when RISC was programmed with either 0.5 nM single-stranded siRNA (Figure 4A), 0.5 nM pre-let-7 mature miRNA, or pre-let-7 hairpin (Figures 4B and 4C).

Importantly, to exclude the possibility that some ATP may be present in the –ATP conditions, additional experiments were performed in which the complex was incubated with hexokinase in a buffer supplemented with 2 mM glucose for 30 min at 37°C prior to the addition of either the single-stranded guide siRNA, the duplex, or the hairpin. The results obtained from ATP depletion by hexokinase were indistinguishable from RISC assays carried out in the absence of ATP (Figures 4A–4C), indicating that the –ATP conditions were devoid of ATP. The fact that, under –ATP conditions, a substantial target-RNA cleavage activity was detected suggests that pre-miRNA processing, RISC assembly, and siRNA-mediated target cleavage do not require ATP.

RISC Is a Multiple-Turnover Enzyme and Does Not Require ATP Hydrolysis

To assess whether RISC could perform multiple rounds of siRNA-mediated target cleavage, we titrated the target RNA from 0.6 to 20 nM using 0.5 nM hairpin as siRNA trigger in the absence of ATP. As Figure 5A attests, increasing target concentrations lead to multiple rounds of target cleavage (nearly 12 rounds of enzyme cleavage at 20 nM of target), evident by about 30% product formation. These results demonstrate that RISC is a multiple-turnover enzyme not requiring ATP for product release.

To assess whether the stimulation of RISC activity by ATP required ATP hydrolysis, we tested the nonhydrolyzable analogs of ATP, ATPγS, AMP-PNP, and AMP-CPP in RISC-mediated targeted cleavage. Surprisingly, the nonhydrolyzable ATP analogs as well as other nucleotides could substitute for ATP in stimulating RISC activity (Figures 5B and 5C and data not shown). These results indicate that the ATP stimulation of RISC activity is not due to ATP hydrolysis and may result from a stimulatory effect of nucleotides in catalysis.

Intriguingly, all nucleotide analogs that we tested resulted in a comparable increase in RISC activity. Next we addressed the question of whether the observed nucleotide stimulation of activity was a specific effect of the nucleotides or was caused by changes in salt concentration and/or pH in the reaction conditions. Since reactions contained 20 mM Tris-HCl (pH 7.9) and the concentration of the nucleotides added was 1 mM, an alteration in pH seemed unlikely. Nevertheless, we measured the pH of the reactions in the absence and presence of ATP and found the pH to be unaltered (data not shown). To rule out a salt effect, we measured RISC activity using a salt titration and found that, under conditions ranging from 80 mM to 160 mM KCl, RISC activity was unaltered (data not shown). These conclusions pointed to a specific stimulatory effect due to a particular common feature of the various different nucleotides that were used. We therefore tested the hypothesis that the presence of multiple phosphate moieties of nucleotides may enhance RISC activity. We compared the effect of 1 mM ATP with 1 mM disodium pyrophosphate ($Na_2H_2P_2O_7$) containing two phosphate moieties. Indeed, addition of either 1 mM ATP or 1 mM disodium pyrophosphate (pH 7.0) resulted in a similar stimulation of RISC activity (see Figure S1A in the Supplemental Data available with this article online). Importantly,

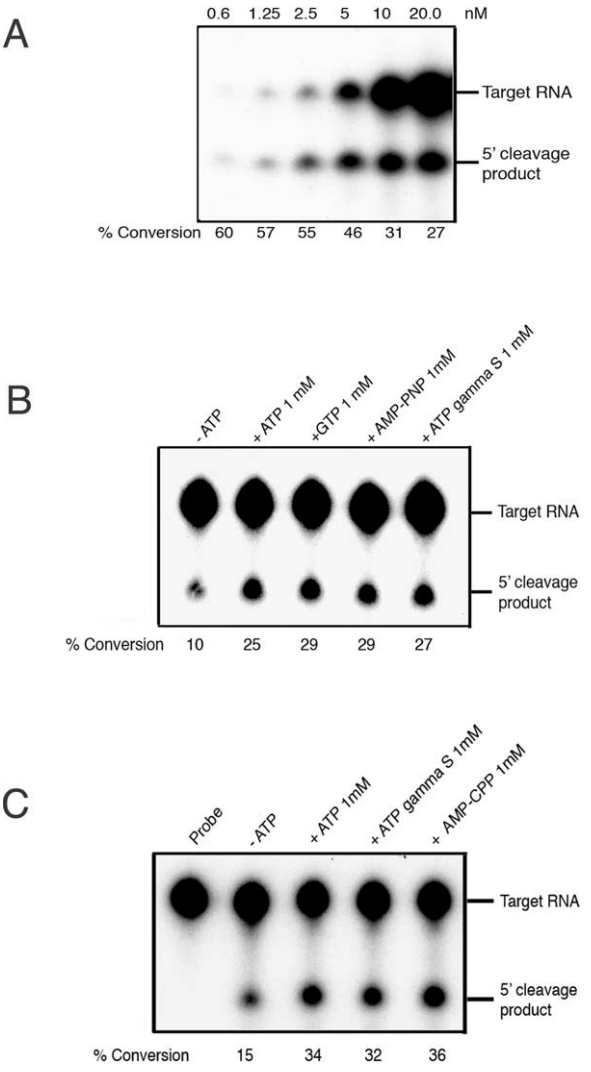


Figure 5. Multiple Rounds of RNA Cleavage in the Absence of ATP

(A) RISC assays performed under multiple-round conditions in the absence of ATP. Purified RISC was incubated with 0.5 nM pre-let-7 RNA for 30 min at 37°C before the addition of increasing amounts (0.6 to 20 nM) of target RNA and was incubated at 37°C for 60 min.

(B) RISC assays performed in the absence of ATP (–ATP), presence of ATP (+ATP), with GTP, or with nonhydrolyzable ATP analogs (AMP-PNP and ATPγS). 0.5 nM pre-let-7 RNA was used to program RISC, and the target RNA concentration was 10 nM.

(C) RISC assays performed as in (B) with 1 mM ATP, ATPγS, or AMP-CPP.

RISC activity was unaltered in reactions containing NaCl, indicating that the stimulatory effect is due to the phosphate moiety (Figure S1A). To further investigate this phosphate-mediated stimulation, we analyzed RISC activity in reactions containing inorganic phosphate (NaH_2PO_4) (pH7.0), which contains a single phosphate. This analysis confirmed that the presence of a single phosphate specifically enhances RISC-mediated target-RNA cleavage, albeit at a higher concentration (Figure S1B). To insure that RISC fractions do not contain nucleoside diphosphate kinase (NDPK) capable of transferring phosphate from one nucleotide to another,

we measured NDPK activity in our RISC fractions. RISC fractions were devoid of any NDPK activity, consistent with their high degree of homogeneity (Figure S1C). Collectively, these data demonstrate that, while nucleotides, including ATP, can stimulate RISC activity, this stimulation is most likely mediated by the presence of phosphates since both pyrophosphate and inorganic phosphate could also enhance RISC activity.

Discussion

We have affinity purified ~500 kDa complex from human cells containing Dicer, TRBP, and Ago2 displaying RISC activity. We demonstrate that this complex processes pre-miRNA and selects the appropriate strand of the siRNA duplex to direct target-RNA cleavage. Furthermore, we show that pre-miRNA processing and RISC assembly are functionally coupled. This contention is supported by the demonstration that RISC can be more effectively programmed using a pre-miRNA Dicer substrate than a 21 nt duplex. Furthermore, RISC programmed with duplex siRNA or pre-miRNA resulted in targeted cleavage of RNA complementary to the guide strand and not the passenger strand. In addition, we demonstrate that RISC assembly—and, therefore, pre-miRNA processing, siRNA duplex strand separation, and multiple rounds of target-RNA cleavage—does not require ATP. However, ATP stimulates RISC activity. Unexpectedly, ATP enhancement of RISC activity does not result from ATP hydrolysis. Our results provide significant insight into the composition of RISC and its mode of assembly and function and demonstrate that miRNA processing by Dicer and Ago2-mediated target-RNA cleavage are coupled.

Our studies suggest that, at least in human cells, a fraction of Ago2 is stably associated with Dicer-TRBP composing the human RISC. This indicates that human cells contain preassembled RISC poised to perform siRNA-directed cleavage of a target RNA. Moreover, we showed previously (Chendrimada et al., 2005) that this complex could associate with the 22 nt duplex product of Dicer cleavage in a gel mobility shift experiment. In this study, we show that, although RISC could utilize the 22 nt duplex as the source of the siRNA, it displays far greater activity once a pre-miRNA, a substrate of Dicer, is used as the source of siRNA. These results strongly support the contention that Dicer cleavage activity is tightly coupled into the effector step of RNAi mediated by Ago2. The coupling of the two enzymatic activities makes ample biological sense since, once the duplex RNA is cleaved by Dicer, it could be unwound and handed over to Ago2 for target-RNA cleavage in a concerted reaction. Our data showing a physical and functional coupling of pre-miRNA processing and RISC assembly also provide a mechanistic framework that explains the observations that 27 nt double-stranded RNAs (Kim et al., 2005) or short hairpin RNAs (Siolas et al., 2005), both of which are Dicer substrates, are considerably more potent triggers of RNAi than the short duplex siRNA (Elbashir et al., 2001) in transfected cells.

Previous studies have identified a variety of different proteins as potential components of the RISC, although few of them have been functionally characterized at the molecular level (Caudy et al., 2001). In reconciliation with our study, some of these proteins were purified as RISC components from *Drosophila* and may be represent species-specific differences in the composition of RISC. However, there are two reports wherein Ago2 was partially purified from human cells using monoclonal antibodies directed to Gemin3 and Gemin4 (Mourelatos et al., 2000, Hutvagner and Zamore 2002). Using anti-Gemin3 antibodies, we failed to detect Gemin3 in our purified RISC. Furthermore, we generated HEK293-derived stable cell lines expressing Gemin3 and affinity-purified Gemin3-containing protein complexes. Using anti-Ago2 polyclonal antibodies, we did not detect Ago2 in the affinity eluate (data not shown). Perhaps the simplest explanation for the absence of Gemin3 is that we may have isolated a "minimal" RISC that, although fulfilling the criteria that define a holo-RISC, may loosely interact with other protein components (including Gemin3 and Gemin4) that, during our stringent purification conditions, become disassociated from the complex. We propose that this Dicer-TRBP-Ago2 complex constitutes an evolutionarily conserved core RISC and predict that, in other organisms, RISC will be composed of a Dicer, a TRBP-like double-stranded RNA binding protein, and an Argonaute. Indeed, it will be interesting to determine whether the DCR-1-RDE-4-RDE-1-containing complex in *C. elegans* and the DCR-2-R2D2-AGO2 and DCR-1-Loquacious-AGO1 complexes in *Drosophila* possess RISC activity (Tabara et al., 2002, Liu et al., 2003, Saito et al., 2005, Jiang et al., 2005).

Interestingly, we show that the unwinding of the siRNA does not require ATP since RISC activity can be obtained using a duplex siRNA in the absence of ATP. This raises the important question of how then the duplex siRNA unwound. We propose the mechanism akin to the action of Dicer-2-R2D2 complex (Tomari et al., 2004) in which the binding of TRBP and Dicer to different ends of the duplex siRNA initiates the unwinding process. This is then followed by the association of the PAZ domain (Lingel et al., 2003; Song et al., 2003; Ma et al., 2004; Yan et al., 2004) of Ago2 with an ensuing single-stranded siRNA. In this model, the energy required for the unwinding of the duplex is generated by the increased ability of the Ago2 in binding single-stranded siRNA as the duplex is unwound, shifting the equilibrium from RNA-RNA interaction to RNA-PAZ interaction (Figure 6). It is also quite likely that Dicer, perhaps through its PAZ domain, and TRBP contribute to this shift in binding activity toward a single-stranded siRNA as recombinant Ago2 is unable to unwind a duplex RNA (Rivas et al., 2005).

In our experiments performed in absence of ATP using 20 nM target RNA and 0.5 nM pre-let-7 hairpin, a conversion of target RNA to cleavage product of up to 30% was achieved. This indicates that each molecule of loaded RISC must cleave ~12 molecules of target RNA, demonstrating that this affinity-purified RISC is indeed a multiple-turnover enzyme. Moreover, this figure is likely an underestimate of RISC turnover, assumes a 100% conversion of pre-let-7 to mature 21 nt let-7, and also assumes that the limiting parameter in these experiments is the hairpin RNA and not the

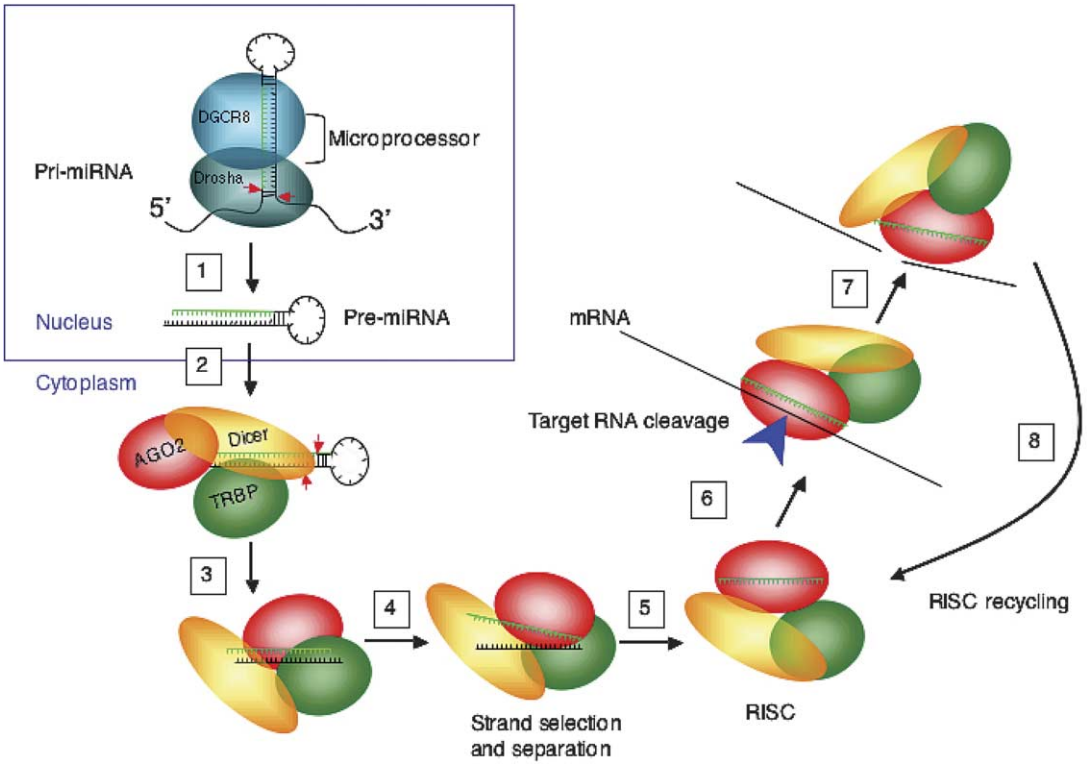


Figure 6. Model of RISC Assembly and Function

Steps of the miRNA biogenesis and RISC assembly pathway are designated by numbers 1 through 8. (1) Primary miRNA transcripts (pri-miRNA) are recognized by the Microprocessor complex (comprised of the RNase III Drosha and the dsRBD protein DGCR8). Microprocessor specifically cleaves (represented by red arrows) the hairpin-shaped RNAs at the base of the stem loop, releasing an ~60–70 nt precursor miRNA (pre-miRNA) that contains a 2 nt 3′ overhang. (2) Pre-miRNAs are transported to the cytoplasm, where they are recognized by the RISC containing Dicer-TRBP-Ago2. (3) The RNase III Dicer cleaves ~22 nt from the Drosha cleavage site, thus generating ~22 nt duplex miRNA (2 nt 3′ overhangs), which remains associated with RISC as a ribonuleoprotein complex. (4) The complex identifies the guide strand of the RNA duplex, perhaps through TRBP's and Dicer's recognizing thermodynamic asymmetry between the two ends of the duplex analogous to *Drosophila* R2D2-Dicer-2 complex. The two RNA strands are separated in an ATP-independent manner. (5) The guide strand of the miRNA remains associated with Ago2 in the active RISC complex. (6) RISC recognizes target mRNAs based on complementarity between the guide miRNA and the mRNA transcript. (7) Ago2 specifically cleaves (blue arrow) the target mRNA for posttranscriptional gene silencing. (8) The cleaved product is released, enabling RISC to catalyze the destruction of another target RNA. Importantly, none of the above steps 1 through 8 requires energy derived from ATP hydrolysis.

amount of RISC. Importantly, such multiple turnover does not require ATP. Moreover, the modest stimulation of RISC activity by ATP is not mediated through ATP hydrolysis since nonhydrolyzable analogs of ATP could also stimulate RISC activity. Although we uncovered a small stimulatory effect on RISC activity by ATP and phosphate analogs in vitro, such effects may not reflect a physiological role for ATP and phosphate analogs in vivo. Future structural and mechanistic analysis of the catalytic sites of RISC should shed further insight into the regulatory action of nucleotides on RISC activity.

Experimental Procedures

Affinity Purification of FLAG-Dicer

FLAG-Dicer expression plasmid was cotransfected with a puromycin resistance plasmid into HEK293 cells. Complexes were purified from 150–450 mg cytoplasmic extract (S100) with anti-FLAG M2 affinity gel (Sigma). After washing twice with buffer A (20 mM Tris-HCl [pH 7.9], 0.5 M KCl, 10% glycerol, 1 mM EDTA, 5 mM DTT, 0.2 mM PMSF, 0.5% NP40) and once with buffer B (20 mM Tris-HCl [pH 7.9], 0.1 M KCl, 10% glycerol, 1 mM EDTA, 5 mM DTT, 0.2 mM PMSF), the affinity column was eluted with FLAG peptide. Analysis of FLAG-Dicer by Superose 6 and Superdex 200 gel filtration was as described previously (Gregory et al., 2004). Size calibration was performed using molecular size markers thyroglubulin (670 kDa) and ferritin (440 kDa). Western blots were performed using anti-TRBP and anti-Ago2 polyclonal antibodies. For functional analysis of column fractions, samples were first dialyzed for 2–3 hr at 4°C against a buffer containing 20 mM Tris-HCl (pH 7.9), 0.1 M KCl, 10% glycerol.

Pre-miRNA Processing

A 60 nucleotide synthetic pre-mRNA corresponding to the *Drosophila* pre-let-7 (UGAGGUAGUAGGUUGUAUAGUAGUAAUUACACAUCAUACUAUACAAUGUGCUAGCUUUCU) was 5′ end labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase (T4 PNK). The labeled pre-let-7 RNA was gel purified from a 15% denaturing polyacrylamide gel by incubating the excised gel slice in H_2O at 65°C for 2 hr followed by phenol:chloroform extraction and RNA precipitation. Processing of pre-miRNA was performed by incubating dialyzed Superose 6 gel filtration column fractions with gel-purified pre-let-7 in a buffer containing 3.2 mM $MgCl_2$, 1 mM ATP, 20 mM creatine phosphate, 0.2 U/μl RNasin, 20 mM Tris-HCl (pH 7.9), 0.1 M KCl, 10% glycerol for 1 hr at 37°C. Reactions were stopped by

the addition of an appropriate volume of 5× gel loading buffer (25% glycerol, 0.5% SDS, 50 mM EDTA, 0.25% bromophenol blue, 0.25% xylene cyanol) and 10 μg Proteinase K; incubated for 30 min at room temperature; and then, after heat denaturation (95°C, 1 min), were loaded directly onto a 15% denaturing polyacrylamide gel. The RNA size marker (Ambion) was prepared according to manufacturer's instructions.

RISC Assays

Affinity-purified protein complexes were assayed for target-RNA cleavage using, where indicated, either single-stranded (ss) guide (UGAGGUAGUAGGUUGUAUAGU) or passenger (UAUACAAUGUG CUAGCUUUCU) 21 nt synthetic RNAs, double-stranded (ds) 21 nt let-7, or 60 nt gel-purified pre-let-7 (UGAGGUAGUAGGUUGUAU AGUAGUAAUUACACAUCAUACUAUACAAUGUGCUAGCUUUCU). All RNAs were 5′ phosphorylated prior to use by incubating 1 μM RNA in a buffer containing 10 units T4 PNK (NEB) and 5 mM ATP for 1 hr at 37°C, followed by G50 (Promega) column purification. Dialyzed column fractions (10–20 μl) were preincubated with the indicated concentration of either ss, ds, or pre-let 5′-phosphorylated RNA in a buffer containing 3.2 mM $MgCl_2$, 1 mM ATP, 20 mM creatine phosphate, 0.2 U/μl RNasin, 20 mM Tris-HCl (pH 7.9), 0.1 M KCl, 10% glycerol for 30 min at 37°C. Subsequently, 5′-labeled target RNA (UAUACAACCUACUACCUCAUU) or antisense RNA (AAAGCUAGCACAUUGUAUAGU) was added to the reaction at the indicated concentrations, and the cleavage reaction was performed for 90 min at 37°C. The final reaction volume was 30 μl. Reactions were stopped by the addition of 5× gel loading buffer (25% glycerol, 0.5% SDS, 50 mM EDTA, 0.25% bromophenol blue, 0.25% xylene cyanol) and Proteinase K; incubated for 30 min at room temperature; and then, after heat denaturation (95°C, 1 min), were loaded directly onto a 15% denaturing polyacrylamide gel. For ATP-dependence experiments, reactions were performed in a buffer containing 3.2 mM $MgCl_2$, 0.2 U/μl RNasin, 20 mM Tris-HCl (pH 7.9), 0.1 M KCl, 10% glycerol supplemented with 1 mM ATP, GTP, AMP-PNP, AMP-CPP, or ATPγS where indicated. For some experiments, samples were preincubated with 1 unit hexokinase (Sigma) in reaction buffer supplemented with 2 mM glucose at 37°C for 30 min prior to addition of 21 nt single-stranded siRNA, duplex siRNA, or pre-let-7 RNA. Gels were exposed to X-ray films, after which the relative band intensities were calculated using Quantity One imaging software (Bio-Rad).

Supplemental Data

Supplemental Data include one figure and can be found with this article online at http://www.cell.com/cgi/content/full/123/4/631/DC1/.

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