Slicer function of *Drosophila* Argonautes and its involvement in RISC formation

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Argonaute proteins play important yet distinct roles in RNA silencing. Human Argonaute2 (hAgo2) was shown to be responsible for target RNA cleavage ("Slicer") activity in RNA interference (RNAi), whereas other Argonaute subfamily members do not exhibit the Slicer activity in humans. In *Drosophila*, AGO2 was shown to possess the Slicer activity. Here we show that AGO1, another member of the *Drosophila* Argonaute subfamily, immunopurified from Schneider2 (S2) cells associates with microRNA (miRNA) and cleaves target RNA completely complementary to the miRNA. Slicer activity is reconstituted with recombinant full-length AGO1. Thus, in *Drosophila*, unlike in humans, both AGO1 and AGO2 have Slicer functions. Further, reconstitution of Slicer activity with recombinant PIWI domains of AGO1 and AGO2 demonstrates that other regions in the Argonautes are not strictly necessary for small interfering RNA (siRNA)-binding and cleavage activities. It has been shown that in circumstances with AGO2-lacking, the siRNA duplex is not unwound and consequently an RNA-induced silencing complex (RISC) is not formed. We show that upon addition of an siRNA duplex in S2 lysate, the passenger strand is cleaved in an AGO2-dependent manner, and nuclease-resistant modification of the passenger strand impairs RISC formation. These findings give rise to a new model in which AGO2 is directly involved in RISC formation as "Slicer" of the passenger strand of the siRNA duplex.

[*Keywords*: RNAi; Argonaute; Slicer, RISC; *Drosophila*]

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RNA interference (RNAi) is a sequence-specific RNA cleavage process triggered by double-stranded RNA (dsRNA) (for review, see Sontheimer 2005; Tomari and Zmore 2005). Long dsRNA is first converted to 21–23-nucleotide (nt) small interfering RNA (siRNA) by Dicer (Bernstein et al. 2001; Elbashir et al. 2001a; Ketting et al. 2001; Knight and Bass 2001). Subsequently, the siRNA duplex goes through an ATP-dependent unwinding process and one strand over the other is often preferentially loaded onto the RNA-induced silencing complex (RISC) that endonucleolytically cleaves target RNA at sites completely complementary to the siRNA (Hammond et al. 2000; Nykanen et al. 2001; Hutvagner and Zamore 2002a; Martinez et al. 2002). Cleavage on the target RNA sequence in RNAi is known to occur between +10 and +11 positions of the guide siRNA (the 5′-end of the guide siRNA being assigned +1) (Elbashir et al. 2001b; Schwarz et al. 2004).

microRNAs (miRNAs) are a large family of 21–22-nt noncoding RNAs that interact with target mRNAs at specific sites to induce cleavage of the message or inhibit translation (Olsen and Ambros 1999; Reinhart et al. 2000; Ambros 2004; Bartel 2004; Bagga et al. 2005). The expression of miRNA itself is often developmentally regulated in a spacious- and/or timing-specific manner, implying an important developmental role for miRNAs in the regulation of endogenous gene expression (Lagos-Quintana et al. 2001; Lau et al. 2001; Lee and Ambros 2001; Reinhart et al. 2002; Brennecke et al. 2003; Leaman et al. 2005; Pillai et al. 2005). Lately, direct relations between miRNA and human diseases such as cancer have been gradually elucidated (Garraway et al. 2005; He et al. 2005; Johnson et al. 2005; Mattick and Makunin 2005). miRNAs are excised in a stepwise process from primary miRNA transcripts (pri-miRNAs) (Bartel 2004; Kim 2005). Then, mature miRNA is loaded onto RISC (miRISC) as siRNA in RNAi (Hutvagner and Zamore 2002b).

A biochemical approach to identifying protein components involved in RISC in the *Drosophila* system led to the identification of a member of the Argonaute family of proteins as an essential factor in RNAi (Hammond et al. 2001). Members of the Argonaute family, defined by the presence of PAZ and PIWI domains, are known to play essential roles, while residing in RISC, in gene silencing triggered by small RNAs (Carmell et al. 2002).

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Argonaute members can be divided into two groups, the Argonaute and the Piwi subfamilies (Sasaki et al. 2003). Even within a subfamily, each member seems functionally distinct. Fly embryos lacking AGO2, a member of the Argonaute subfamily in fly, are siRNA-directed RNAi defective, but still capable of miRNA-directed target RNA cleavage (Okamura et al. 2004). AGO1, another Argonaute protein in fly (Kataoka et al. 2001), is dispensable for siRNA-directed RNA cleavage, but is necessary for miRNA-directed target RNA cleavage (Okamura et al. 2004). Depletion of AGO2, but not AGO1, from *Drosophila* Schneider2 (S2) cells results in transgene mRNA stabilization with concomitant shortening of the mRNA poly(A) tail (Siomi et al. 2005). In humans, Ago2 (hAgo2) is associated with both siRNA and miRNA, and mediates RNA cleavage targeted by small RNAs; however, other Argonaute subfamily members, such as hAgo1, hAgo3, and hAgo4, do not mediate such RNA cleavage, although all can associate with siRNA and miRNA (Liu et al. 2004; Meister et al. 2004a) and show high similarities to hAgo2 at peptide sequence levels (Sasaki et al. 2003). In plants, specific members of the Argonaute subfamily also show distinct biochemical activities (Qi et al. 2005).

Structural analysis of full-length *Pyrococcus furiosus* AGO-related protein revealed that the PIWI domain, one of the common domain structures in the Argonautes, shows an RNaseH-like structure, and that the essential residues for the endonucleolytic activity of RNaseH are conserved in the Argonaute-PIWI (AGO-PIWI) domain (Song et al. 2004). Mutagenizing the conserved residues in hAgo2 abolished the RNA cleavage activity targeted by small RNAs (Liu et al. 2004). Furthermore, Slicer, the endonucleolytic enzyme residing in RISC activity can be reconstituted with recombinant hAgo2 and single-stranded siRNA (Rivas et al. 2005). These observations indicate that hAgo2 acts as Slicer in RNAi.

The crystal structure of *Archaeoglobus fulgidus* Piwi protein (AfPiwi) associated with an siRNA-like small RNA has been determined (Ma et al. 2005; Parker et al. 2005). AfPiwi, utilized as a model for understanding the eukaryotic AGO-PIWI domain, was shown to bind to the 5′-end of the guide strand of the siRNA duplex, although previous structural and biochemical studies implied that the conserved PAZ domain in Argonaute proteins was the guide siRNA-binding module through its 3′-end (Yan et al. 2003; Lingel et al. 2004; Ma et al. 2004; Song et al. 2004). The crystal structure of *Aquifex aeolicus* Argonaute (Aa-Ago), a site-specific DNA-guided RNA endonuclease, was recently solved (Yuan et al. 2005) and an Ago-mediated target RNA cleavage cycle model proposed. According to this model, the Ago-PAZ domain binds to the 3′-end of siRNA at the beginning, but once target RNA comes and starts to anneal to the siRNA, the 3′-end of the guide strand is released from the PAZ domain. This action consequently allows target RNA to form a full-length guide siRNA-target RNA duplex before the target RNA cleavage really occurs. However, crystallographic characterization of Argonaute from organisms furnishing genuine RNAi machinery will be necessary to create a fully elaborated biochemical model of the small RNA-directed target RNA cleavage pathway.

In *Drosophila*, AGO2 was reported to be the sole protein required for target RNA cleavage activity in RNAi (Rand et al. 2004). While AGO2 may indeed function as the Slicer, AGO1 is also thought responsible for cleaving target RNA since it is more similar to hAgo2 at the peptide sequence level than is AGO2; in addition, AGO1 is also required for efficient miRNA-directed target RNA cleavage in fly embryo (Okamura et al. 2004). In humans, hAgo1 does not show Slicer activity as opposed to hAgo2. This is quite striking since hAgo1 and hAgo2 show strong similarities at both the peptide sequence level and in their biochemical properties, such that both proteins bind siRNA and miRNA (Liu et al. 2004; Meister et al. 2004a). They have even been shown to coimmunoprecipitate from human culture cells (Sen and Blau 2005). Although the function of hAgo1 has yet to be elucidated, it seems quite unlikely that *Drosophila* AGO1 is not simply the counterpart of hAgo1.

In this study, we show that in *Drosophila*, unlike in humans, not only AGO2, but also AGO1 has the capacity to show Slicer activity. Recombinant full-length AGO1 and the PIWI domains of AGO1 (AGO1-PIWI) and AGO2 (AGO2-PIWI) were successfully produced in *Escherichia coli*. All recombinants were found capable of cleaving RNA targets when associated with small guide RNA. Our data indicate that the PAZ domain is not strictly necessary for the Argonautes to exhibit Slicer activity. It has been reported that recombinant RISC does not require ATP for cleaving target RNA and releasing the RNA products from itself (Rivas et al. 2005). In this study, using a *Drosophila* system, we showed that each step indeed occurs without ATP and that they are not stimulated by the addition of ATP. Of the siRNA duplex programmed in S2 lysate, the passenger strand was cleaved in an AGO2-dependent manner and the AGO2 cleavage-resistant passenger strand clearly impaired RISC formation in S2 cell lysate. These findings give rise to a new model in which AGO2 is directly involved in RISC formation as "Slicer" of the passenger strand of the siRNA duplex. Finally, reconstitution of Slicer activity was accomplished with recombinant full-length AGO1 together with the siRNA duplex and its target RNA.

Results

Immunopurified AGO2 from siRNA-programmed S2 lysate shows target RNA cleavage activity

We assessed whether *Drosophila* AGO2 immunopurified from S2 cells could show target RNA cleavage activity. S2 lysate was incubated with the *luc* siRNA duplex, from which AGO2 was immunoprecipitated under a high-salt condition using an anti-AGO2 antibody raised specifically for the N terminus of the protein. Silver-staining of the protein contents in the immunoprecipitate showed that AGO2 was nearly uniform in the

fraction (Fig. 1A). Western blot analysis confirmed that the immunoprecipitate contains AGO2, but not AGO1, as an undesired contaminant (Supplementary Fig. S1). Northern blotting revealed that the immunopurified AGO2 associates with *luc* guide siRNA (Fig. 1B). This data agreed well with previous reports showing that Argonaute proteins are very tightly bound to single-stranded siRNA within RISC (Martinez et al. 2002; Rand et al. 2004). *luc* target RNA radiolabeled with ^{32}P at the 5′-end was then incubated with the AGO2-*luc* guide siRNA complex immobilized on beads. Figure 1C shows

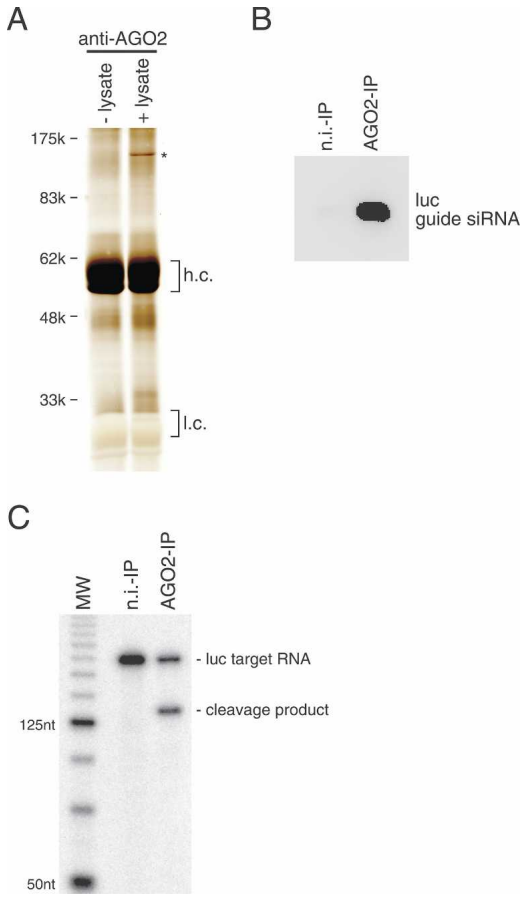

Figure 1. AGO2 immunopurified from siRNA-programmed S2 cells is associated with guide siRNA and exhibits target RNA cleavage activity. (*A*) Silver-staining of the immunoprecipitate using a specific monoclonal antibody against AGO2. The lane indicated as –lysate shows the proteins that originated from the antibody. The lane indicated as +lysate contains a band corresponding to AGO2 (shown with an asterisk). Other protein bands were not observed in the +lysate lane compared with the –lysate lane, indicating that the AGO2 immunoprecipitated was nearly uniformly purified. (h.c.) Heavy chains of the antibody; (l.c.) light chains of the antibody. (*B*) Northern blot shows that *luc* guide siRNA is specifically associated with the AGO2 immunopurified in *A*. The lane indicated as n.i.-IP shows that *luc* guide siRNA is not in the immunoprecipitate with nonimmune IgG used as a negative control. (*C*) In vitro target RNA cleavage assay using *luc* target RNA. Immunopurified AGO2, as shown in *A*, shows ability for cleaving the target RNA (luc180; 5′-radiolabeled with ^{32}P), whereas the control (n.i.-IP) does not. that the complex is able to cleave *luc* target RNA. AGO2 immunopurified from naïve S2 lysate under the same conditions did not show such activity (data not shown). These data demonstrate that after incubation in S2 lysate, the siRNA duplex was properly processed and single-stranded guide siRNA was loaded onto AGO2, and further, that AGO2 associated with guide siRNA is capable of cleaving the RNA target harboring a sequence completely complementary to the siRNA. Previously it was revealed in a biochemical approach that the sole protein required for RISC activity in S2 cells was AGO2 (Rand et al. 2004). Our current data confirm that AGO2 is indeed an element required for the target RNA cleavage activity in *Drosophila*.

AGO1 also functions as Slicer in Drosophila

Drosophila AGO1 shows a higher similarity than *Drosophila* AGO2 to hAgo2 at the amino acid sequence level (Supplementary Fig. S2). We previously showed that even in AGO2-lacking embryo lysate, target RNA cleavage takes place in a miRNA-dependent manner (Okamura et al. 2004); therefore, it was assumed that AGO1 in *Drosophila* should also have the ability to cleave the RNA target as well as AGO2. Immunoprecipitation was carried out from *luc* siRNA-programmed S2 lysate with an anti-AGO1 antibody under a high-salt condition (Fig. 2A), and cleavage assay was performed using *luc* target RNA as in Figure 1C. The fact that the immunoprecipitate contains AGO1 but not AGO2 was determined by Western blotting (Supplementary Fig. S1). Unlike AGO2 (Fig. 1C), immunopurified AGO1 did not show *luc* target RNA cleavage activity (data not shown). Northern blotting revealed that immunopurified AGO1 was not associated with *luc* guide siRNA (Fig. 2B, top). We then assessed whether the immunopurified AGO1 was associated with miRNAs by performing Northern blotting using an oligo DNA probe for mi-RNA bantam (miR-ban), one of the miRNAs known to be expressed in S2 cells (Okamura et al. 2004). Immunopurified AGO1, but not AGO2, associates with miR-ban (Fig. 2B, bottom). In vitro target RNA cleavage assay was then performed using an RNA containing a sequence completely matched to miR-ban (bantam38). We observed the expected cleavage product when bantam38 was incubated with immunopurified AGO1 (Fig. 2C), indicating that AGO1 also shows Slicer activity depending on the miRNA with which AGO1 is associated. Under the same conditions, immunopurified AGO2 not associating with miR-ban (Fig. 2B) was unable to cleave bantam38 (Fig. 2C). In the next experiment, the same assay as in Figure 2C was performed, but after incubation the supernatant was separated from the beads on which AGO1-miR-ban was immobilized. RNAs were then isolated from both fractions and analyzed. About 65% of the cleavage product was observed in the supernatant fraction under the condition where ATP was not added exogenously to the reaction mixture. Depletion of ATP (treatment of AGO1-miR-ban complex with hexokinase and glucose prior to the cleavage reaction; see Materials and Methods) (Ny-

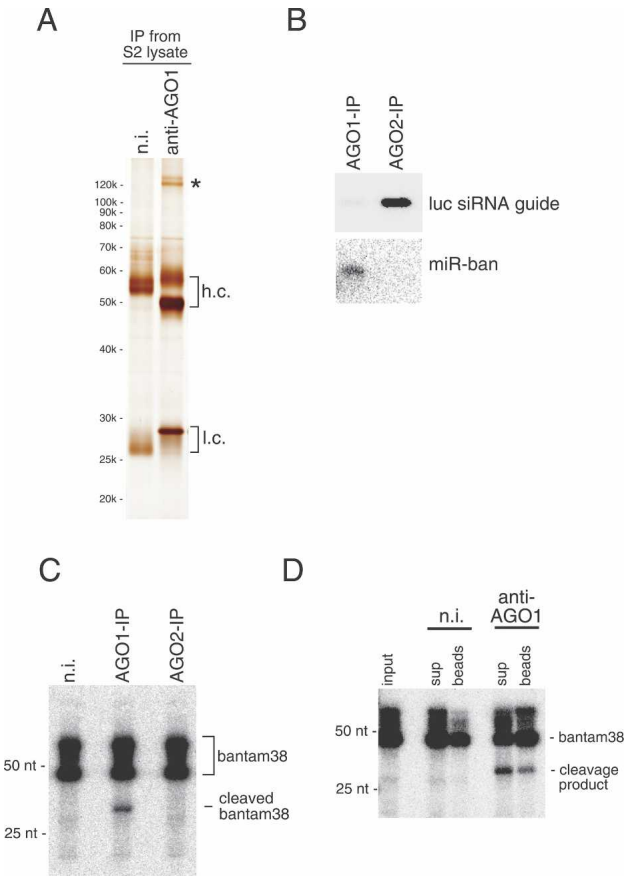

Figure 2. AGO1 immunopurified from naïve S2 cells is associated with endogenous miRNA and cleaves target RNA harboring a sequence completely complementary to the miRNA. (*A*) Silver-staining of the immunoprecipitate with anti-AGO1 monoclonal antibody. The lane indicated as anti-AGO1 shows the protein components in the immunoprecipitate with anti-AGO1 antibody. The n.i. lane contains the protein components in the immunoprecipitate with negative control nonimmune IgG. A doublet corresponding to AGO1 is shown with an asterisk. The same protein band is not observed in the n.i. lane. (h.c.) Heavy chains of the antibody; (l.c.) light chains of the antibody. (*B*, *top*) Northern blot shows that *luc* guide siRNA is not associated with AGO1 immunopurified from *luc* siRNA-programmed S2 lysate. (*Bottom*) miR-ban, a miRNA known to be expressed in S2 cells, is associated with immunopurified AGO1. (*Bottom*) Under the same conditions, the AGO2 immunoprecipitated was not associated with miR-ban. (*C*) In vitro target RNA cleavage assay using miR-ban target RNA (bantam38), which was radiolabeled with ^{32}P at the 5′-end. Immunopurified AGO1 (AGO1-IP) (*A*) and AGO2 (AGO2-IP) (Fig. 1A) was incubated with bantam38, and then the reaction RNAs were prepared and analyzed on a gel. AGO1-IP shows the ability to cleave bantam38, whereas the control (n.i.) and AGO2-IP showed no such activity for cleaving bantam38. (*D*) The in vitro target RNA cleavage assay shown in *C* was repeated. The supernatant and the bead fractions were separated after reaction and RNAs were prepared and analyzed. The cleaved product is observed in the supernatant fraction, meaning that the product was released in buffer after cleavage. The same was observed even under conditions without ATP (data not shown); indicating that the releasing of cleaved target RNA from AGO1 occurs in an ATP-independent manner.

kanen et al. 2001) or addition of ATP did not affect the result (data not shown), indicating that the target RNA-cleaved products were released from AGO1 in solution in an ATP-independent manner. This is not a unique property of *Drosophila* AGO1, since the same phenomenon was also observed with *Drosophila* AGO2-guide siRNA (data not shown) and recombinant hAGO2-guide siRNA (Rivas et al. 2005) complexes. Nonetheless, ATP hydrolysis may accelerate the product release in RNAi occurring in vivo (Haley and Zamore 2004).

Reconstitution of Slicer activity with recombinant full-length AGO1 and the PIWI domains of AGO1 and AGO2

Even though immunoprecipitation was performed under stringent conditions, in which almost all protein–protein interaction was presumably abolished, AGO1 and AGO2 immunopurified from S2 lysate may still contain tiny amounts of contaminants that could participate to some degree in target RNA cleavage activity. To verify whether Argonaute was indeed the Slicer, recombinant Argonaute expressed in a system that does not furnish the RNAi machinery (i.e., bacteria) was required. In our study, recombinant *Drosophila* AGO1 fused to glutathione S-transferase (GST-AGO1) was successfully produced in *E. coli* (Supplementary Fig. S3). This prompted us to question whether the Slicer activity can be reconstituted with GST-AGO1. Purified GST-AGO1 was incubated with *luc* guide siRNA, and then *luc* target RNA radiolabeled at the 5′-end was added to the reaction mixture. The target RNA was efficiently cleaved by GST-AGO1 (Fig. 3A). Addition of EDTA at 10 mM to the reaction abolished the activity (Fig. 3A). Without *luc* guide siRNA, target RNA cleavage was not observed (Fig. 3A). A similar experiment was carried out using miRNA let-7 (let-7) and let-7 target RNA, where the let-7 target RNA was cleaved in an AGO1-dependent manner (Fig. 3B). In both cases, GST itself did not show target RNA cleavage activity. These results clearly demonstrate that AGO1 does indeed function as Slicer. In these experiments, recombinant AGO1 did not distinguish siRNA or miRNA to associate with. siRNA was not loaded onto AGO1 when the siRNA duplex was programmed into S2 lysate (Fig. 2B). These results suggest that a well-ordered, programmed mechanism distinguishing siRNA from miRNA being loaded onto AGO2 exists in the lysate. It has been already shown that R2D2 bridges the initiation and effector steps of the *Drosophila* RNAi pathway by facilitating siRNA passage from Dicer2 to AGO2 (Liu et al. 2003; Tomari et al. 2004b). Similarly, mature miRNA loading onto AGO1 is likely restricted by specific, consecutive interactions of pre-miRNA with the processing complex including Dicer1/Loquacious (R3D1), and then of the complex with AGO1 (Forstemann et al. 2005; Jiang et al. 2005; Saito et al. 2005).

The PIWI domain conserved in Argonaute proteins adopts an RNaseH fold (Liu et al. 2004; Song et al. 2004) and provides the binding pocket for the 5′-end of guide RNA (Ma et al. 2005; Parker et al. 2005). We then sought

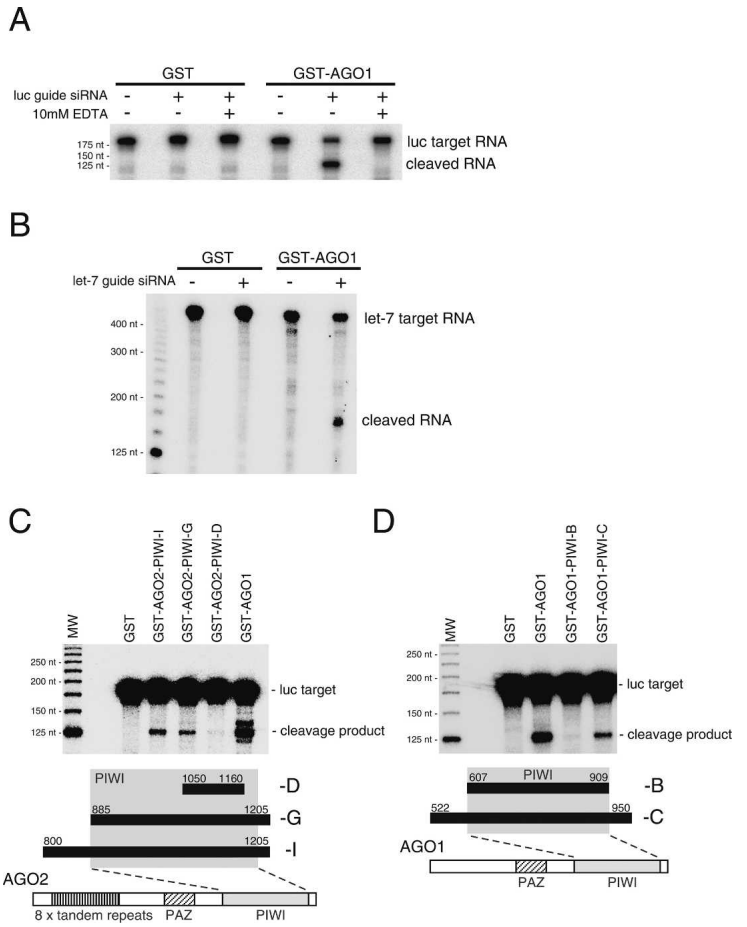

Figure 3. Recombinant *Drosophila* full-length AGO1 and the PIWI domains of AGO1 and AGO2 cleave target RNA, depending on the small RNA sequences with which they are associated. (*A*) *luc* target RNA was efficiently cleaved by GST-AGO1 associated with *luc* guide siRNA. Purified GST-AGO1 (Supplementary Fig. S3) was first incubated with *luc* guide siRNA and then *luc* target RNA (luc180) radiolabeled at the 5′-end was added. Addition to the reaction of EDTA at 10 mM abolished the activity. Without siRNA addition, the target RNA cleavage is not observed. GST itself does not show the activity. (*B*) A similar experiment to *A* was carried out using miRNA let-7 (miR-let-7) and the miR-let-7 target RNA (Okamura et al. 2004). Target RNA is cleaved in an AGO1-miR-let-7-dependent manner. (*C*) Reconstitution of Slicer activity with the PIWI domain of AGO2. All of the recombinants used were first incubated with *luc* guide siRNA and then *luc* target RNA (luc180) radiolabeled at the 5′-end was added and incubated. GST-AGO2-PIWI-I and GST-AGO2-PIWI-G cover the PIWI domain of AGO2, but GST-AGO2-PIWI-D contains only a portion of the PIWI domain as indicated below. (*D*) The PIWI domain of AGO1 preassociated with guide siRNA also shows activity to cleave target RNA.

to test whether the PIWI domain alone of *Drosophila* Argonautes would be sufficient for binding to guide RNA and for cleaving its RNA target. Recombinant peptides harboring the PIWI domains of both *Drosophila* AGO1 and AGO2 tagged with GST were purified from *E. coli* (Supplementary Fig. S4), and target RNA cleavage assays were performed. *luc* target RNA was cleaved by GST-AGO2-PIWI-G, GST-AGO2-PIWI-I (Fig. 3C), and GST-AGO1-PIWI-C (Fig. 3D) preassociated with *luc* guide siRNA. A small portion of the PIWI domain of AGO2 tagged with GST (GST-AGO2-PIWI-D) did not exhibit the activity (Fig. 3C). These data demonstrate that the PIWI domain is itself sufficient, and that the PAZ domain is dispensable for initiating guide siRNA-dependent target RNA cleavage in vitro. GST-AGO1-PIWI-B showed much less Slicer activity compared with GST-AGO1-PIWI-C (Fig. 3D), suggesting that the amino acid residues adjacent to the PIWI domain are necessary for, for instance, supporting the domain structure.

AGO1/AGO2 associated with guide siRNA is able to cleave passenger siRNA

Endonucleolytically cleaved target RNAs were released from AGO1 and AGO2 in a manner independent of ATP (Fig. 2D; data not shown). It has previously been shown that without AGO2, the siRNA duplex was not unwound even after 1 h incubation in fly embryo lysate, and as a result, RISC formation did not occur (Okamura et al. 2004; Tomari et al. 2004b). We hypothesized that AGO2 might be involved in the siRNA duplex unwinding process as an enzyme cleaving the passenger strand while it is still in a duplex form with guide siRNA. After this process, the cleaved passenger could be thrown away in buffer and, consequently, AGO2 would be able to bind only with the remaining single-stranded guide siRNA. To test this hypothesis, we first examined whether AGO2 associated with guide siRNA is capable of cleaving passenger siRNA. AGO2 immunopurified from siRNA-programmed S2 lysate under a high-salt condition was incubated with *luc* passenger siRNA radiolabeled at the 5′-end. A band corresponding to the cleavage product (9 nt) was clearly observed, indicating that the passenger siRNA was cleaved as expected by AGO2 associated with guide siRNA (Fig. 4A). This result is consistent with the previous observation that RISC is able to recognize a target RNA molecule as short as 15 nt (Martinez and Tuschl 2004). We next questioned whether the cleaved form of the passenger strand (9 nt in length) could be detected in lysates programmed with siRNA duplex. In this experiment, two kinds of siRNA duplex (the nucleotide sequences are the same, but only the pas-

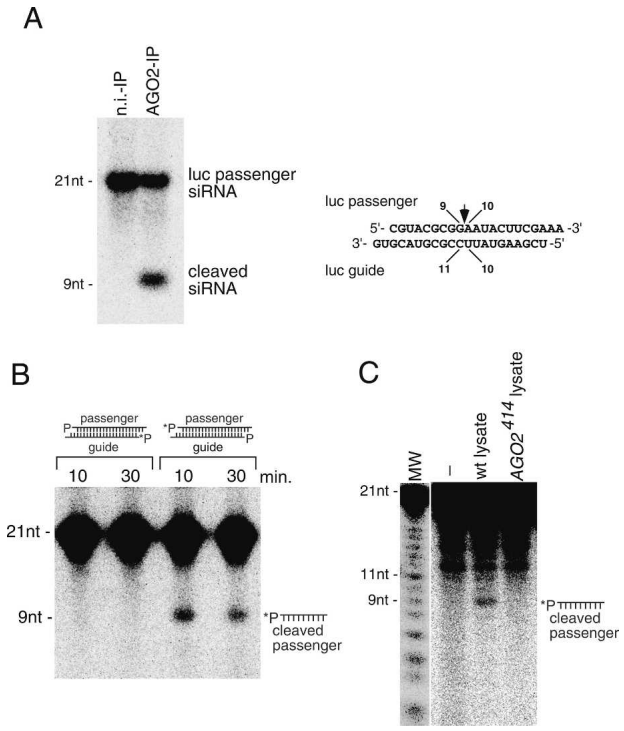

Figure 4. Cleavage of the passenger strand of siRNA duplex by AGO2. (*A*) AGO2 immunopurified from siRNA-programmed S2 lysate under high-salt conditions was incubated with *luc* passenger siRNA radiolabeled at the 5′-end. The passenger strand is cleaved by AGO2 preloaded with *luc* guide siRNA. *luc* guide and passenger strand sequences are shown on the *right*. The cleavage site on the passenger is indicated with an arrow. (*B*) Detection of the cleaved passenger strand of siRNA duplex programmed in S2 lysates. Two sets of *luc* siRNA duplex (the nucleotide sequences are the same, but only passenger or guide strand of the duplex was radiolabeled at the 5′-end) were used. The passenger strand, but not guide siRNA being cleaved (9 nt) is detected. (*C*) In AGO2-lacking embryo lysate (*AGO2*^{414} lysate) (Okamura et al. 2004), the cleaved passenger is not detected, as opposed to the wild-type embryo lysate, which indicates that cleavage of the passenger strand of the siRNA duplex occurs in an AGO2-dependent manner. The *luc* siRNA duplex (the passenger was radiolabeled at the 5′-end) was used.

senger or guide strand of the duplex was radiolabeled at the 5′-end) were prepared. We could detect the passenger strand but not the guide siRNA being cleaved in S2 lysate (Fig. 4B). In AGO2-lacking embryo lysate (*AGO2*^{414} lysate) (Okamura et al. 2004), the cleaved passenger was not detected as opposed to the wild-type embryo lysate (Fig. 4C), indicating that cleavage of the passenger strand of the siRNA duplex in lysates occurs in an AGO2-dependent manner.

Nuclease-resistant modification of the passenger strand blocks RISC formation

However, it could be possible that the siRNA duplex programmed in lysates comes apart right after being added to the lysate; then, single-stranded guide siRNA is loaded onto RISC, which then captures the passenger as a target and cleaves it. Thus, a similar assay as in Figure 4B was performed using a nuclease-resistant-modified oligonucleotide. The presence of a single-stranded passenger modified with 2′-O-methyl groups, which is known to act as competitive inhibitors of RISC by binding to guide siRNA within RISC (Haley and Zamore 2004; Hutvagner et al. 2004; Meister et al. 2004b), did not block the production of 9 nt (Fig. 5A). The result demonstrates that the cleavage of the passenger occurs while it is still in the initial duplex form with guide siRNA. In the next experiment, we used the siRNA duplex, in which only one nucleotide of the passenger strand (the ninth nucleotide from the 5′-end, see Fig. 4A) was modified by the 2′-O-methyl group (OMe-9). This single modification is known to block target RNA cleavage in RNAi (Martinez and Tuschl 2004). The modified passenger strand, but not the guide strand, was radiolabeled with ^{32}P at the 5′-end for visualization. After incubation in the lysate, we did not observe cleaved passenger of si-duplex (OMe-9), as expected (Fig. 5B). Native agarose gel electrophoresis showed that RISC formation was clearly impaired when si-duplex (OMe-9) (in this particular experiment the unmodified guide strand was labeled with ^{32}P at the 5′-end) was added to S2 lysate (Fig. 5C, left panel). The target RNA cleavage activity of S2 lysate was also markedly impaired when si-duplex (OMe-9) was used in the target RNA cleavage assay (Fig. 5C, right panel). These results indicate that the cleavage of the passenger strand of the siRNA duplex is required for efficient formation of active RISC. These findings give rise to a new model, in which AGO2 is directly involved in RISC formation as Slicer of the passenger strand of the siRNA duplex.

RISC formation was clearly impaired but not completely blocked with the 2′-O-methyl-modified passenger (Fig. 5C), implying that activities other than AGO2 Slicer function might be cooperatively involved in the siRNA duplex unwinding process and concomitant RISC formation with AGO2. A most likely candidate for such activities is the helicase activity of, for example, a DEAD-box RNA helicase. To distinguish whether or not such an RNA helicase is indeed involved in RISC formation, as has been presumed for a long time, we re-examined ATP requirements in RISC formation by native agarose gel electrophoresis. S2 lysate was prepared and divided in half. One-half was treated with hexokinase in the presence of glucose as previously reported (Nykanen et al. 2001). The other half of the lysate was also incubated, but hexokinase and glucose were not added prior to incubation. Both portions of the lysate were then used for the RISC formation with the *luc* siRNA duplex, in which the guide strand was radiolabeled with ^{32}P at the 5′-end and the passenger was phosphorylated with cold ATP. After annealing, the siRNA duplex was gel-purified to avoid any contamination of residual ATP from the phosphorylation reaction. ATP, GTP, creatine phosphate, and creatine kinase were omitted for –ATP reaction. Even in ATP-depleted lysate, RISC was formed as well with ATP (Fig. 5D). Furthermore, addition of a com-

petitive inhibitor of ATP, AMP-PNP, at 1μM to ATP-depleted lysate did not affect the result (data not shown). RISC formed in ATP-depleted lysate is active, since target RNA cleavage occurred quite similarly in both lysate portions (Fig. 5E). Taken together, it is suggested that enzyme(s) requiring ATP as a cofactor for their functions, such as a typical ATP-dependent DEAD-box RNA helicase, are not strictly required for the siRNA duplex unwinding process in concert with AGO2.

Reconstitution of Slicer activity using recombinant full-length AGO1 together with siRNA duplex and its target RNA

Reconstitution of Slicer activity has so far been demonstrated using single-stranded siRNA or miRNA (Fig. 3A,B; Rivas et al. 2005). Next, we asked whether Slicer activity could be reconstituted with a sole protein, recombinant Argonaute, and the siRNA duplex. We first checked whether GST-AGO1 has the capacity to cleave passenger strands still in a duplex form with guide siRNA. The siRNA duplex (both strands phosphorylated, but only the passenger radiolabeled with ^{32}P) was incubated in buffer containing GST-AGO1 or GST. Cleaved passenger was observed with GST-AGO1, but not with GST (Fig. 6A), indicating that the passenger was cleaved by AGO1 even in a duplex form. Then, a similar reaction was set up using the cold *luc* siRNA duplex. After incubation to allow GST-AGO1 to process the siRNA duplex, the target RNA (*luc* target radiolabeled with ^{32}P at the 5′-end; 130 nt in length) was added to the reaction mixture. As a control, GST-AGO1 preassociated with single-stranded *luc* guide siRNA was also used. Although it was not as robust compared with GST-AGO1 preassociated with guide siRNA, GST-AGO1 preincubated with the siRNA duplex clearly showed Slicer activity (Fig. 6B). These results demonstrate that Slicer activity was reconstituted with recombinant full-length AGO1 together with siRNA duplex and its target RNA.

Discussion

In this study, we showed that endogenous AGO1 and AGO2 immunopurified with specific monoclonal antibodies under harsh conditions in which almost all protein–protein interactions were disrupted exhibited target RNA cleavage activity. We proceeded to reconstitute Slicer activity with recombinant full-length AGO1 and the PIWI domains of both AGO1 and AGO2 purified from *E. coli*. These experiments made a compelling argument that both AGO1 and AGO2 function as Slicer in *Drosophila*.

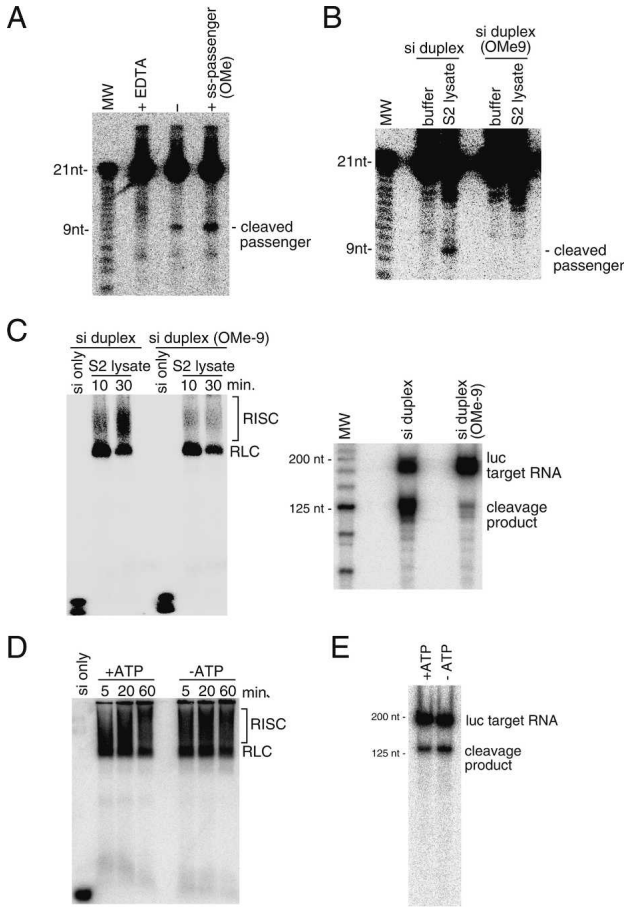

Figure 5. Nuclease-resistant passenger of the siRNA duplex blocks RISC formation, but ATP depletion does not. (*A*) The reaction shown in Figure 4B was repeated. Either EDTA (to 10 mM) or 2′-OMe-modified passenger was added when the reactions were initiated. Total incubation time was 15 min. Addition of EDTA blocks the cleavage of the passenger; in contrast, 2′-O-methyl-modified passenger does not. (*B*) The siRNA duplex, of which the passenger strand was modified with the 2′-O-methyl group at the ninth nucleotide (OMe-9) and radiolabeled with ^{32}P at the 5′-end, was programmed in S2 lysate. In si-duplex (OMe-9), the cleaved passenger is not observed, unlike with nonmodified si-duplex. (*C*, *left*) Native agarose gel electrophoresis shows that even after 30 min incubation, RISC formation is strongly impaired with si-duplex (OMe-9) (the passenger was modified with the 2′-O-methyl group at the ninth nucleotide, and the guide was labeled with ^{32}P at the 5′-end). (*Right*) The target RNA cleavage activity of S2 lysate was also markedly impaired when the si-duplex (OMe-9) was preprogrammed to the lysate. These results suggest that cleavage of the passenger strand of the siRNA duplex by AGO2 is required for efficient formation of active RISC. A target RNA cleavage assay was performed as described previously (Okamura et al. 2004). luc180 target RNA was used as in Figure 1C. (*D*) Even in ATP-depleted lysate (indicated as –ATP), RISC is formed as well as that with ATP (+ATP). To create –ATP conditions, S2 lysate was treated with hexokinase in the presence of glucose as previously reported (Nykanen et al. 2001). RISC formation was performed with gel-purified *luc* siRNA duplex (the guide strand was radiolabeled with ^{32}P at the 5′-end and the passenger was phosphorylated with cold ATP). ATP, GTP, creatine phosphate, and creatine kinase were omitted for the –ATP reaction. (*E*) RISC formed in ATP-depleted lysate is active. ATP-depleted S2 lysate was prepared as in *D*. Target RNA cleavage assay was performed with luc180 target RNA.

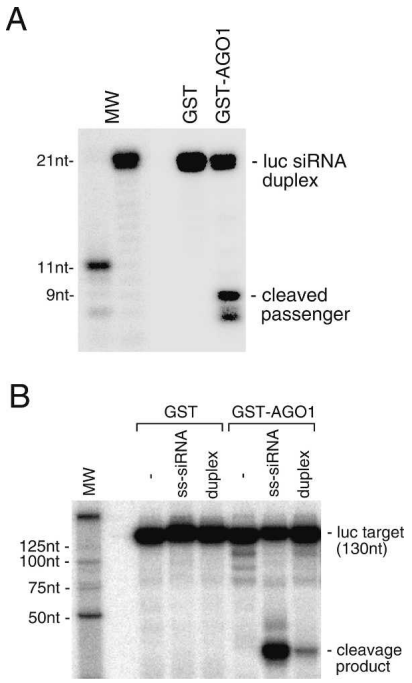

Figure 6. (*A*) Recombinant GST-AGO1 was incubated with *luc* siRNA duplex. A 9-nt RNA product is observed in the GST-AGO1 lane, but not in the GST-only lane. (*B*) Reconstitution of Slicer activity with recombinant GST-AGO1, the siRNA duplex, and its target RNA in vitro. GST-AGO1 preprogrammed with the siRNA duplex shows Slicer activity, although it is not as robust compared with GST-AGO1 preassociated with guide siRNA.

Structural and mutagenesis analyses on hAgo2 revealed that an Asp–Asp–His (DDH) motif is the catalytic triad coordinating Mg^{2+} ions at the active site of the protein (Rivas et al. 2005). At a peptide sequence level, the DDH motif is well conserved in AGO1 and AGO2 in *Drosophila* (Supplementary Fig. S2). In contrast, the Gln–His (Q-H) sequence that has been shown to be required in hAgo2 Slicer function (Liu et al. 2004) is only conserved in AGO1, while in AGO2, Arg–Gly (R-G) corresponds to it. The hAgo2 mutant (H634A; His in the Q-H sequence is changed to Ala) shows no Slicer activity (Liu et al. 2004). The peptide sequence of the entire PIWI domain in AGO1 is highly similar to that in hAgo2 (~88% identity), but AGO2 only shows ~43% identity (Supplementary Fig. S2). Taken together, in *Drosophila*, AGO1 is more closely related to hAgo2 than AGO2; thus, it is reasonable that AGO1 also has Slicer activity in *Drosophila*.

Drosophila AGO1 functions in miRNA processing (Okamura et al. 2004; Saito et al. 2005) and potentially in a translational repression pathway mediated by miRNAs without altering levels of the messenger. Why then does AGO1 have to contain the PIWI domain that can exhibit Slicer activity? There is as yet no clear answer to this, but it can be speculated that there may be unidentified, but endogenous miRNAs existing in flies that can lead target mRNA degradation as siRNA in the RNAi pathway. Recently, it was found that ALG-1, a *Caenorhabditis elegans* Argonaute family protein that is part of miRISC (Grishok et al. 2001), interacts with AIN-1 (Ding et al. 2005). AIN-1 shares homology with GW182 (Eystathioy et al. 2002), a protein recruited in cytoplasmic loci called processing bodies (P-bodies) that are known to be sites of mRNA decapping and degradation (Sheth and Parker 2003). It was also shown that human Argonautes (hAgo1 and hAgo2) are localized in P-bodies in cultured cells and the localization is linked to siRNA/miRNA function (Liu et al. 2005; Sen and Blau 2005). We found that when Flag-tagged AGO1 is expressed in S2 cells, it localizes in P-bodies (K. Miyoshi and M.C. Siomi, unpubl.), suggesting that even in *Drosophila*, AGO1 may function in degrading mRNA targeted by miRISC after being sequestered in P-bodies.

We produced recombinant AGO1-PIWI and AGO2-PIWI domains and showed that they are active in RISC assays, indicating that other Argonaute domains, including the PAZ domain, are not strictly necessary for siRNA binding and target RNA cleavage. However, cleavage of AGO1-PIWI (GST-AGO1-PIWI-C) was less efficient compared with that of recombinant full-length AGO1 (GST-AGO1) (Fig. 3C), although the concentrations of proteins used in assays were equal to each other. Thus, we cannot exclude the possibility that other regions of AGO1, including the PAZ domain, contribute to Slicer activity to some extent. According to the target RNA cleavage cycle model recently proposed by Yuan et al. (2005), 5′- and 3′-ends of siRNA are first bound to the PIWI and the PAZ, respectively, domains of Argonautes. Once the cycle moves forward, the 3′-end is released from the PAZ domain and a siRNA/target RNA duplex is formed, while the 5′-end of siRNA is still fixed on the Argonaute-PIWI domain. The action of binding and releasing the 3′-end of guide siRNA by the PAZ domain itself, and/or protein conformational change made through such action, may accelerate Argonaute Slicer activity.

One of the crucial issues for elucidating the molecular mechanisms underlying the RNAi pathway is to identify the siRNA unwinding enzyme(s). We previously showed that without AGO2, the siRNA duplex is not unwound and RISC is not formed in fly embryo lysate (Okamura et al. 2004). As in the case of embryo lysate, S2 lysate without AGO2 also has no ability to form RISC (H. Tsukumo and M.C. Siomi, unpubl.). In this study, we found that AGO2 associated with guide siRNA is able to cleave passenger siRNA (Fig. 4A) and that cleaved passenger strands originating from the siRNA duplex programmed into the lysates can be detected as 9 nt in an AGO2-dependent manner (Fig. 4B,C). A 2′-O-methyl modification of passenger strands of the siRNA duplex, which shows resistance to AGO2 Slicer activity, did not completely abolish, but clearly impaired RISC formation in S2 lysate (Fig. 5C, left panel). The target RNA cleavage activity of S2 lysate was also markedly impaired when si-duplex (OMe-9) was preprogramed to the lysate (Fig. 5C, right panel). Taken together, it can be speculated that AGO2 is directly involved in the siRNA duplex un-

winding process as an enzyme-cleaving passenger strand forming a duplex with guide siRNA and discarding the cleaved passenger in solution. However, it might not be the only mechanism for conversion of siRNA duplex into single-stranded siRNA. Although the unwinding of the siRNA duplex is totally dependent upon the AGO2 activity, other factors likely function cooperatively with AGO2 or act as a substitute, yet with low efficacy, for AGO2 when AGO2 cannot cleave the passenger strand of the siRNA duplex for some reason, such as the passenger showing AGO2-cleavage resistance. miRNA/miRNA* duplexes usually contain small bulge/internal loops in the middle of the duplexes (Bartel 2004; Kim 2005), which likely interfere with AGO Slicer activity. Thus, supposedly our model can apply only to an siRNA duplex but not to a miRNA/miRNA* duplex. How, then, can a miRNA duplex be converted to single-stranded miRNA? We observed that the bantam/bantam* duplex is unstable and easily becomes single-stranded in solution (K. Miyoshi and M.C. Siomi, unpubl.). Therefore, one might imagine that the miRNA/miRNA* duplex may come apart upon release from the precursor by Dicer.

We depleted ATP from S2 cell lysate by treating it with hexokinase in the presence of glucose, and performed RISC formation assay by adding siRNA duplex with presumably no ATP contamination. Surprisingly, RISC was formed even under such conditions, as in the case with an energy-regenerating system and exogenous ATP and GTP (Fig. 5D). Addition of a nonhydrolyzable ATP analog (AMP-PNP) to ATP-depleted lysate did not affect the result (data not shown). The simple implication is that RISC formation occurs ATP independently; however, there is the possibility that a trace of ATP molecules remaining in the lysate, even after the hexokinase treatment (~1 nM) and even in the presence of AMP-PNP (1 μM), is just enough to form RISC in the lysate. If Argonaute is indeed directly involved in the siRNA duplex unwinding process, and this process does not require ATP, where (in which step) in RISC formation is ATP hydrolysis required? siRNA duplex programmed in lysate is promptly incorporated in RLC and then active RISC is gradually formed (Tomari et al. 2004a). Although RLC components other than R2D2 and Dicer2 are not yet fully understood, it can be imagined that the siRNA duplex is entirely covered with protein components in the complex, and if so, the siRNA duplex in RLC would not be accessed by AGO2. ATP would stimulate the remodeling of the siRNA–RLC complex in vivo, by which the siRNA duplex becomes accessible to AGO2.

Materials and methods

Immunoprecipitation and silver-staining

Monoclonal antibodies against *Drosophila* AGO1 and AGO2 were produced against both N termini of AGO1 (300 amino acids) and AGO2 (300 amino acids). Immunoprecipitation from S2 lysate was performed using anti-AGO1 or anti-AGO2 antibodies immobilized on Gamma-Bind beads (Amersham Bioscience). Sodium chloride was added to the lysates to 800 mM just before immunoprecipitation was started. The reaction mixtures were rocked at 4°C for at least 1 h, and the beads washed extensively with a buffer containing 30 mM HEPES (pH 7.4), 800 mM sodium chloride, 2 mM magnesium acetate, 2 mM DTT, 0.1% NP40, 2 μg/mL pepstatin, 2 μg/mL leupeptin, and 0.5% aprotinin. The final wash was carried out with a cleavage reaction buffer (30 mM HEPES at pH 7.4, 40 mM potassium acetate, 5 mM magnesium acetate, and 5 mM DTT). Figures 1A and 2A show proteins eluted with SDS sample buffer without DTT. After elution, DTT was added to the eluates to 100 mM, which were then boiled and loaded onto SDS–acrylamide gels. After electrophoresis, protein bands were visualized using SilverQuest (Invitrogen).

Northern blot analysis

RNAs were isolated from the immunoprecipitates with ISOGEN (Nippon Gene). Northern blot was performed as described (Ishizuka et al. 2002). The probes used for detecting *luc* guide siRNA and miR-ban were 5′-CGUACGCGGAAUACUUC GAAA-3′ and 5′-CAGCTTTCAAAATGATCTCAC-3′, respectively.

In vitro target RNA cleavage assays

Target RNA cleavage assays using the immunoprecipitates were performed in a reaction buffer containing 30 mM HEPES (pH 7.4), 40 mM potassium acetate, 5 mM magnesium acetate, 5 mM DTT, 0.5 μg of yeast RNA (Ambion), and 40 U RNasin (Promega). After reaction at room temperature, RNAs were isolated from the whole-reaction mixtures, run on gels, and visualized on the BAS 2500. In Figure 2D, after the cleavage reaction, the supernatant and the beads were separated by centrifugation and RNAs isolated from each fraction. Target RNAs were transcribed with a Megascript T7 kit (Ambion) and then radiolabeled at the 5′-G cap by guanylyltransferase (Ambion). In each reaction 2000 to ~5000 cpm of cap-labeled target RNA was used. To prepare a template to make *luc* target RNA (luc180), PCR was performed to amplify a portion of *luc* cDNA (160 nt from the 5′-end). Forward and reverse primers used in the PCR reaction contain T7 and T3 promotor sequences, respectively. To make miR-ban target RNA harboring a sequence completely complementary to miR-ban (bantam38), a PvuII–EcoRI fragment excised from the pBS-miR-ban-target plasmid was used as a template. The pBS-miR-ban-target plasmid was constructed as follows: a KpnI–EcoRI fragment containing a sequence completely complementary to miR-ban was produced by annealing a set of oligo DNAs (5′-CCAGCTTTCAAAATGATCTCACG -3′ and 3′-CATGGGTCGAAAGTTTTACTAGAGTGCTTAA-5′), which was then inserted into pBluescript digested with KpnI and EcoRI. Production of target RNA for let-7 (let-7 target RNA) has been described previously (Okamura et al. 2004). To make *luc* target RNA (luc130) harboring a sequence completely complementary to *luc* siRNA (Fig. 6B), a PCR fragment was amplified with T7 and T3 primers from the pBS-*luc*-target plasmid as a template, and in vitro transcription was performed. The pBS-*luc* target plasmid was constructed as follows: a KpnI–EcoRI fragment containing a sequence completely complementary to *luc* siRNA was produced by annealing a set of oligo DNAs (5′-CCACGTACGCGGAATACTTCGAG-3′ and 5′-AATTCTCGAAGTATTCCGCGTACGTGGGTAC-3′), which was inserted into pBluescript digested with KpnI and EcoRI. *luc* passenger strand (Fig. 4A) was labeled with T4 polynucleotide kinase (PNK; TaKaRa) in the presence of [γ-^{32}P]ATP.

Recombinant protein concentrations used in each target RNA cleavage assay was ~100 nM. One microgram of BSA was also added together with recombinant proteins in each reaction. Single-stranded siRNA or miRNA was preincubated with GST fusion proteins at ~100 nM. In Figure 6B, GST-AGO1 or GST itself was first incubated with a *luc* siRNA duplex (100 nM) for 1.5 h at room temperature and *luc* target RNA (luc130) was added and then incubated for another 1.5 h at room temperature.

Production of recombinant Argonaute proteins

To obtain cDNA encoding AGO1, poly(A)$^+$ RNAs were purified from S2 cells and RT–PCR was carried out using a ProSTAR Ultra-HF RT–PCR system (Stratagene). To produce a GST-AGO1 fusion protein, full-length AGO1 cDNA was subcloned into a pGEX-5X expression vector (Amersham Bioscience). GST-AGO1-PIWI-B and GST-AG01-PIWI-C were generated by cloning PCR fragments covering a portion of AGO1 that included the PIWI domain (as shown in Fig. 3D) into pGEX-5X. GST-AGO2-PIWI-D, GST-AG02-PIWI-G, and GST-AGO2-PIWI-I were generated by cloning PCR fragments covering a portion of AGO2 (as shown in Fig. 3C) into pGEX-5X. All of the GST-tagged proteins and GST itself were expressed in BL21(DE3). After incubation to A_{600} of ~0.8 at 37°C, IPTG was added to the medium to 1 mM and cells were grown overnight at 16°C. Cells were then lysed by sonication and GST and GST fusion proteins were bound onto glutathione-Sepharose 4B resins according to the manufacturer's instructions (Amersham Bioscience). After extensive washing with PBS, GST, and GST fusion proteins were eluted from the beads with PBS containing 10 mM glutathione diluted with PBS containing 5 mM magnesium acetate and then concentrated on spin columns.

Detection of cleaved passenger strand of siRNA duplex in lysates

S2 and embryo lysates were prepared as described previously (Okamura et al. 2004). siRNA duplexes were gel-purified before use. Then, siRNA duplex (~20,000 cpm), 5× cleavage buffer (150 mM HEPES at pH 7.4, 200 mM potassium acetate, 25 mM magnesium acetate, 25 mM DTT), 0.5 µg of yeast RNA, and 40 U RNasin were added to lysates and incubated at room temperature. Reactions were stopped by the addition of stop buffer (50 mM sodium chloride, 50 mM EDTA, 1% SDS, and 100 µg/mL proteinase K). RNA was then extracted using phenol/chloroform/isoamyl alcohol (25:24:1), precipitated with isopropanol, and analyzed. *luc* passenger strands modified with 2′-O-methyl group(s) at the ninth nucleotide (the 5′-end of the passenger being assigned first) (OMe-9) and at all of the nucleotides (OMe) were purchased from Hokkaido System Science. In Figure 5A, the modified passenger strand (OMe) was added to the reaction mixture at 150 nM.

Native agarose gel electrophoresis

Native gel electrophoresis of RLC and RISC was performed as described previously (Okamura et al. 2004; Tomari et al. 2004a). ^{32}P-radiolabeled *luc* siRNA duplex was incubated with S2 lysate at room temperature. After incubation, samples were adjusted to 6% (w/v) glycerol and resolved by submarine native agarose gel electrophoresis. Gels were dried under a vacuum onto Hybond-N+ nylon membrane (Amersham Bioscience) and exposed to an imaging plate of a BAS 2500. For depleting ATP from the lysate, hexokinase and glucose were added and incubated as described previously (Nykanen et al. 2001). ATP concentration of lysate was monitored using an ATP Bioluminescent Assay kit (Sigma). A nonhydrolyzable ATP analog (AMP-PNP) was purchased from CALBIOCHEM.

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