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In vitro RNA Cleavage Assay for Argonaute-Family Proteins

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Summary

Recent studies have revealed that Argonaute proteins are crucial components of the RNA-induced silencing complexes (RISCs) that direct both small interfering RNA (siRNA)- and microRNA (miRNA)-mediated gene silencing. Full complementarity between the small RNA and its target messenger RNA (mRNA) results in RISC-mediated cleavage ("Slicing") of the target mRNA. A subset of Argonaute proteins directly contributes to the target cleavage ("Slicer") activity of the RISC. We describe *in vitro* Slicer assays using endogenous Argonaute protein immunopurified from animal cells and recombinant Argonaute protein produced in and purified from *Escherichia coli*.

Key Words: RNAi; Argonaute; Slicer; RISC; *Drosophila*.

1. Introduction

Although RNA interference (RNAi) has been enthusiastically viewed as a new therapeutic modality, it is important to keep in mind that RNAi is but one aspect of a larger web of sequence-specific cellular responses to RNA now known collectively as "RNA silencing" ***(1)***. In essence, it is a sequence-specific RNA cleavage process triggered by double-stranded (ds) RNA from a variety of sources (*see* **Fig. 1** in **Chapter 4**) ***(1,2)***. One such source is long dsRNA exogenously introduced or endogenously expressed in cells; this is first converted to 21–23-nucleotide (nt) small RNAs termed "small interfering RNAs" (siRNA) by Dicer in the cytoplasm of cells ***(3–6).*** Subsequently, the siRNA duplex

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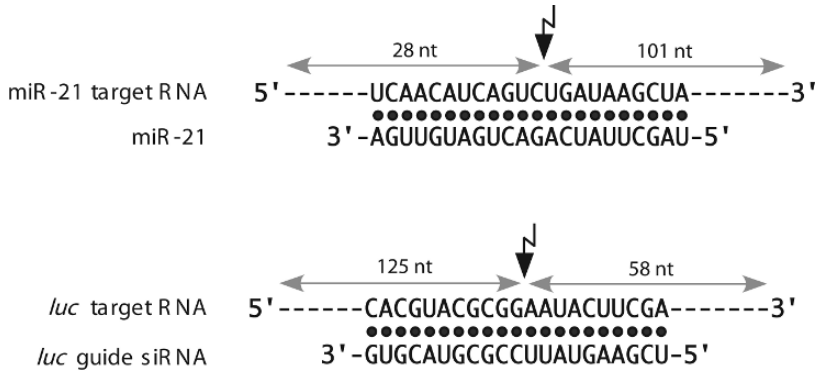

Fig. 1. Sequences of RNA targets, miR-21 target RNA and *luc* target RNA, and potential base pairings with miR-21 and *luc* guide siRNA. Cleavage sites are indicated with lightning bolts. RNA targets that bear complete complementarity to the small RNAs are cleaved at a position across from their middle (i.e., between the 10th and 11th nucleotides of small RNAs).

goes through an unwinding process, and one strand over the other is preferentially loaded onto the RNA-induced silencing complex (siRISC), which in turn endonucleolytically cleaves target RNA at sites completely complementary to the siRNA ***(7–10)***. Another source of dsRNA is transcripts of a large family of cellular genes encoding self-complementary RNA molecules that form imperfect hairpins, termed "primary microRNA" (pri-miRNA). Pri-miRNAs are first processed by the Drosha-Pasha/DGCR8 complex in the nucleus to generate pre-miRNA (precursor of miRNA). The pre-miRNA is exported to the cytoplasm by exportin-5 and further processed by Dicer into an approximately 22-nt miRNA duplex; then, one strand over the other is loaded onto RISC (miRISC) as siRNA in RNAi ***(11)***. Mature miRNAs residing in the miRISC anneal to target mRNAs at specific sites, and miRISC induce cleavage of the messages or inhibit their translation ***(12–16)***. By regulating endogenous gene expression, miRNAs are known to play crucial roles in development ***(17–21)***.

Chromatographic purification of RISC nuclease or Slicer activity from *Drosophila* cells revealed several RISC components ***(2)***. However, since the discovery of RNAi, the central question of which RISC component actually catalyzes the hydrolytic cleavage reaction has remained unanswered. Argonaute-family proteins, categorized by two characteristic domains, PAZ and PIWI, were the first identified component of RISC. In 2004, Joshua-Tor and colleagues solved the crystal structure of the Argonaute protein from *Pyrococcus furiosus,* which revealed that the PIWI domain shows an RNaseH-like structure and that essential residues for the endonucleolytic activity of

RNaseH are conserved in the Argonaute-PIWI domain ***(22)***. These data strongly indicate that Argonaute (Ago) might be the "Slicer," the endonucleolytic enzyme residing in RISC. Indeed, Hannon and colleagues showed that epitope-tagged human Ago2 (hAgo2) expressed in and purified from 293T cells was able to catalyze target RNA cleavage when the immunopurified hAgo2 was pre-incubated with single-stranded siRNA, but not with single-stranded small DNA ***(23)***. Mutagenizing the conserved residues in hAgo2 abolished target RNA cleavage activity ***(23)***. Later, Slicer activity was reconstituted with recombinant hAgo2 produced in *E. coli* and single-stranded siRNA ***(24)***. Taking all of the above into consideration, it was concluded that hAgo2 is Slicer in RNAi.

In humans, there are four closely related Argonaute-family members, Ago1–4 (hAgo1–4), all of which have been associated with both siRNA and miRNA; however, only hAgo2 mediates RNA cleavage targeted by the small RNAs ***(23–25)***. hAgo1, hAgo3, and hAgo4 show high similarities with hAgo2 at peptide sequence levels, and the amino acid residues required for hAgo2 Slicer activity are well conserved among the three other proteins ***(26)***: Thus, it is somewhat anomalous that only hAgo2 has Slicer activity.

In *Drosophila*, the Argonaute family consists of five members (AGO1, AGO2, AGO3, Piwi, and Aubergine) ***(27)***. Among these, AGO1 and AGO2 are the most closely related to hAgo1–4. Our previous studies have focused on the functional contribution of AGO1 and AGO2 in RNA silencing and revealed that AGO2 is required for the siRNA-directed target RNA cleavage process, while AGO1, the closest relative to AGO2 among the Argonaute members in fly, is dispensable for such reaction ***(28)***. Thus, we assessed if AGO2 isolated from *Drosophila* cells could show target RNA cleavage activity, as hAgo2 did from human cells. Unlike Hannon and colleagues, we decided to utilize endogenous AGO2 protein since we could easily obtain purified AGO2 by immunoprecipitation from *Drosophila* Schneider 2 (S2) cells using a monoclonal antibody we raised against the N-terminal region of AGO2 ***(29)***. We first confirmed by Northern blot analysis that the immunopurified AGO2 from S2 lysate preprogrammed with *luc* siRNA duplex was indeed associated with *luc* guide siRNA, as expected. Later, by performing *in vitro* target RNA cleavage assay, we found that the immunopurified AGO2 associated with *luc* siRNA was capable of cleaving *luc* target RNA. These data strongly implicated that in *Drosophila,* as with hAgo2 in human, AGO2 functions as Slicer. Through the course of our studies, we became aware that miRNA-directed target RNA cleavage occurred even without AGO2. Further investigation revealed that AGO1 is the protein essential for miRNA-directed target RNA cleavage reaction. Analysis of amino acid sequences showed that AGO1 more resembles hAgo2 than it does AGO2. Therefore, we next assessed if AGO1 also functions as Slicer. We prepared AGO1 protein by two independent methods. The first

was endogenous AGO1 immunopurified from S2 cells using the monoclonal antibody against AGO1. The second was recombinant GST-tagged full-length AGO1 produced in and purified from *E. coli*. Target RNA used in the assay was an RNA molecule containing a sequence completely matched to miR-bantam. That AGO1 immunopurified from S2 cells was associated with miR-bantam was confirmed in advance by Northern blotting. GST-AGO1 was incubated with miR-bantam prior to the target RNA cleavage assay. Both forms of AGO1 were able to efficiently cleave the target RNA; thus, it was concluded that AGO1 also has Slicer activity.

In this chapter, we describe *in vitro* target RNA cleavage assay using purified Argonaute proteins from both animal cells and *E. coli*. While this *in vitro* assay will clearly be useful for elucidating the Slicer function of Argonaute proteins from a variety of organisms, it will also be useful for other applications. Indeed, this assay allowed us to ascertain that AGO2 Slicer function is needed for facilitating siRNA duplex unwinding by cleaving and discarding the passenger strand of siRNA duplex, as if AGO2 cleaves a target RNA annealed to guide siRNA residing in siRISC ***(29)***. Finally, we address important issues as well as suggest useful techniques to optimize the assay.

2. Materials

1. RNA extraction reagent (ISOGEN, or ISOGEN-LS, Nippon Gene, Toyama, Japan).
2. Cloning vectors harboring phage promoters: pBluescript SK (Stratagene, La Jolla, CA) or equivalent.
3. MEGAscript kit (Ambion, Austin, TX).
4. Pellet Paint® Co-precipitant (EMD Bioscience, Darmstadt, Germany).
5. 6% acrylamide denaturing gel: 1X Tris-borate-ethylenediaminetetraacetic acid, 6 *M* of urea, 6% acrylamide.
6. 8% acrylamide denaturing gel: 1X Tris-borate-ethylenediaminetetraacetic acid, 6 *M* of urea, 8% acrylamide.
7. RNA elution buffer: 0.5 *M* of ammonium acetate, 1 m*M* of ethylenediaminetetraacetic acid (EDTA), and 0.2% sodium dodecylsulfate.
8. Guanylyltransferase, 10X Capping reaction buffer, and S-adenosyl methionine (Ambion).
9. [α-^{32}P]GTP (3000 Ci/mmol) (PerkinElmer, Boston, MA).
10. Gel-filtration columns: Micro Bio-Spin Columns P-30 Tris, RNase-Free (Bio-Rad, Hercules, CA).
11. *luc* siRNA (guide strand: 5′-UCGAAGUAUUCCGCGUACGUG-3′, passenger strand: 5′-CGUACGCGGAAUACUUCGAAA-3′).
12. Lithium salt of ATP, 100 m*M*, pH 7 (Roche, Basel, Switzerland).
13. T4 Polynucleotide Kinase (T4PNK) and 10X PNK reaction buffer (TaKaRa, Shiga, Japan).

14. HeLa cells.
15. Dulbecco's modified Eagle's medium (SIGMA, St. Louis, MO) supplemented with 10% fetal calf serum.
16. Phosphate-buffered saline (PBS).
17. Monoclonal anti-hAgo2 antibody (4G8), against the N-terminus of hAgo2 (120 amino acids).
18. Binding buffer: 30 m*M* HEPES, pH 7.4, 150 m*M* KOAc, 2 m*M* $Mg(OAc)_2$, 5 m*M* dithiothreitol (DTT), 0.1% Nonidet P-40, 2 μg/mL pepstatin, 2 μg/mL leupeptin, and 0.5% aprotinin.
19. GammaBind G Sepharose (GE Healthcare Bio-Sciences, Piscataway, NJ).
20. 5X cleavage buffer: 125 m*M* Hepes-KOH, pH 7.5, 250 m*M* KOAc, 25 m*M* $Mg(OAc)_2$, 25 m*M* DTT.
21. RNasin® Plus RNase Inhibitor (Promega, Madison, WI).
22. *Escherichia coli* (*E. coli*) BL21 (DE3).
23. 1 *M* isopropyl thiogalactoside (IPTG).
24. Complete, Mini, EDTA-free (Roche).
25. Glutathione Sepharose™ 4B (GE Healthcare Bio-Sciences).
26. GST elution buffer: 10 m*M* reduced glutathione, 50 m*M* Tris-HCl, pH 8.0, 10% glycerol, and 5m*M* $Mg(OAc)_2$.
27. Protein assay (Bio-Rad).
28. 5 mg/mL yeast RNA (Ambion).
29. Image plates: BAS-MS2040 (Fujifilm, Tokyo, Japan).
30. BAS-2500 imaging system (Fujifilm).

3. Methods

3.1. Preparation of Target RNA

Both siRNAs and miRNAs are able to guide mRNA degradation or translational repression depending on the complementarity of the target RNAs to the small RNAs. Namely, even the Slicer function of Argonaute that is naturally associated with miRNAs, for example *Drosophila* AGO1, can be assessed with this *in vitro* target RNA cleavage assay when a particular target RNA containing a sequence completely matching the miRNA is prepared. Target RNAs that appear in this chapter are miR-21 target RNA and *luc* target RNA **(Fig. 1)**.

3.1.1. Preparation of Template DNA of Target RNA

To make miR-21 target RNA harboring a sequence fully complementary to miR-21, PCR was performed to amplify a sequence between the T7 and T3 promoter regions of the pBS-miR-21-target plasmid, which was constructed as follows: A *Kpn*I-*Eco*RI fragment containing a sequence fully complementary to miR-21 was produced by annealing a set of DNA oligos

(5′-CTCAACATCAGTCTGATAAGCTAG-3′ and 5′-AATTCTAGCTTATCA-GACTGATGTTGAGGTAC -3′), which was then inserted into pBluescript digested with *Kpn*I and *Eco*RI.

To prepare a template DNA to make *luc* target RNA, PCR was performed to amplify a portion of luciferase cDNA. The forward and reverse primers used for this PCR reaction were forward 5′-TAATACGACTCACTATAGG-GCTATCCTCTAGAGGATGGAAC-3′, reverse 5′-AATTAACCCTCACTAA-AGGGCATAGCTTCTGCCAACCGAAC-3′. It should be noted that the forward and reverse primers contain the T7 and T3 promoter sequences, respectively.

3.1.2. Target RNA Transcription

Target RNA is transcribed according to the manufacturer's instructions (MEGAscript, Ambion) with minor modifications. The detailed protocol is as follows:

1. Combine the following transcription reaction mixture in a total volume of 20 µL:
    a. 2 µL each of 75 m*M* ATP, CTP, GTP, and UTP.
    b. 2 µL of 10X reaction buffer.
    c. Roughly 0.5 µg of template DNA.
    d. 2 µL of enzyme mix.
    e. Nuclease-free water.
2. Incubate the transcription reaction solution at 37 ºC overnight.
3. Add 1 µL of DNase I and incubate at 37 ºC for 15 min.
4. Add 15 µL of 3 *M* NaOAc, 115 µL of nuclease-free water, and 150 µL of phenol/chloroform, and mix thoroughly. Recover the aqueous phase and transfer to a new tube.
5. Precipitate the RNA by adding an equal volume of isopropanol and 1 µL of pellet-paint® co-precipitant and mixing well.
6. Chill the mixture for at least 15 min at –80 ºC. Centrifuge at 4 ºC for 20 min at maximum speed (20,000 x g) to pellet the RNA.
7. Isolate the target RNA by 8% acrylamide denaturing gel electrophoresis as follows:
    a. Separate the RNA by 8% acrylamide denaturing gel electrophoresis.
    b. Excise the gel piece containing the target RNA from the gel.
    c. Crush the gel piece into small pieces with a disposable pipette tip.
    d. Add 400 µL (> two gel volumes) of RNA elution buffer.
    e. Rotate at 4 ºC overnight to elute the RNA.
    f. Remove the gel pieces and collect the supernatant.
    g. Purify the target RNA by phenol extraction and isopropanol precipitation in the presence of a precipitation carrier.

8. Cap labeling of the target RNA (5′-terminal phosphate of the 5′-triphosphate target RNA): Combine the following capping reaction mixture in a total volume of 15 µL:
    a. 1.5 µL of 10X Capping reaction buffer.
    b. 3 µL of [α-^{32}P]GTP (3000 Ci/mmol).
    c. 1 µL of S-adenosyl methionine.
    d. 0.5 µg of target RNA.
    e. 1 µL of guanylyltransferase enzyme.
9. Incubate the reaction at 37 ºC for 60 min.
10. Gel-filter with a P-30 column, according to the manufacturer's instructions, to remove any unincorporated radioactive GTP.
11. Isolate the target RNA by 8% acrylamide denaturing gel electrophoresis as below.
12. Dissolve RNA with nuclease-free water to a working concentration of 2,000–5,000 cpm/µL.

3.2. Preparation of Phosphorylated siRNA

The processing of dsRNA, pri-miRNA, or pre-miRNA by Dicer or Drosha leaves 5′-phosphate groups at the end of the resultant small RNAs. This feature has been proposed as an important quality check during RISC assembly ***(3,4,7, 30)***, and the function has been attributed to the recognition of those phosphate groups within the RISC loading complex (RLC), in part by the asymmetry determining factor, R2D2 ***(31)***. However, in the reconstitution system using recombinant hAgo2, the siRNA 5′-phosphate was shown to be unnecessary for the Slicer activity, although it was important for the stability and the fidelity of the RISC ***(24)***. In this chapter, 5′-phosphorylated siRNA is used to ensure the cleavage activity for purified Argonaute proteins.

When synthetic single-stranded siRNA, purchased commercially, is not 5′-phosphorylated, it can be phosphorylated at the 5′ ends using T4 polynucleotide kinase (PNK) in the presence of ATP:

1. Combine the following reaction mixture in a total volume of 30 µL:
    a. 1 µL of single-stranded siRNA (100 µ*M*).
    b. 1 µL of ATP (100 m*M*).
    c. 1 µL of T4PNK.
    d. 3 µL of 10X PNK reaction buffer.
    e. 24 µL of H_2O.
2. Incubate the reaction at 37 ºC for 60 min.
3. Add 15 µL of 3 *M* NaOAc, 105 µL of nuclease-free water, and 150 µL of phenol/chloroform, and mix thoroughly. Recover the aqueous phase and transfer to a new tube.

4. Precipitate the RNA by adding 1 volume of isopropanol and 1 µL of pellet-paint® co-precipitant, mixing well.
5. Chill the mixture for at least 15 min at –80 °C. Centrifuge at 4 °C for 20 min at maximum speed (20,000 x g) to pellet the RNA.
6. Wash with 70% ethanol.

3.3. Purification of Argonaute Proteins

In this section, we perform *in vitro* target RNA cleavage assay using two particular types of Argonaute proteins. One is recombinant *Drosophila* AGO1 produced in *E. coli*. Although recombinant Argonaute proteins are notorious for their difficulty to produce in *E. coli*, partially because of their low solubility and high toxicity, we were able to obtain GST-tagged, full-length recombinant *Drosophila* AGO1. The other is endogenous hAgo2 immunopurified from HeLa cells. The monoclonal antibody against hAgo2 was originally made in our laboratory by immunizing mice with the N-terminal region (about 120 amino acids) of hAgo2 expressed in and purified from *E. coli* and fusing their lymphocytes with myeloma cells. Details of the expression of GST-AGO1 in *E. coli* and its purification procedures, and hAgo2 immunoprecipitation from HeLa cells, are indicated below.

3.3.1. Production of Recombinant Argonaute Proteins

1. Inoculate an *E. coli* BL21(DE3) colony containing the transformed plasmid into 2 mL of LB media with 100 µg/mL of Ampicillin (Amp); grow at 37 °C overnight.
2. Inoculate 100 mL of LB/Amp in a 500-mL flask. Grow for 6–8 h at 37 °C.
3. Inoculate 500 mL of LB/Amp in a 2-L flask. Grow for 1–2 h at 37 °C. Check OD_{600}. Aim for a final OD_{600} between 0.8 and 1.0.
4. Add 1 *M* of IPTG to a 1 m*M* concentration to induce fusion protein expression, and incubate culture at 16 °C overnight (*see* **Note 1**).
5. Spin down cells for 25 min at 3,500g.
6. Discard media and remove the cells into 50 mL tubes.
7. Spin down cells for 15 min at 4,500g.
8. Discard media strictly. (At this point cells can be stocked at –80 °C for later use.)
9. Resuspend cells in 25 mL of PBS containing protease inhibitor (Complete, Mini, EDTA-free). Keep cells on ice at all times.
10. Sonicate cells for 15 sec five times using 50% output (Branson Sonifier or equivalent).
11. Add 10% Triton X-100 detergent to 1% final concentration to lysis cells.
12. Place tube in rotary shaker at 4 °C for 20 min.
13. Spin cells for 30 min at 12,000g. Separate supernatant.
14. Set the column containing Glutathione Sepharose beads and wash with PBS.
15. Load the cell lysate into the column and wash the beads three times with PBS.

16. Add 250 µL of GST elution buffer to the column, to elute GST proteins, and collect the flowthrough. Repeat this step nine times.
17. Electrophoresis 10 µL of each sample and check for the expression of the fusion protein (**Fig. 2a**). Assay protein concentration of each fraction using Protein Assay.

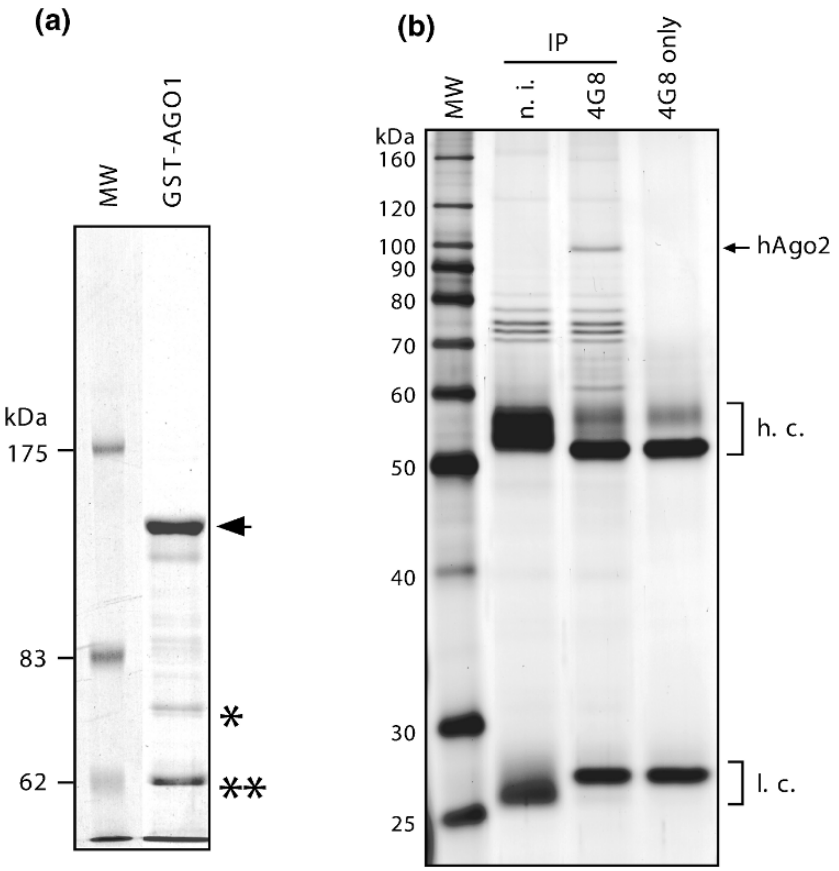

Fig. 2. Purification of Argonaute proteins. (a) Purification of bacterially expressed *Drosophila* AGO1. GST-AGO1 was purified with Glutathione Sepharose beads, resolved on an SDS-PAGE gel, and visualized by Coomassie Brilliant Blue staining. The preparation contained two additional proteins (indicated with * and **) other than GST-AGO1 (indicated by an arrow); identified by mass spectrometry as *E. coli* proteins, Dnak (*) and GroEL (**) (*see* **Note 1**). The left lane represents molecular markers (MW). (b) Purification of hAgo2 from HeLa cell lysates by immunoprecipitation with a specific monoclonal antibody (4G8). Endogenous hAgo2 immunopurified with 4G8 and immunoprecipitates with a control non-immune antibody (n.i.) were resolved on SDS-PAGE and visualized by silver staining. 4G8 itself bound on beads was also visualized (4G8 only). The farthest left lane shows molecular markers (MW). h.c. and l.c. show heavy and light chains, respectively, of the antibodies.

3.3.2. Immunopurification of hAgo2 from HeLa Cells

So far, the expression of the full-length recombinant hAgo2 in *E. coli* has been unsuccessful in our laboratory. Thus, we instead obtained immunopurified endogenous hAgo2 from HeLa cell lysates as follows and used it in the assay.

1. Culture HeLa cells. Harvest 5–10 × 10^7 cells and wash twice with PBS.
2. Suspend the cells in 500 µL of Binding buffer.
3. Incubate on ice for 5 min.
4. Sonicate briefly (three times, 5 sec each) using 30% output (Branson Sonifier or equivalent).
5. Centrifuge at maximum speed (20,000g) for 25 min.
6. Incubate supernatant with anti-hAgo2 (4G8) antibody immobilized on Gamma-Bind beads. Add NaCl to the lysates to 1 *M* just before immunoprecipitation is started (*see* **Note 2**). Rock the reaction mixtures at 4 ºC for at least 60 min.
7. Wash the beads four times with binding buffer containing 1 *M* of NaCl buffer and then twice with 1X cleavage buffer (IP-hAgo2 bound with miRNAs) **(Fig. 2b)**.

3.4. In vitro Target RNA Cleavage (Slicer) Assay

Previously, Hannon and colleagues showed that immunopurified siRNA-programmed hAgo2 has Slicer activity *in vitro* ***(23)***. Several other groups also showed quite similar data from *in vitro* cleavage assays ***(25,32)***. However, these experiments were performed with tagged (mostly FLAG-tagged) proteins overexpressed from transfected DNA constructs. Here, we show for the first time that endogenous hAgo2 exhibits Slicer activity **(Fig. 3b)**. *In vitro* target RNA cleavage assay was performed according to previously reported methods ***(23,24,33)***, but with some modifications.

3.4.1. Slicer Assay Using Recombinant Full-Length Drosophila AGO1

Joshua-Tor and colleagues demonstrated an RISC reconstitution system using recombinant hAgo2 and siRNA ***(24)***. In our laboratory, *in vitro* target RNA cleavage assay with recombinant GST-AGO1 was performed according to their method ***(24)***, with some modifications.

1. For a 20-µL reaction, assemble on ice:
   a. ~100 n*M* of recombinant Argonaute protein (GST-AGO1) (*see* **Note 3**).
   b. 4 µL of 5X cleavage buffer.
   c. 100 n*M* of single-stranded, phosphorylated siRNA.
   d. 1 µL of RNasin plus (40 U/µL).
2. Incubate the reaction at 26 ºC for 90 min.
3. Add the target RNA (3,000–5,000 cpm) and 0.5 µg of yeast RNA.
4. Incubate the reaction at 26 ºC for 90 min.
5. Purify the RNA.

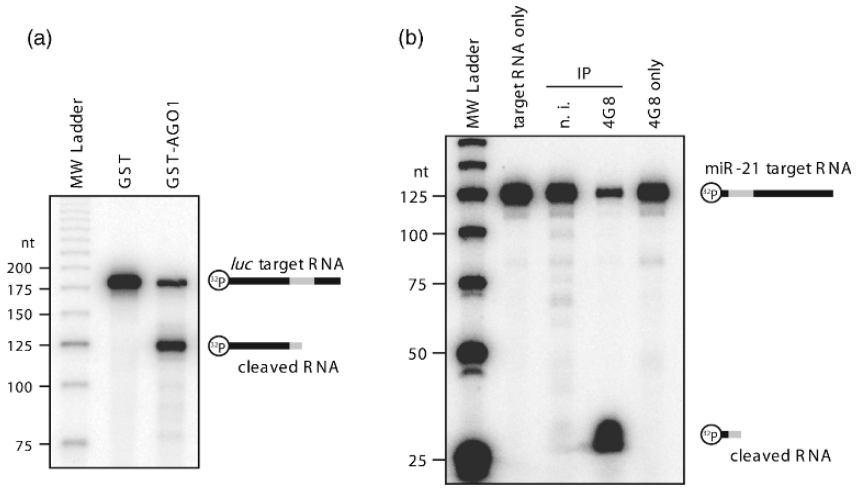

Fig. 3. *In vitro* target RNA cleavage assay (a) *luc* guide siRNA-directed target RNA cleavage using recombinant *Drosophila* AGO1. The 5′-end radiolabeled RNA target, *luc* target RNA (also *see* **Fig. 1**), was incubated with purified GST-AGO1 pre-bound with *luc* guide siRNA or with GST itself as a negative control. Schematic drawings of *luc* target RNA and its cleaved product (cleaved RNA) are indicated on the right, which show where the RNA bands migrated on the gel. (b) miRNA-directed target RNA cleavage using immunopurified hAgo2 from HeLa cells. The 5′-end radiolabeled RNA target, miR-21 target RNA (also *see* **Fig. 1**), was incubated with hAgo2 immunopurified with 4G8, 4G8 itself (a negative control), and immunoprecipitates with non-immune IgG (n.i.: another negative control). Schematic drawings of the RNA target and its cleaved product (cleaved RNA) are indicated on the right, which show where they migrated on the gel.

6. Separate in a 6% acrylamide denaturing gel.
7. Expose the gel to an imaging plate and visualize the signals using the BAS-2500 system (**Fig. 3a**).

3.4.2. Slicer Assay Using Immunopurified hAgo2 from HeLa Cells

1. For a 30-μL reaction, assemble on ice:
   a. Immunopurified hAgo2 on beads.
   b. 6 μL of 5X cleavage buffer.
   c. 1 μL of RNasin plus (40 U/μL).
   d. 1 μL of yeast RNA (0.5 mg/mL).
   e. 1 μL of miR-21 target RNA (3,000–5,000 cpm/μL).
   f. 21 μL of H_2O.
2. Incubate the reaction at 37 ºC for 90 min.

3. Purify the RNA using ISOGEN-LS.
4. Separate in a 6% acrylamide denaturing gel.
5. Expose the gel to an imaging plate and visualize the signals using the BAS-2500 system (**Fig. 3b**).

4. Notes

1. In our case, GST-AGO1 was copurified with heat-shock proteins, DnaK (Hsp70) and GroEL ***(29)***, both known to be chaperonins. It is known that many newly synthesized bacterial proteins avoid aggregation by folding with the aid of chaperonins; thus, the AGO1 protein may be fortuitously solubilized by DnaK and GroEL. Expression of recombinant hAgo2 in *E. coli* was also successfully done through the coexpression of human HSP90 ***(24,33)***. Interestingly, a potential connection between heat-shock proteins and the RNAi pathway has been reported: (a) A recent genome-wide screen for identifying components of the RNAi pathway in *Drosophila* cells revealed seven genes that affect the RNAi response, two were the heat-shock proteins Hsc70-3 and Hsc70-4 ***(34);*** and (b) human HSP90 was shown to be associated with hAgo2 in coimmunoprecipitation studies ***(23)*** (also *see* **Note 2**). These findings suggest the possibility that some heat-shock proteins may not only be chaperonins for Argonaute proteins but also are critical regulators for Argonaute functions.
2. We intentionally purified hAgo2 under harsh conditions with a salt concentration of 1 *M* of NaCl, where most of the proteins associated with hAgo2 in cells would ideally be stripped away from hAgo2. Actually, as indicated in **Fig. 2a**, under such conditions we could immunoprecipitate a good amount of hAgo2 but did not observe any clear protein bands specific in the 4G8-IP lane. Even a band that most likely would correspond to HSP90 was not detected. It could be argued that 1 *M* of NaCl could lead to the dissociation of small RNAs from hAgo2; however, Northern blotting analysis clearly revealed that this was not the case, as we could still detect miR-21 association with hAgo2 immunopurified from HeLa cells under the harsh conditions (data not shown). Indeed, it has been noted that the potassium chloride concentration in the wash steps of the affinity column purification could be increased up to 2.5 M without loss of RISC activity ***(36)***. Thus, it is critical to selectively adjust immunoprecipitation conditions (salt and/or detergent concentrations, incubation time for binding, and so on) according to the specific aim.
3. We normally add Mg^{2+} to buffers at a final concentration of 2–5 m*M* when Argonautes are purified from cells. It has been reported that the Slicer activity of Argonaute proteins is Mg^{2+}-dependent and that Argonautes use an active site Asp-Asp-His (DDH) motif in the PIWI domain for metal ion coordination ***(35)***. Through our experiments, we now understand that Mg^{2+}may also be needed for stabilizing Argonaute proteins. In other words, *in vitro* target RNA cleavage assay does not go well when Argonautes purified in buffers lacking Mg^{2+} are used.

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