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Passenger-Strand Cleavage Facilitates Assembly of siRNA into Ago2-Containing RNAi Enzyme Complexes

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Summary

In the *Drosophila* and mammalian RNA interference pathways, siRNAs direct the protein Argonaute2 (Ago2) to cleave corresponding mRNA targets, silencing their expression. Ago2 is the catalytic component of the RNAi enzyme complex, RISC. For each siRNA duplex, only one strand, the guide, is assembled into the active RISC; the other strand, the passenger, is destroyed. An ATP-dependent helicase has been proposed first to separate the two siRNA strands, then the resulting single-stranded guide is thought to bind Ago2. Here, we show that Ago2 instead directly receives the double-stranded siRNA from the RISC assembly machinery. Ago2 then cleaves the siRNA passenger strand, thereby liberating the single-stranded guide. For siRNAs, virtually all RISC is assembled through this cleavage-assisted mechanism. In contrast, passenger-strand cleavage is not important for the incorporation of miRNAs that derive from mismatched duplexes.

Introduction

RNA interference (RNAi) begins with the production of ~21 nt, double-stranded RNAs called small interfering RNAs (siRNAs; Hamilton and Baulcombe, 1999; Hammond et al., 2000; Zamore et al., 2000; Elbashir et al., 2001a, 2001b; Bernstein et al., 2001). siRNAs must be dissociated into their component single strands in order to act as guides for RNA-induced silencing complexes (RISCs), the protein complexes that repress gene expression (Nykänen et al., 2001; Martinez et al., 2002). In both humans and flies, Argonaute2 (Ago2) is the sole siRNA-guided Argonaute protein able to act as an RNA-guided, Mg^{2+}-dependent RNA endonuclease that cleaves a single phosphodiester bond in the target RNA, triggering its destruction (Hammond et al., 2001, Okamura et al., 2004; Meister et al., 2004b; Rand et al., 2004; Liu et al., 2004; Song et al., 2004; Rivas et al., 2005). In *Drosophila melanogaster*, dissociation of the two siRNA strands requires the Dicer-2 (Dcr-2)/R2D2 protein heterodimer, components of the RISC-loading complex (RLC), as well as Ago2 itself (Liu et al., 2003; Lee et al., 2004; Pham et al., 2004; Okamura et al., 2004; Tomari et al., 2004b). In *Drosophila* embryo lysate lacking Ago2, siRNAs remain double stranded (Okamura et al., 2004) and stably bound to Dcr-2 and R2D2 (Tomari et al., 2004b), suggesting that siRNA strand separation is initiated only after Ago2 interacts with the siRNA bound to Dcr-2 and R2D2.

Previous models have proposed that an ATP-dependent helicase first separates the siRNA duplex, and then the resulting single-stranded guide binds Ago2 (Zamore et al., 2000; Nykänen et al., 2001; Bartel, 2004; Meister and Tuschl, 2004; Sontheimer, 2005; Tomari and Zamore, 2005). Here, we present experimental data supporting an alternative model. Our data indicate that the RLC loads an siRNA duplex, not an siRNA single strand, into Ago2. Which 5′ end of the siRNA duplex is lodged in the phosphate binding pocket of the Ago2 Piwi domain (Ma et al., 2005; Parker et al., 2005) would be determined by the RLC. Once bound to the siRNA duplex, Ago2 cleaves the passenger strand, triggering its dissociation from the complex and the concomitant maturation of the active RISC. Passenger-strand cleavage is not obligatory but is the normal mechanism for loading siRNA into Ago2. For standard siRNAs, passenger-strand cleavage follows rapidly after Ago2 binds the double-stranded siRNA, but when cleavage is blocked by chemical modification or by mismatches between the siRNA guide and passenger strands, a slower "backup" pathway dissociates and destroys the passenger strand, liberating mature RISC. This bypass pathway explains the loading of miRNAs into RISC and the loading of silencing RNAs into complexes that are not capable of Ago-mediated cleavage.

Results

Passenger, but Not Guide, Strand Cleavage Accompanies RISC Assembly

Is the passenger strand cleaved during RISC assembly? Human and *Drosophila* Ago2 cleave their target RNAs at the phosphodiester bond that lies across from nucleotides 10 and 11 of the guide strand (Elbashir et al., 2001b, 2001c; Martinez et al., 2002; Schwarz et al., 2002; Haley and Zamore, 2004; Meister et al., 2004b; Liu et al., 2004; Rivas et al., 2005). The equivalent phosphodiester bond lies between nucleotides 9 and 10 of the passenger strand. Cleavage of the passenger strand, directed by the siRNA guide strand and catalyzed by Ago2, is therefore predicted to yield a 9 nt 5′ cleavage product. To determine if the siRNA passenger strand is cleaved during RISC assembly, we 5′-^{32}P-radiolabeled each strand of a highly asymmetric siRNA targeting the human *sod1* mRNA (Figure 1A). This siRNA loads one strand (red) to the near exclusion of the other (blue) because it contains an unpaired nucleotide at the 5′ end of the guide (Schwarz et al., 2003). Upon incubation in *Drosophila* embryo lysate, the radiolabeled passenger strand was converted to a 9 nt, 5′-radiolabeled

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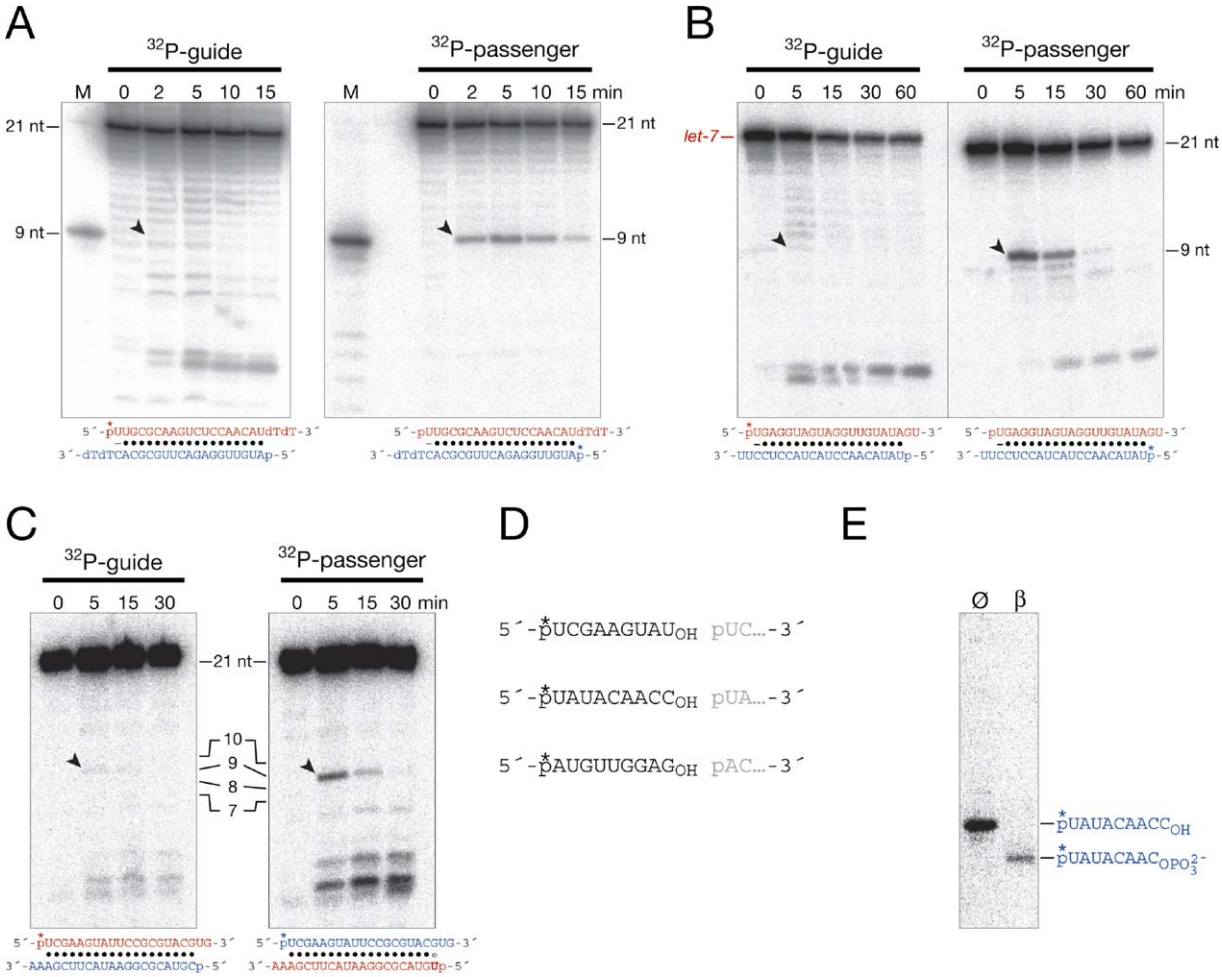

Figure 1. The siRNA Passenger Strand, but Not the Guide, Is Cleaved between Nucleotides 9 and 10 during RISC Assembly in *Drosophila* Embryo Lysate

(A) An siRNA targeting human superoxide dismutase 1 (*sod1*), 5′-^{32}P-radiolabeled on either the guide or the passenger strand was incubated in an in vitro RNAi reaction. For the passenger strand, a 9 nt 5′ cleavage product accumulated early in the reaction, then disappeared.

(B) A similar experiment, using an siRNA in which the guide strand had the sequence of the *let-7* microRNA and the passenger strand was antisense to *let-7*. Again, a 9 nt 5′ cleavage product was detected only for the passenger.

(C) Two variants of an siRNA targeting firefly luciferase were incubated in an in vitro RNAi reaction. For both, the antisense strand was ^{32}P-radiolabeled. When the radiolabeled antisense strand served as passenger, a 9 nt 5′ cleavage product was observed, but not when the antisense strand acted as the guide.

(D) The three different passenger cleavage site sequences suggest that cleavage is not sequence specific.

(E) The 3′ termini of the purified *let-7* siRNA passenger-strand cleavage product were mapped by reaction with sodium periodate followed by β elimination; the 5′ passenger-strand cleavage product bears 2′,3′ hydroxy termini, just as do the products of Ago2-mediated target RNA cleavage. Ø, gel-isolated 9 nt passenger-strand cleavage product; β, the purified passenger-strand cleavage product reacted with sodium periodate followed by β elimination.

species whose accumulation peaked at 5 min, then disappeared (Figure 1A). In contrast, no 9 nt product was detected for the guide strand. The 9 nt passenger-strand cleavage fragment was also produced when this siRNA was incubated in S2 cell lysate that supports in vitro Ago2 RISC assembly and RNAi (see Figure S1 in the Supplemental Data available with this article online).

Next, we 5′-radiolabeled each strand of a second highly asymmetric siRNA in which the guide (red) had the sequence of the microRNA *let-7*. Again, when the siRNA was incubated in an in vitro RNAi reaction, we detected a 9 nt, 5′-radiolabeled product for the passenger strand (blue), but not the guide (Figure 1B). We also examined an siRNA targeting the firefly luciferase mRNA, 5′-^{32}P-radiolabeled on its antisense strand (red; Figure 1C). When this siRNA is fully base paired, the local thermodynamic difference between the two 5′ ends favors assembly of the antisense strand into RISC (Schwarz et al., 2003), making the radiolabeled strand the guide. Changing a C to a U at position 19 of the sense strand (blue) inverts the asymmetry of the siRNA, converting the radiolabeled strand into the passenger. When incubated in the RNAi reaction, only the siRNA in which the radiolabeled strand served as passenger

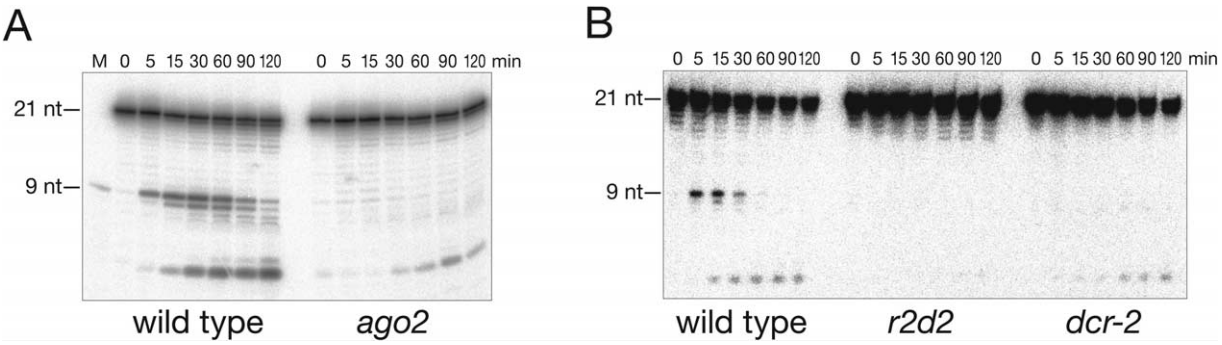

Figure 2. Passenger-Strand Cleavage Requires the RISC Assembly Machinery

(A) Ago2 is required for passenger-strand cleavage. *ago2*^{414} mutant ovary lysates produced little or no passenger-strand cleavage product compared to a wild-type ovary lysate control.

(B) Ovary lysates prepared from *dcr-2*^{L811fsX} and *r2d2* mutant females do not cleave the passenger strand. These mutants cannot assemble the RISC-loading complex (RLC).

produced a 9 nt, 5′ product. These data indicate that cleavage is restricted to the passenger strand of the siRNA duplex.

The siRNAs in Figure 1 include examples in which the nucleotide 5′ to the cleavage site is a purine or a pyrimidine and in which the nucleotide 3′ to the cleavage site is a purine or a pyrimidine (Figure 1D), suggesting that passenger-strand cleavage does not depend on siRNA sequence. Treatment of the purified 9 nt cleavage product with sodium periodate followed by β elimination generated an 8 nt RNA bearing a 3′ phosphate, demonstrating that the 9 nt passenger-strand product contains a 2′,3′ hydroxy terminus (Figure 1E). A 3′ hydroxy terminus is consistent with passenger-strand cleavage by Ago2, which cleaves target RNAs to yield 5′ cleavage products bearing a 3′ hydroxyl group (Martinez and Tuschl, 2004; Schwarz et al., 2004), but not by the RISC component Tudor-SN, which is a member of a nuclease family that leaves a 3′ phosphate on its products (Caudy et al., 2003).

Passenger-Strand Cleavage Requires Ago2 and RISC Assembly Factors

Consistent with the view that Ago2 cleaves the passenger strand, the 9 nt cleavage product was not produced when an siRNA, 5′-radiolabeled on the passenger strand, was incubated in ovary lysate from *ago2*^{414} mutant flies (Figure 2A), which contain little (Deshpande et al., 2005) or no (Okamura et al., 2004) detectable Ago2 protein. These data also indicate that Dcr-2, which, like Ago2, produces 5′ cleavage products bearing 3′ hydroxy termini, is not the nuclease that cleaves the passenger strand, because *ago2*^{414} mutants contain normal levels of Dcr-2 protein (Tomari et al., 2004b), and Dcr-2 needs no protein cofactors to act as an endonuclease (Liu et al., 2003).

Our model postulates that passenger-strand cleavage occurs as a normal step in Ago2 RISC loading and not as a consequence of association of the siRNA with Ago2 through a mechanism independent from the RLC. Passenger-strand cleavage should therefore require the core components of the RLC, Dcr-2, and R2D2 (Lee et al., 2004; Pham et al., 2004; Tomari et al., 2004a). Indeed, both Dcr-2 and R2D2 were required for cleavage of the passenger strand, in that ovary lysates prepared from *dcr-2*^{L811fsX} and *r2d2* mutant flies do not cleave the passenger strand (Figure 2B).

The Passenger Strand Is Cleaved before the siRNA Strands Dissociate

We can envision two distinct mechanisms in which an siRNA passenger strand might act as a substrate for Ago2 cleavage: in *trans*, after the strands separate and mature RISC has been formed, or in *cis*, before the two strands dissociate. A *trans* model predicts that passenger-strand cleavage requires the function of mature RISC, so cleavage should be blocked by an antisense oligonucleotide that binds siRNA-programmed Ago2. In contrast, in a *cis* model, an antisense inhibitor of RISC should have no effect on passenger-strand cleavage, although it would block the subsequent ability of RISC to cleave a bona fide target mRNA in *trans*. To distinguish between these two possibilities, RISC was programmed by combining *Drosophila* embryo lysate, a double-stranded siRNA, 5′-^{32}P-radiolabeled on the passenger strand, and a 29 nt long, 2′-*O*-methyl oligonucleotide in a standard in vitro RNAi reaction (Figure 3A). The 2′-*O*-methyl oligonucleotide contained a central 21 nt region complementary to the guide strand of the siRNA so that it could bind to and block the function of mature RISC. Such 2′-*O*-methyl oligonucleotides act as stoichiometric inhibitors of RISC function in vitro and in vivo (Hutvágner et al., 2004; Meister et al., 2004a; Leaman et al., 2005). We monitored the production of cleaved passenger strand for both the experimental and the control reactions. After 30 min, RISC assembly was quenched by treatment with *N*-ethylmaleimide (Nykänen et al., 2001), then a ^{32}P-radiolabeled target RNA was added to the reaction, and we monitored target cleavage, a measure of the concentration of active RISC. Neither 2′-*O*-methyl oligonucleotide had any detectable effect on passenger-strand cleavage (Figure 3B). Yet, in the same reaction, the 2′-*O*-methyl oligonucleotide complementary to the siRNA

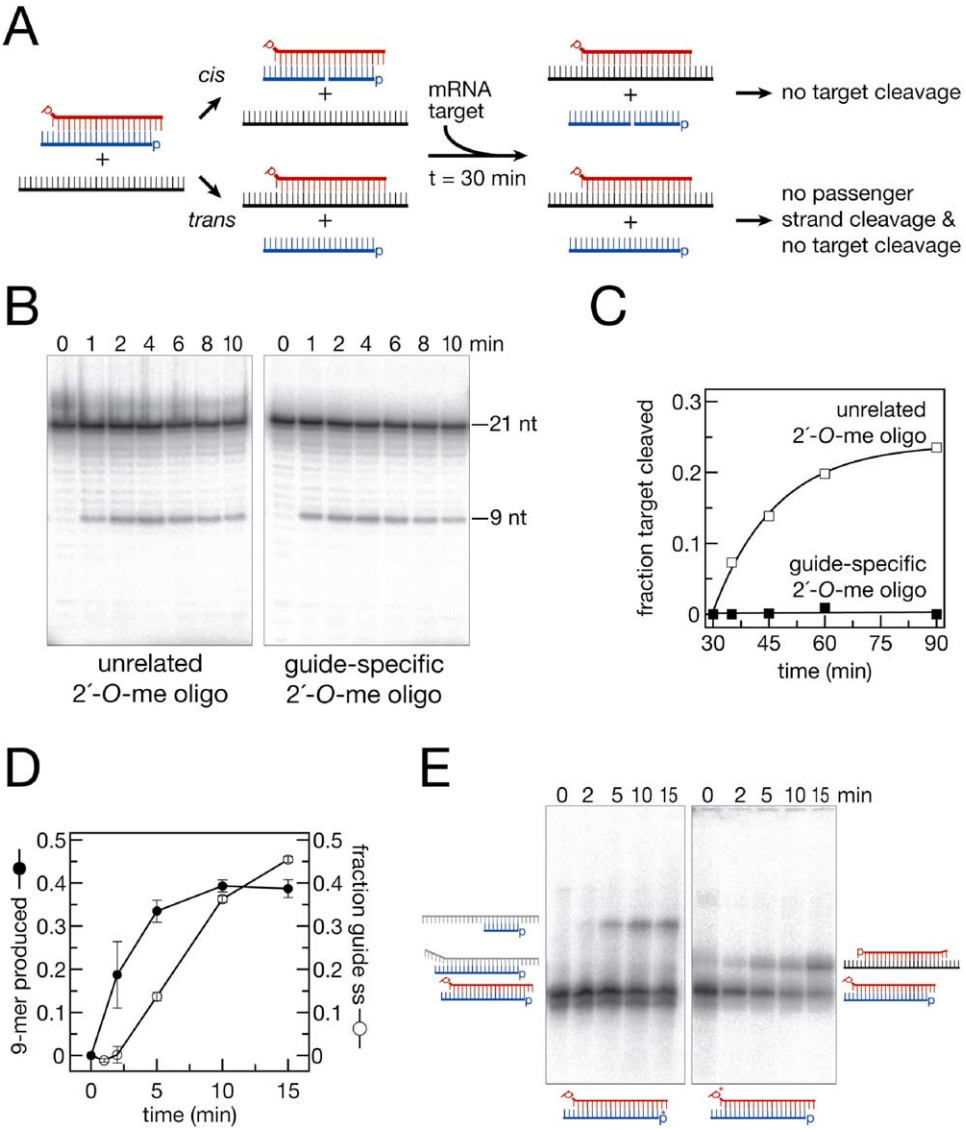

Figure 3. The Passenger Strand Is Cleaved before the Two siRNA Strands Dissociate

(A) Experimental design. A 2′-*O*-methyl oligonucleotide (black) bearing the same sequence as the target of the guide strand (red) or an unrelated control 2′-*O*-methyl oligonucleotide was incubated with double-stranded siRNA in an in vitro RISC assembly reaction. Passenger-strand cleavage was monitored for the first 10 min. RISC assembly was continued until 30 min, when assembly was quenched and target RNA added. Target cleavage was monitored for an additional 60 min, i.e., 90 min after initiating RISC assembly. The sequence of the siRNA appears in Figure 1A.

(B) Passenger-strand cleavage was unaffected by the guide-specific 2′-*O*-methyl oligonucleotide.

(C) In contrast, the guide-specific 2′-*O*-methyl oligonucleotide, but not the control oligonucleotide, inactivated mature RISC. The data suggest that the passenger strand was cleaved in *cis*, before the two siRNA strands dissociated to liberate mature RISC.

(D) The rates of passenger-strand cleavage and of the production of single-stranded guide strand—a measure of mature RISC production—were monitored for the siRNA used in (B) and (C). Each data point represents the average of three trials ± standard deviation. To prevent 9-mer degradation, a 100-fold excess of 2′-*O*-methyl oligonucleotide, relative to siRNA, was included in the reaction.

(E) When RISC was assembled under standard in vitro conditions, full-length (21 nt) guide strand was readily trapped by a complementary 2′-*O*-methyl oligonucleotide. In contrast, we could not detect the production of full-length passenger strand when a passenger-complementary 2′-*O*-methyl oligonucleotide trap was included. Instead, we observed accumulation of the 9 nt 5′ passenger-strand cleavage product. A small amount of single-stranded full-length passenger strand, bound to the 2′-*O*-methyl oligonucleotide, was detected at the beginning of the reaction and was, therefore, not a consequence of RISC assembly. The identity of the bands was assigned by their comigration with synthetic RNA:RNA or 2′-*O*-methyl oligonucleotide:RNA duplexes as diagrammed.

guide strand, but not a control oligonucleotide of unrelated sequence, completely blocked target cleavage (Figure 3C). These data indicate that passenger-strand cleavage occurs in *cis*, during RISC assembly.

In further support of the *cis* cleavage model, we found that passenger-strand cleavage of a standard, double-stranded siRNA preceded the production of single-stranded guide strand, i.e., the production of

mature RISC (Figure 3D). In order to accurately measure the rate of production of cleaved passenger strand, the reaction included a 2′-*O*-methyl oligonucleotide trap complementary to the 5′-^{32}P-radiolabeled passenger strand. The trap oligo can bind single-stranded, cleaved passenger strand, helping to protect it from subsequent degradation (Figure S2 and below). At each time point, cleaved passenger strand was resolved from full-length passenger strand by denaturing gel electrophoresis. Single-stranded guide production was measured in parallel by native gel analysis using the same siRNA duplex, except the siRNA was 5′-^{32}P-radiolabeled on the guide.

These two assays revealed that nearly one-fifth of the passenger strand was cleaved after 2 min incubation, a time when little or no single-stranded guide could be detected (Figure 3D). This observation was counter to the *trans* model of passenger-strand cleavage, in which single-stranded guide would need to form prior to 9-mer production, but it concurred with the *cis* model, in which single-stranded guide must appear after or concurrently with 9-mer production. The observed lag between 9-mer production and single-stranded guide formation suggests that passenger-strand fragments remain paired to the guide strand for several minutes after cleavage. Only after these fragments dissociate is the guide strand liberated and available for detection as single-stranded.

Control experiments (not shown) demonstrated that although the passenger-strand-specific trap oligonucleotide significantly improved capture of the 9 nt species produced by passenger-strand cleavage, the 9-mer was eventually degraded even in the presence of the trap. Thus, our method underestimated the amount of 9-mer produced from the passenger strand and therefore likely underestimated the speed of this cleavage event relative to the production of mature RISC containing a single-stranded guide. We conclude that, within the limits of detection of our assay, one molecule of passenger strand was cleaved for every molecule of guide strand assembled into RISC.

Supporting this view, we have been unable to detect the production of any full-length, single-stranded passenger strand during RISC assembly (Figure 3E). We incubated an siRNA, ^{32}P-radiolabeled on its passenger strand, in a standard RNAi reaction that contained a 29 nt, 2′-*O*-methyl oligonucleotide complementary in its central 21 nts to the passenger strand. At each time point, the samples were deproteinized at room temperature and then analyzed by native gel electrophoresis using a gel system containing 1.5 mM Mg^{2+} in both the gel and the running buffer. In parallel, the analysis was performed using the same siRNA, but radiolabeled on the guide strand and using a 2′-*O*-methyl oligonucleotide complementary to the guide. We readily detected the production of single-stranded, full-length guide strand, but were unable to detect any full-length, single-stranded passenger strand, observing only the production of single-stranded, 9 nt passenger-strand cleavage fragment.

A New Model for Assembly of siRNA into Ago2 RISC

Together, the results presented in Figures 1, 2, and 3 suggest a revised model for the assembly of Ago2-containing RISC (Figure 4A). This model predicts the existence of a new siRNA species in the Ago2 RISC assembly pathway: a double-stranded siRNA bearing a nick on the passenger strand (yellow box 2, Figure 4A). To determine if such a species exists, we assembled RISC in vitro using an siRNA containing a radiolabeled passenger strand. At each time point, a sample of the reaction was deproteinized at room temperature in the presence of a 20-fold excess of a 2′-*O*-methyl oligo nucleotide containing at its center the 21 nt sequence of the guide-strand RNA, designed to prevent reannealing of the original passenger strand with the guide strand. Next, the reaction was analyzed by native gel electrophoresis. Like the gel system used in Figure 3E, this gel system could resolve the double-stranded siRNA from the heteroduplex formed between the 2′-*O*-methyl oligonucleotide and the 9 nt passenger-strand cleavage product and from the heteroduplex formed between the 2′-*O*-methyl oligonucleotide and the full-length passenger strand. During the first five minutes of the reaction, most of the labeled passenger strand migrated in the native gel as double-stranded siRNA, although some 9 nt passenger-strand cleavage fragment released from the siRNA duplex was also detected (Figure S2).

To examine whether some of the double-stranded RNA represented the nicked duplex predicted by the model, the double-stranded siRNA population was eluted from the native gel and analyzed by denaturing gel electrophoresis to resolve full-length passenger strand from the 9 nt, 5′ passenger-strand cleavage fragment (Figure 4B). For comparison, the total reaction was analyzed in parallel. We found that at early times in RISC assembly, the double-stranded siRNA was a mixture of guide strand bound to full-length passenger strand and guide strand bound to cleaved passenger strand. Moreover, a 2′-*O*-methyl oligonucleotide trap intended to capture and protect the 9 nt cleavage product increased only slightly the amount of 9-mer recovered at early times ($\leq$5 min) in RISC assembly but dramatically increased the recovery of this species later ($\geq$10 min) (Figures 4C and S1B). These data suggest that early in RISC assembly, the 9 nt fragment of the passenger strand is protected from degradation even in the absence of the 2′-*O*-methyl oligonucleotide trap. The simplest explanation is that early in RISC assembly, the 9 nt passenger-strand fragment remains bound to the guide strand, in the target RNA binding cleft of Ago2, and is therefore naturally protected from nucleolytic destruction.

A Bypass Mechanism for siRNA Unwinding

Our data suggest that, under normal conditions, passenger-strand cleavage is the dominant mechanism for initiating siRNA strand dissociation during the production of Ago2-containing RISC. To assess more quantitatively the contribution of passenger-strand cleavage to Ago2 RISC assembly, we sought an siRNA chemical modification that would inhibit passenger-strand cleavage while leaving intact the essential structure of the siRNA. Phosphorothioate modification of the scissile phosphodiester bond inhibits Ago2-catalyzed cleavage of an mRNA target, and this inhibition is partially but

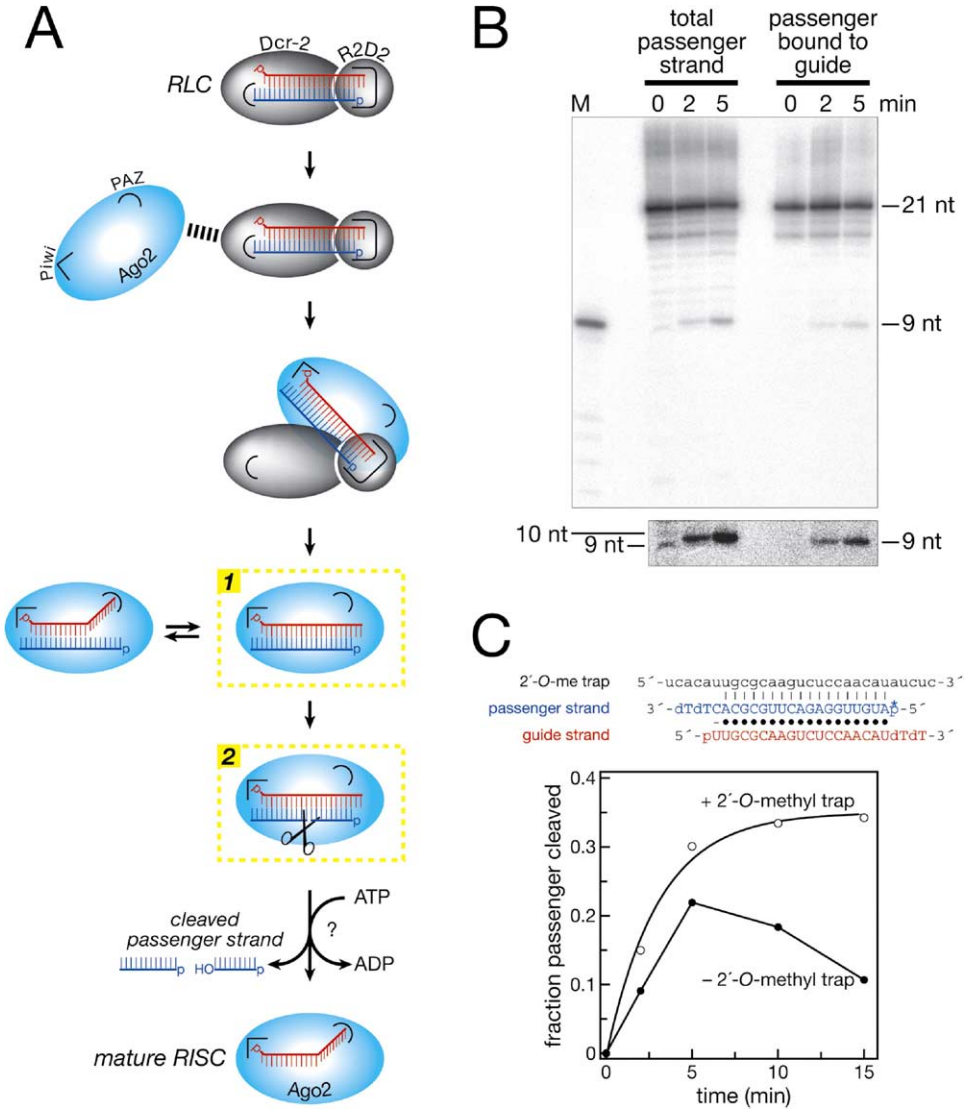

Figure 4. A Revised Model for RISC Assembly

(A) Dcr-2 and R2D2 are envisioned to recruit Ago2 directly to the double-stranded siRNA. Ago2 exchanges first with Dcr-2, with whom it makes a protein-protein contact (dashed line; Tahbaz et al., 2004), then with R2D2. Finally, Ago2 cleaves the passenger strand (blue), thereby liberating the guide (red) from the siRNA duplex and producing active RISC. Release of the passenger-strand cleavage products may be facilitated by an ATP-dependent cofactor, much as release of the products of target cleavage facilitated by ATP (Haley and Zamore, 2004). We have included conformational rearrangements in Ago2 proposed to be associated with target RNA cleavage (Tomari and Zamore, 2005).

(B) Two-dimensional analysis of the double-stranded population provides support for the model. Double-stranded siRNA, 5′-^{32}P-radiolabled on the passenger strand, was incubated in a RISC assembly reaction in the presence of a 29 nt 2′-*O*-methyl oligonucleotide complementary to the passenger strand, then the trap bound and free RNA species were separated by native gel electrophoresis as diagrammed in Figure S2. The region of the gel corresponding to double-stranded siRNA was excised and its RNA constituents analyzed by denaturing electrophoresis. The double-stranded siRNA population comprised both species *1* and species *2* postulated by the model in (A). That is, a portion of the "double-stranded" siRNA contains a cleaved passenger strand. The lower panel is a digital overexposure of the region of the gel corresponding to the 9 nt passenger-strand cleavage product. The sizes of passenger-strand nonspecific hydrolysis products are indicated for reference. These hydrolysis products were present before incubation in lysate in the total siRNA population, but not in the sample that had been purified by native gel electrophoresis. The 9 nt passenger-strand cleavage product bears a 3′ hydroxyl group, whereas hydrolysis is expected to yield a mixture of 3′, 2′, and 2′,3′ cyclic phosphate termini. Thus, the 9 nt cleavage product migrates between the 9 and 10 nt long hydrolysis products.

(C) Passenger-strand 5′ cleavage product is disproportionately stable early in RISC assembly, consistent with its still being bound to the guide strand. *sod1* siRNA containing 5′-^{32}P-labeled passenger was incubated in a standard in vitro RNAi reaction, in the absence or presence of a 2′-*O*-methyl oligonucleotide complementary to the passenger strand, and the fraction of siRNA passenger strand recovered as a 9 nt cleavage product was plotted versus time.

not fully rescued by Mn^{2+} (Schwarz et al., 2004; Rivas et al., 2005). We surmised that a single phosphorothioate at the scissile phosphate of the passenger strand of an siRNA should inhibit passenger-strand cleavage (Figure 5A). Phosphorothioate linkages are chiral, with sulfur substitution of each of the two non-bridging oxygens designated Rp or Sp. Consistent with the idea that both target-RNA and siRNA passenger-strand cleavage are catalyzed by the Mg^{2+}-dependent, endonuclease activity of Ago2, a racemic mixture of Rp and Sp phosphorothioate linkages between nucleosides 9 and 10 of the siRNA passenger strand decreased the rate of siRNA strand separation by ~3-fold, relative to the rate for the all-phosphodiester siRNA or to phosphorothioate substitution at the adjacent passenger-strand phosphodiester bonds (Figure S3).

We resolved the mixture of Rp and Sp phosphorothioate-substituted siRNA passenger strands by reverse-phase HPLC and prepared two diastereomerically pure siRNA duplexes, one with an Rp phosphorothioate and one with an Sp phosphorothioate at the passenger strand scissile phosphate (Figure S4). Each siRNA was examined for passenger-strand cleavage during incubation in a standard RISC assembly reaction, in the absence and the presence of a 2′-*O*-methyl oligonucleotide trap designed to protect the 9 nt passenger-strand cleavage fragment from degradation (Figure 5B). Substitution of the scissile phosphate of the passenger strand of the siRNA duplex reduced the rate of 9-mer production ~10-fold with the Rp diastereomer and >30-fold with the Sp diastereomer (Figure 5B and data not shown). Inhibition of endonucleolytic cleavage by a phosphorothioate linkage at the scissile phosphate can be partially rescued by supplementing the reaction with Mn^{2+}, a thiophilic cation (Verma and Eckstein, 1998; Schwarz et al., 2004). Similarly, supplementing the RISC assembly reaction with 2.5 mM Mn^{2+}, but not Mg^{2+}, rescued cleavage of the Rp phosphorothioate-containing passenger strand (Figures 5C, 5D, and S5). Mn^{2+} did not detectably rescue passenger-strand cleavage for the Sp phosphorothioate-substituted siRNA (Figures 5D and S5). Together, these data are consistent with the partial rescue by Mn^{2+} of Ago2-directed cleavage of a target RNA substituted with a racemic mixture of phosphorothioate diastereomers at the scissile phosphate (Schwarz et al., 2004; Rivas et al., 2005).

The production of single-stranded guide strand from the siRNA duplex, a measure of mature RISC production, was slower for an siRNA containing an Rp or Sp phosphorothioate at the scissile phosphate of the passenger strand relative to an all-phosphodiester passenger strand (Figure 5E). When supplemental Mn^{2+} was provided, the production of single-stranded guide strand was enhanced for the Rp phosphorothioate-substituted siRNA, relative to the unmodified siRNA, but not for the Sp (Figure 5F). In Figure 5E, supplemental Mg^{2+} was included so that the divalent cation concentration was equivalent to the total divalent cation concentration when supplemental Mn^{2+} was added. The higher-than-standard Mg^{2+}, which was likely much greater than the intracellular free Mg^{2+} concentration, enhanced RISC assembly, as monitored by the production of single-stranded guide (Figure 5E) and RISC activity (data not shown), relative to that observed for the phosphodiester and phosphorothioate-substituted siRNAs under standard conditions.

Substitutions within the phosphodiester backbone known to inhibit Ago2-catalyzed RNA cleavage inhibit both passenger-strand cleavage and maturation of the RISC. Divalent metal ions that partially rescue cleavage also partially rescue RISC maturation. Nonetheless, when viewed quantitatively, the results of these experiments, which where performed at a nonstandard, high divalent cation concentration, suggest that inhibition of passenger-strand cleavage reduced RISC assembly but did not prevent it. That is, using an siRNA duplex for which very little cleavage product could be detected (Figure 5D) resulted in a reduced but readily detectable amount of mature RISC, as measured by single-stranded guide formation (Figure 5E). Part of this difference can be explained by the finite lifetime of the 9-mer cleavage product, even in the presence of the 2′-*O*-methyl oligonucleotide trap. However, we suspect that much of this difference is due to a bypass mechanism for RISC maturation that is more efficient at elevated divalent cation concentrations. The existence of such a bypass mechanism implies that passenger-strand cleavage is the normal mechanism by which siRNA strand separation is initiated, but it is not obligatory.

How big a role does passenger-strand cleavage play in the production of active, Ago2-containing RISC for siRNAs? To assess the effect of blocking passenger-strand cleavage during RISC assembly, we compared the amount of RISC activity produced for the all-phosphodiester siRNA and the two diastereomerically pure phosphorothioate siRNAs after 5 and after 10 min of RISC assembly (Figure 6). RISC was assembled in a standard reaction, quenched by treatment with *N*-ethylmaleimide, and then a radiolabeled target RNA was added and target cleavage monitored. When RISC was assembled for just 5 min, considerable RISC was produced from the all-phosphodiester siRNA, but little or no RISC activity was detected for the two siRNAs bearing an Rp or an Sp phosphorothioate at the passenger strand scissile phosphate. In contrast, when RISC was assembled for 10 min, RISC activity was detected for all three siRNAs. The all-phosphodiester siRNA was most active; the siRNA bearing an Rp phosphorothioate at the passenger-strand scissile phosphate produced an intermediate amount of RISC activity; the Sp siRNA showed the least activity. These results show that RISC activity is rapidly assembled when the passenger strand can be cleaved. When it cannot be cleaved, RISC is slower to form because it must rely on a bypass mechanism in which the passenger strand is dissociated intact from the guide. Because the bypass mechanism is so much slower than the cleavage-assisted mechanism, we conclude that nearly all of the Ago2 RISC generated with a standard siRNA duplex is formed through the cleavage-assisted mechanism.

Passenger-Strand Cleavage Facilitates Assembly of Human Ago2 RISC

The RISC assembly pathway has been studied largely in extracts from *Drosophila* but is presumed to be similar in human cells, in which an RLC-like complex has

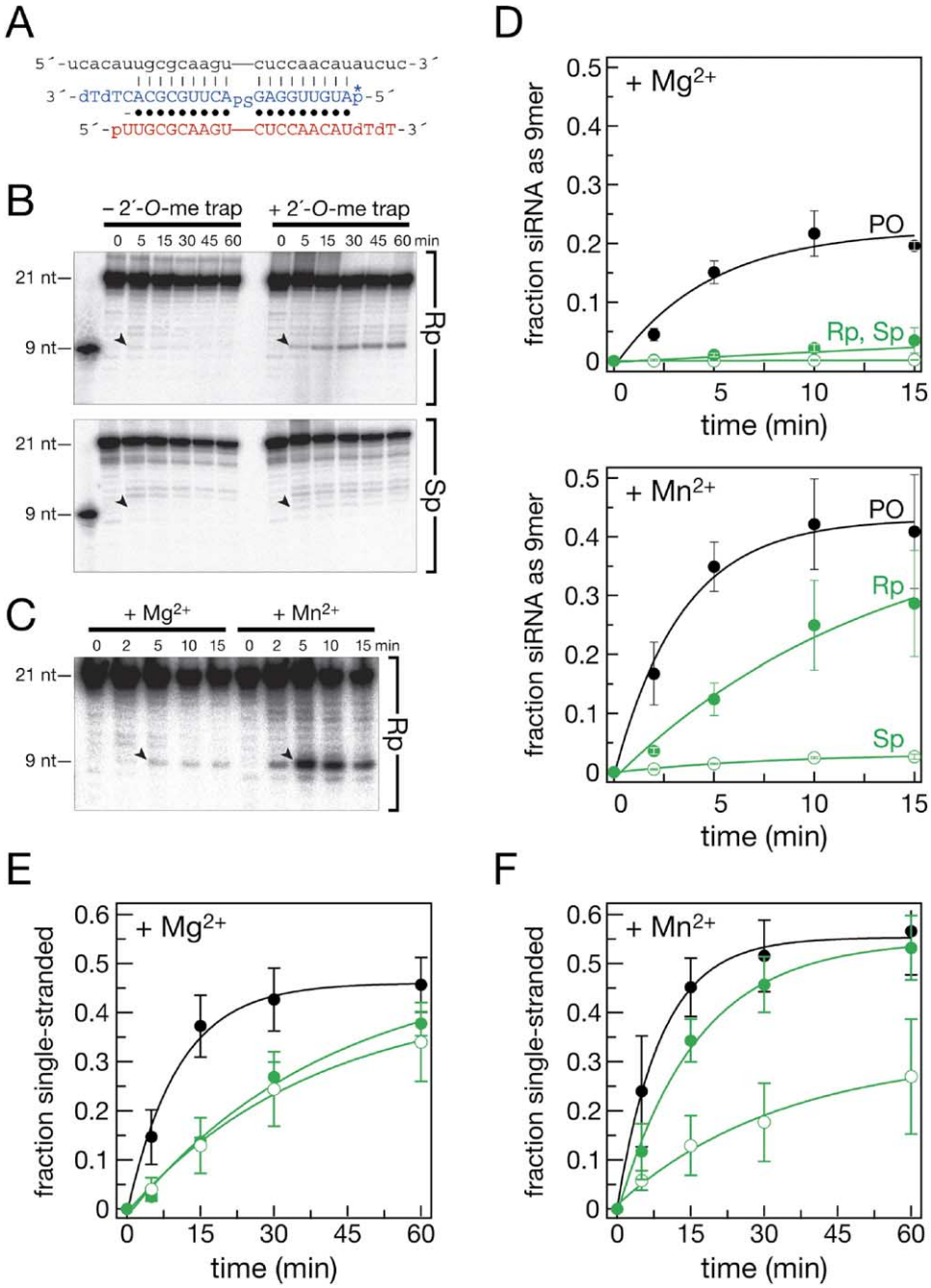

Figure 5. A Phosphorothioate (PS) Linkage at the siRNA Scissile Phosphate Inhibits Passenger Cleavage and Slows Dissociation of the Two siRNA Strands

(A) siRNA were synthesized with a single PS modification at the passenger-strand scissile phosphate and the diastereomers resolved by reverse-phase HPLC (Figure S4).

(B) Both Rp and Sp PS diastereomers inhibited passenger-strand cleavage. When a 2′-*O*-methyl oligonucleotide complementary to the passenger was included in the reaction, small amount of 9 nt passenger-strand cleavage product was detected for the Rp, but not the Sp, passenger strand.

(C) The inhibitory effect of the Rp phosphorothioate linkage could be overcome when the thiophilic divalent cation Mn^{2+} was included in the reaction. A 2′-*O*-methyl oligonucleotide was not included in this experiment.

(D) The rate of passenger-strand cleavage was measured in the presence of a 2′-*O*-methyl oligonucleotide trap for the all-phosphodiester (PO), Rp and Sp PS siRNAs in the presence of 2.5 mM supplemental Mg^{2+} or Mn^{2+}.

(E and F) The rate of production of single-stranded guide siRNA was measured using a radiolabeled guide strand for siRNAs containing a phosphodiester, Rp, or Sp PS-modified passenger strand in the presence of supplemental Mg^{2+} or Mn^{2+}. In (D), (E), and (F), each data point is the average of three trials ± standard deviation.

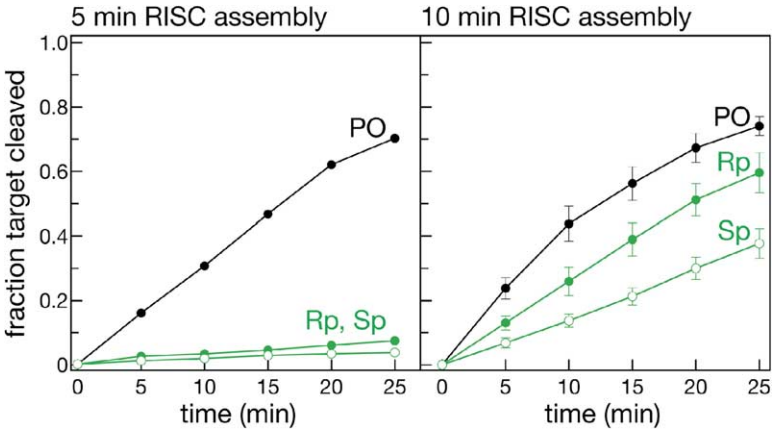

Figure 6. Passenger-Strand Cleavage Is Required for Rapid RISC Assembly, but a Slower Bypass Mechanism Dissociates the Two siRNA Strands When Passenger-Strand Cleavage Is Inhibited

The assembly of active RISC was assayed by measuring the production of target-cleaving activity. RISC was assembled for 5 or 10 min, quenched, then a cap-radiolabeled target RNA was added to the reaction and the rate of target cleavage monitored. In the right panel, each data point is the average of three trials ± standard deviation.

recently been proposed to load RISC containing Ago2 (Chendrimada et al., 2005). Because Ago2 is the only human Argonaute protein with known cleavage activity, this silencing complex is thought to be the one that mediates target-strand cleavage in humans (Meister et al., 2004b; Liu et al., 2004; Rivas et al., 2005). Does passenger-strand cleavage play a role in human Ago2 RISC assembly? Supporting the view that it does, the passenger strand, but not the guide, of an siRNA duplex in which the guide strand corresponds to miR-1 was cleaved when incubated in human HeLa cell S100 extract under conditions that support RNAi in vitro (Figure 7A). As observed for RISC assembly in *Drosophila* embryo lysate, a racemic mixture of Rp and Sp phosphorothioate linkages between nucleosides 9 and 10 of the passenger strand inhibited RISC assembly in human cell extracts, and this inhibition was substantially greater with phosphorothioate substitution at this position compared to substitution of the adjacent phosphodiester bonds (Figure S6). Furthermore, assembly was inhibited by both the Sp and Rp diastereomers but far more by the Sp than by the Rp, as was characteristic of the *Drosophila* RISC (Figures 7B and 7C). In this experiment, RISC was assembled for 20 or 60 min with the *sod1* siRNAs used in Figure 5. Then assembly was quenched and a radiolabeled *sod1* RNA added to the reaction in order to monitor target cleavage as a measure of Ago2 RISC activity. Thus, multiple lines of evidence support the conclusion that passenger-strand cleavage facilitates assembly of the human Ago2 RISC, just as it does for the *Drosophila* Ago2 RISC. Indeed, the virtually undetectable assembly with the Sp substitution, even after a 60 min assembly incubation (Figures 7B and 7C), implicates the cleavage-assisted mechanism for nearly all of the Ago2 RISC assembly occurring with a standard siRNA duplex.

The Bypass Mechanism Appears Sufficient for Assembling MicroRNAs

If siRNAs are assembled into human Ago2 RISC by a passenger-strand cleavage-assisted mechanism, how are miRNAs assembled? To begin to address this question, we examined assembly of the muscle-specific miRNA, miR-1, which is not present in HeLa cells. We prepared three small RNA duplexes (Figure 8A) predicted to load miR-1 into RISC: (1) a miR-1/miR-1* duplex, like that predicted to result from Dicer processing of pre-miR-1 from the human mir-1-1 gene; (2) a functionally asymmetric siRNA in which miR-1 was paired to antisense miR-1 with a "frayed" miR-1 5′ end (miR-1 siRNA); and (3) an siRNA in which miR-1 was paired to antisense miR-1, but for a G:U wobble at position 2 of miR-1 (miR-1 "GU" siRNA). All three duplexes mediated efficient Ago2 RISC loading, as measured functionally by target cleavage (Figure 8B, PO time courses). For each duplex, we synthesized a passenger strand with a racemic phosphorothioate at the scissile phosphate (Figure 8A). Substitution of the passenger-strand scissile phosphate with a phosphorothioate strongly inhibited the assembly of active RISC for the miR-1 siRNA, had a more modest effect for the miR-1 "GU" siRNA, and had no effect for the miR-1/miR-1* duplex. These data suggest that passenger-strand cleavage facilitates RISC assembly for the standard miR-1 siRNA, plays a supportive role for the miR-1 "GU" siRNA, but plays no role for the miR-1/miR-1* duplex. Analogous results were obtained when miR-1 was presented as the authentic pre-miRNA hairpin or in the context of a perfectly paired short-hairpin RNA (shRNA); again, the phosphorothioate inhibited assembly when the passenger strand was fully paired to miR-1 but not when it was within the miR-1/miR-1* duplex (Figures 8A and 8C). These results support the idea that miRNAs, when paired to their natural passenger strands, the miRNA* strands, are loaded into the Ago2 RISC without cleavage of the miRNA*. This preference for the bypass mechanism is because of the multiple mismatches typical of miRNA/miRNA* duplexes, which presumably inhibit the cleavage step of the cleavage-assisted mechanism while accelerating the bypass mechanism, obviating the need for guide-strand cleavage to enhance strand separation.

Discussion

Our data suggest a new model for Ago2 RISC assembly in which siRNAs are initially loaded into Ago2 as duplexes, and then Ago2 cleaves the passenger strand of the siRNA, facilitating its displacement and leaving the siRNA guide strand bound stably to Ago2 (Figure 4A). In support of this cleavage-assisted assembly model,

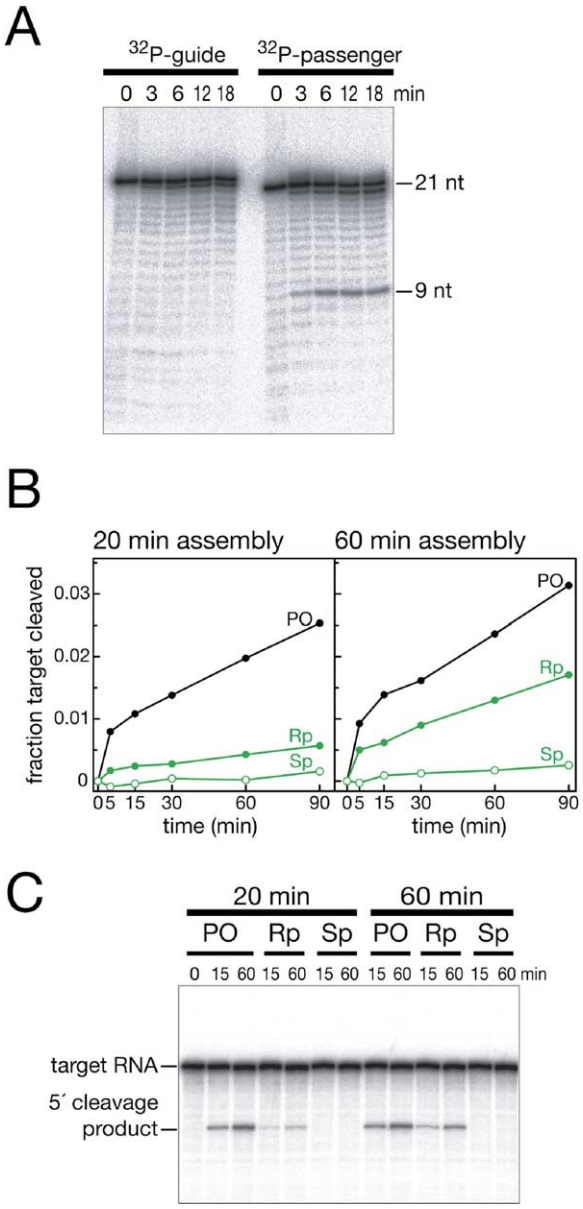

Figure 7. Passenger-Strand Cleavage Is Required for Rapid Assembly of the Ago2-Containing Human RISC

(A) The passenger strand, but not the guide, is cleaved when human RISC is assembled. An siRNA in which the guide strand corresponded to miR-1 was used. A 2′-*O*-methyl oligonucleotide complementary to the 5′-^{32}P-radiolabeled strand was included in each reaction.

(B and C) All-phosphodiester (PO), Rp, or Sp phosphorothioate (PS)-containing *sod1* siRNA (diagrammed in Figure 5A) were used to program RISC assembly in human HeLa cell S100 extract for 20 or 60 min, then RISC assembly was quenched, a target RNA added, and the production of Ago2-containing RISC activity measured by monitoring target cleavage.

we find that the passenger strand is cleaved during RISC assembly. The position of the cleavage site on the passenger strand and the stereospecificity of inhibition by phosphorothioates are diagnostic of siRNA-directed, Ago2-catalyzed cleavage (Figures 1, 5, and 7). Both Ago2 and the RLC are required for this cleavage (Figure 2), and cleavage occurs before the passenger strand has dissociated from the guide strand—before the formation of active RISC (Figure 3).

A cleavage-assisted assembly mechanism is also consistent with our previous finding that early (5 min) in assembly, RISC contains considerable amounts of double-stranded siRNA (Tomari et al., 2004b). When the asymmetry rules for siRNA loading were first uncovered, they evoked the idea of a nonprocessive helicase that separated the two strands of the duplex, starting from the end that was less stably paired (Khvorova et al., 2003; Schwarz et al., 2003). However, subsequent studies showed that the R2D2/Dcr-2 heterodimer, the core of the RLC, binds asymmetrically to double-stranded siRNA (Tomari et al., 2004b), suggesting that a nonprocessive helicase does not sense siRNA thermodynamic asymmetry during RISC loading. Unlike Ago2 loaded via the RISC assembly pathway, affinity-purified (Liu et al., 2004) or recombinant Ago2 (Rivas et al., 2005) can only be programmed with single-, not double-stranded siRNA, suggesting that the essential function of the RLC is to facilitate the directional loading of a double-stranded siRNA into Ago2. The idea that Ago2-mediated passenger-strand cleavage triggers siRNA strand dissociation also accounts for the previous observation that one passenger strand appears to be destroyed for every cycle of assembly of a guide strand into target-cleaving RISC (Schwarz et al., 2003). Passenger-strand cleavage would strongly reinforce siRNA functional asymmetry by coupling passenger-strand destruction to RISC assembly.

Blocking Ago2-mediated passenger-strand cleavage by substituting a phosphorothioate for the scissile phosphate revealed a slower bypass mechanism that dissociates the two siRNA strands. Of course, phosphorothioate-substituted siRNAs do not occur in nature, but miRNA/miRNA* duplexes do often contain central mismatches predicted to block cleavage of the miRNA* strand, the analog of the siRNA passenger strand (Bartel, 2004). Most, if not all, miRNAs are efficiently loaded into Ago2 in cultured human cells (Meister et al., 2004b; Liu et al., 2004; Rivas et al., 2005), and miR-127, miR-136, miR-196, miR-431, miR-433, and miR-434 are known to cleave their targets in vivo (Yekta et al., 2004; Davis et al., 2005) and are therefore presumed to function in an Ago2-containing RISC.

We envision that in the bypass pathway—as in the cleavage-assisted pathway—Argonaute proteins are loaded with double- rather than single-stranded siRNAs. Dissociation of the full-length passenger strand would then require breaking its interaction with the "seed" region of the guide strand (nucleotides 2–7), a region proposed to mediate miRNA target pairing (Lewis et al., 2003; Bartel, 2004). For metazoan miRNAs, conserved Watson-Crick pairing to the seed is necessary and sufficient for accurate target prediction (Lewis et al., 2003, 2005; Brennecke et al., 2005; Krek et al., 2005). Similarly, Watson-Crick pairing to the seed can be sufficient for miRNA-mediated repression (Doench and Sharp, 2004; Lim et al., 2005; Brennecke et al., 2005), and pairing to this 5′ region of the guide strand makes a far greater contribution to target binding affinity than does pairing to the 3′ end (Haley and Zamore, 2004). The

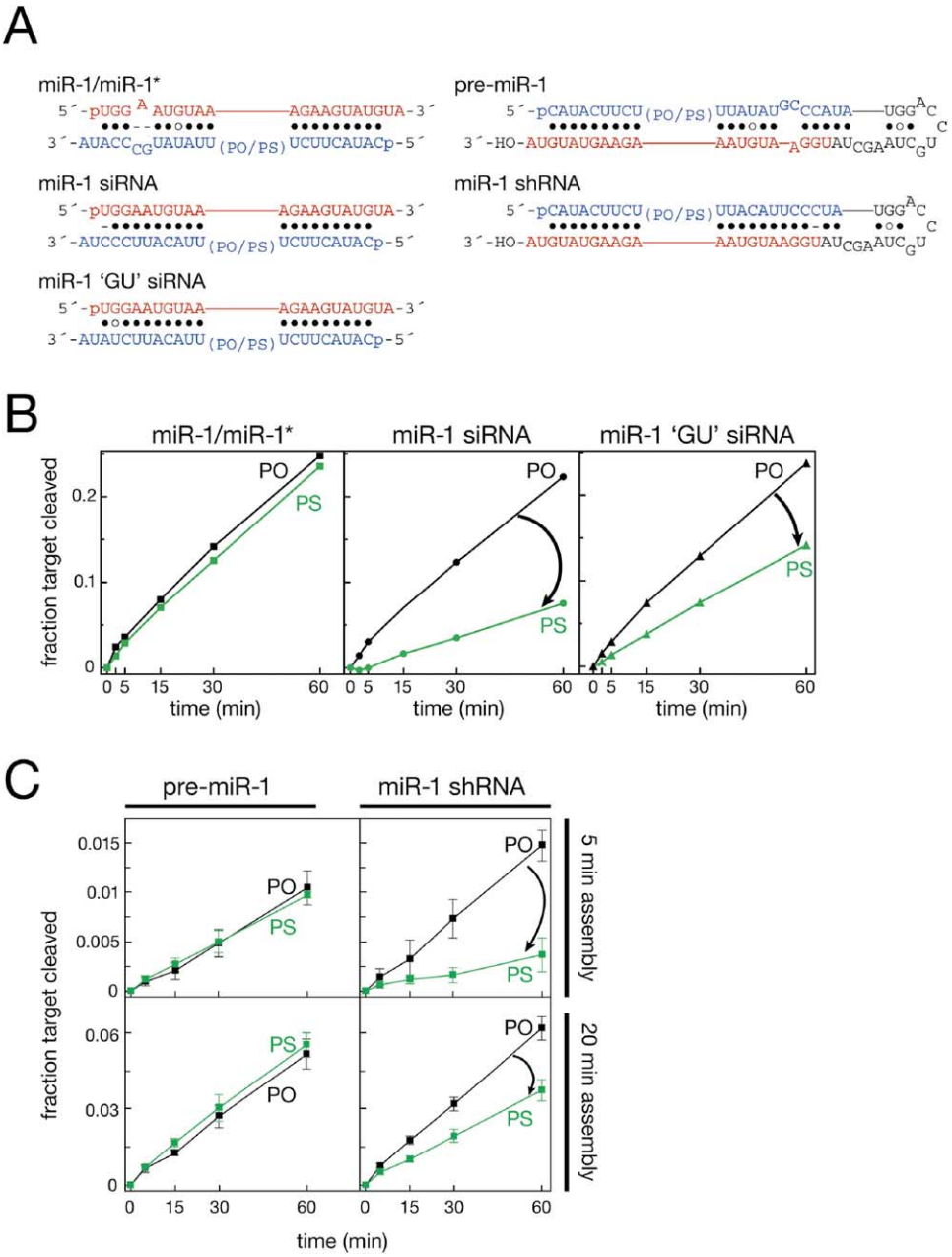

Figure 8. Phosphorothioate Modification of the Scissile Phosphate of a miR-1 siRNA Passenger Strand and of the *pro*-Passenger Strand of a miR-1 shRNA, but not of the miR-1* Strand of a miR-1/miR-1* Duplex nor of *pro*-miR-1* of Authentic pre-miR-1-1, Inhibited the Production of Active miR-1-Programmed RISC

(A) siRNAs and hairpin RNAs used in this study. "PO/PS" indicates linkages at which the effect of a racemic phosphorothioate modification was compared to a phosphodiester bond.

(B) The three siRNAs in (A) were prepared either with a phosphodiester linkage (PO) or a phosphorothioate (PS) at the passenger-strand scissile phosphate. RISC was assembled for 5 min, quenched, then a cap-radiolabeled RNA added and the production of Ago2-containing RISC measured by monitoring target cleavage. The phosphorothioate inhibits assembly of active Ago2-containing RISC most strongly for the miR-1 siRNA, less so for the miR-1 "GU" siRNA. No phosphorothioate inhibition was detected for the miR-1/miR-1* duplex, suggesting that passenger-strand cleavage is important for the rapid assembly of Ago2 RISC for siRNAs, but not for at least some miRNAs.

(C) Target cleavage activity was assayed using the hairpin precursors diagrammed in (A). RISC was programmed using a either pre-miR-1 RNA containing all phosphodiester (PO) linkages or a single, racemic phosphorothioate (PS) linkage at the predicted scissile phosphate of *pro*-miR-1* or was programmed using a miR-1 shRNA with all PO linkages or a PS linkage at the predicted scissile phosphate for the *pro*-passenger strand. HeLa S100 extract was programmed for the indicated times, quenched with *N*-ethylmaleimide, and then a ^{32}P-radiolabeled target RNA containing a 21 nt sequence complementary to miR-1 was added. Each data point is the average of three trials ± standard deviation.

tight binding to the 5′ portion of the guide strand is what presumably prevents an appreciable amount of a standard siRNA duplex from being assembled into Ago2 through the bypass mechanism. In contrast, when presented to Ago2 as a miR-1/miR-1* duplex, miR-1 loads efficiently without need for passenger-strand cleavage, because a Watson-Crick pairing to the miRNA seed is disrupted (Figure 8). Indeed, far less disruption of seed pairing might be sufficient to enable the bypass mechanism to begin to play a substantial role, as hinted by our results using the miR-1 "GU" siRNA, which has a single G:U wobble disrupting perfect Watson-Crick pairing to the seed (Figure 8B). Passenger-strand cleavage appeared less important for this duplex than for the standard siRNA duplex (Figure 8B), a result consistent with computational and experimental studies of miRNA target specificity imply that single G:U wobble pairs in the seed disproportionately perturb small RNA binding (Lewis et al., 2003,2005; Doench and Sharp, 2004; Brennecke et al., 2005).

In addition to loading miRNAs, the bypass mechanism might be used to load siRNAs into Argonaute proteins that have lost their catalytic amino acids, such as human Ago1, Ago3, and Ago4 (Meister et al., 2004b; Liu et al., 2004; Rivas et al., 2005). Conversely, *Arabidopsis thaliana* Argonaute1, a miRNA-guided plant Argonaute protein with a functional endonuclease domain (Baumberger and Baulcombe, 2005; Qi et al., 2005), might be loaded by a passenger-strand cleavage-assisted pathway. Although human Ago1 and Ago3 can bind standard siRNAs, endogenous Ago1 and Ago3 cannot support siRNA-directed RNAi in Ago2-knockout mouse embryonic fibroblasts (Liu et al., 2004). The function of Ago1, Ago3, and Ago4-containing RISC is not yet known. Perhaps the capacity for loading small RNAs via the cleavage-assisted pathway confers a degree of specificity to the function of different silencing complexes, with Ago2 able to use a broader range of small RNAs than those Argonaute proteins incapable of RNA cleavage.

Experimental Procedures

General Methods

Drosophila Schneider 2 (S2) cell, embryo, and ovary lysates and in vitro RNAi assays were as described (Haley et al., 2003; Tomari et al., 2004a; Förstemann et al., 2005). siRNAs were deprotected according to manufacturer's protocol (Dharmacon). RNAi reactions with HeLa S100 extract were incubated at 30°C (Schwarz et al., 2002) using 20 nM siRNA, 1 mM ATP, 0.2 mM GTP, 5 mM $MgCl_2$, 25 mM creatine phosphate, and 20 U/ml RNasin (Promega). RISC assembly was quenched with 10 mM (f.c.) *N*-ethylmaleimide (Pierce) for 10 min at 4°C, followed by 11 mM DTT for 10 min at 4°C before the addition of radiolabeled target RNA.

Passenger-Strand Cleavage

siRNA passenger strands were 5′-^{32}P-radiolabeled with T4 polynucleotide kinase, isolated from a denaturing gel, annealed to a slight excess of unlabeled, phosphorylated guide strand, then the duplex was isolated from a 15% native polyacrylamide gel. Ten nanomolar radiolabeled siRNA was incubated in a standard in vitro RNAi reaction; the reactions were quenched by adding 2× Proteinase K buffer (200 mM Tris-Cl [pH 7.5], 25 mM EDTA, 300 mM NaCl, 2% [w/v] SDS), 2 mg/ml Proteinase K, and 1 μg glycogen, incubated for 30 min at 65°C, extracted with an equal volume of phenol/chloroform (1:1), and the RNA precipitated with 3 volumes absolute ethanol. RNAs were resolved by electrophoresis through a 20% denaturing (19:1) polyacrylamide gel, the gel dried under vacuum, and detected with a FLA 5000 phosphorimager (Fuji). To quantify the rate of passenger-strand cleavage, a 2′-*O*-methyl oligonucleotide (200 nM or 1 μM) complementary to the passenger strand was added to the reaction to stabilize the cleavage product.

Native and Two-Dimensional RNA Analysis

Production of single-stranded guide siRNA strand (Figures 3, 5, and S3) was measured as previously described (Nykänen et al., 2001; Okamura et al., 2004; Tomari et al., 2004b). For all native gel analyses, reactions were stopped with 2× Proteinase K buffer containing 1.5 mM $Mg(OAc)_2$, 2 mg/ml Proteinase K, and 1 μg glycogen. After incubation for 30 min at 25°C, 3 volumes absolute ethanol were added, and the RNA allowed to precipitate for an additional 30 min at 25°C. The precipitates were collected by centrifugation, washed with 80% (v/v) ethanol, then dissolved in 2 mM Tris-Cl (pH 7.5), 3% (w/v) Ficoll-400, 0.04% (w/v) bromophenol blue, 100 mM KOAc, 30 mM HEPES, 2 mM $Mg(OAc)_2$, and resolved by electrophoresis through a 15% native polyacrylamide gel (19:1 acrylamide:bis; 89 mM Tris-Borate [pH 8.3], 2 mM EDTA, 2.5 mM $Mg(OAc)_2$). In Figure S2, Mg^{2+} was not included in gel or buffer. For the two-dimensional analysis in Figure 4B, the reactions were first resolved by native electrophoresis, the region of the gel corresponding to double-stranded siRNA was excised and the RNA eluted overnight in 2× Proteinase K buffer. The samples were then extracted with phenol:chloroform (1:1), precipitated with 3 volumes of ethanol, the precipitate collected by centrifugation, washed with 80% (v/v) ethanol, the pellets dissolved in 98% deionized formamide, 10 mM EDTA (pH 8.0), 0.025% (w/v) xylene cyanol, 0.025% (w/v) bromophenol blue, and then resolved on a 20% denaturing urea-polyacrylamide sequencing gel.

Supplemental Data

Supplemental Data include six figures and Supplemental Experimental Procedures and can be found with this article online at http://www.cell.com/cgi/content/full/123/4/607/DC1/.

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