Molecular Requirements for RNA-Induced Silencing Complex Assembly in the Drosophila RNA Interference Pathway*

John W. Pham and Erik J. Sontheimer

Department of Biochemistry, Molecular Biology and Cell Biology
Northwestern University, 2205 Tech Drive, Evanston, Illinois 60208-3500 U.S.A.

Correspondence: erik@northwestern.edu, Tel. (847) 467-6880, Fax (847) 491-2467

Complexes in the Drosophila RNA-induced silencing complex (RISC) assembly pathway can be resolved using native gel electrophoresis, revealing an initiator called R1, an intermediate called R2, and an effector called R3 (now referred to as holo-RISC). Here we show that R1 forms when the Dicer-2/R2D2 heterodimer binds short interfering RNA (siRNA) duplexes. The heterodimer, alone, can initiate RISC assembly, indicating that other factors are dispensable for initiation. During assembly, R2 requires Argonaute2 to convert into holo-RISC. This requirement is reminiscent of the RISC-loading complex (RLC), which also requires Ago2 for assembly into RISC. We have compared R2 to the RLC and show that the two complexes are similar in their sensitivities to ATP and to chemical modifications on siRNA duplexes, indicating that they are likely to be identical. We have examined the requirements for RISC formation and show that the siRNA 5’ termini are repeatedly monitored during RISC assembly, first by the Dcr-2/R2D2 heterodimer and again after R2 formation, before siRNA unwinding. The 2′ position of the 5′-terminal nucleotide also affects RISC assembly because an siRNA strand bearing a 2′-deoxyribose at this position can inhibit the cognate strand from entering holo-RISC; in contrast, the 2′-deoxyribose-modified strand has enhanced activity in the RNA interference (RNAi) pathway.

In many eukaryotic organisms, double-stranded RNAs (dsRNAs) elicit an innate gene-silencing phenomenon known as RNAi (1). The RNAi response occurs in two phases. In the first phase, the dsRNAs are cleaved by the RNase III enzyme, Dicer, into double-stranded fragments called siRNAs (2-4). In the second phase, these siRNAs are conveyed into a large ribonucleoprotein complex known as the RISC, where they act as guides, base-pairing to complementary mRNAs and triggering RISC-mediated mRNA cleavage (2,4-10) and destruction (11-13). Exactly how the dsRNAs make the transition, from the targets of the first phase to the triggers of the second, is the subject of recent and continuing investigation.

The siRNAs produced by Dicer may determine the outcome of this transition. Newly generated siRNAs have characteristic 5′-phosphorylated ends that are recognized by the RNAi machinery (8) and facilitate their entry into the RISC (14-16). To get there, the siRNA duplexes have to be separated into their constituent strands (8) and only one of the two strands is assembled into the RISC (10). When synthetic siRNAs are used to initiate RNAi, the siRNA strand whose 5′ end is less stably base-paired is more frequently incorporated into the RISC (17,18), while the cognate strand is more frequently degraded.

We previously showed that siRNAs are channeled into the RISC in an ordered pathway that links the early and late phases of RNAi (14). The pathway begins when a group of pre-associated factors binds the siRNA to form an initiation complex known as R1. R1 is an ATP-independent complex that cannot form on siRNAs that lack 5′-phosphorylated ends. It contains at least two protein factors: Dicer-2 (Dcr-2), the Drosophila enzyme responsible for generating siRNAs, and presumably R2D2, a Dcr-2-associated factor that is a key player in bridging
the initiation and effector phases of RNAi (19). The R1 complex converts into the intermediate complex R2 by the ATP-independent addition of unidentified factors. Eventually, the siRNA in R2 is unwound, leading to the formation of a large, ATP-dependent, ~80S complex known as holo-RISC (formerly R3). This complex can cleave targeted mRNAs, likely acting as the RNAi effector in Drosophila (14).

Zamore and coworkers have proposed a similar yet distinct assembly pathway that begins when siRNAs first bind to a complex known as B (15). Complex B contains neither Dcr-2 nor R2D2, but is proposed to be a direct precursor to the RLC (formerly complex A), which harbors both of these factors and reportedly requires ATP to form (15). Dcr-2 and R2D2 crosslink to opposite ends of the siRNA duplex in the RLC, and the orientation of this interaction is strongly influenced by the relative thermodynamic stabilities of the two siRNA ends (16). R2D2 prefers to bind the more stably base-paired end whereas Dcr-2 typically binds to the less stably base-paired of the two ends. Since the siRNA strand that enters RISC is the one whose 5’ end is less stably base-paired, R2D2 is thought to sense siRNA asymmetry, distinguishing the strand that enters the RISC from the one destined for degradation (16).

Although there are parallels between the two proposed RISC assembly models, there are also key differences. For example, it is unclear how complex B and the RLC fit into the R1→R2→holo-RISC assembly scheme. We aim to resolve the discrepancies in the two models and to develop a coherent, unified understanding of RISC assembly in Drosophila. To that end, we have defined in greater detail the molecular events of the assembly pathway, providing a model that includes multiple 5’-recognition events and reconciles the reported differences in the R2 and RLC complexes.

EXPERIMENTAL PROCEDURES

Synthetic siRNAs - Single-stranded siRNAs were obtained either from IDT or Dharmacon. The siRNAs were radiolabeled with γ-32P-ATP (MP Biomedicals) and T4 Polynucleotide kinase (NEB) according to the manufacturer’s instructions. The single-stranded siRNAs were annealed as previously described (14).

Chromatography - 200 µL standard RNAi reaction mixtures (20) containing either Drosophila embryo extract [prepared as described (20)] (15%) or rR2D2/Dcr-2 heterodimer (107 nM) and 32P-radiolabeled Pp-luc siRNA were incubated at 25° C for 45 minutes. The purified, baculovirus-expressed rR2D2/Dcr-2 heterodimer was generously provided by Y. Tomari and P. Zamore. The mixtures were loaded onto a Superdex-200 HR 10/30 gel filtration column (Amersham) and fractionated as previously described (14). 200 µL fractions were collected and analyzed by Cerenkov counting.

To partially purify R2D2/Dcr-2 fractions, 200 µL of embryo extract was loaded directly onto a Superdex-200 column and chromatographed as described above. The R1-forming fractions were collected (fractions 18-28), supplemented with carrier insulin (final concentration 0.25 mg/mL) (Sigma), and concentrated to ~180 µL in a Microcon YM-30 spin column (Millipore).

Native gel electrophoresis - RNAi reaction mixtures for native gel experiments were assembled as previously described (14), except reaction volumes were 5 µL rather than 10 µL, and the mixtures contained 15% embryo lysate. The mixtures were quenched with 1 µL of a 4 mg/mL heparin mix and analyzed on 4% polyacrylamide native gels (10.8 mm x 17 cm x 23 cm) that were run at 10W at 4° C in 1× TBE as previously described (14). Agarose native gels were prepared and run as previously reported (15) with modifications (21).

ATP depletion was performed by pre-incubating reaction mixtures with glucose (20mM) and hexokinase (Calbiochem, 0.5U) for 20 minutes at 25° C. The concentration of ATP after depletion was measured using an ATP bioluminescence assay kit (Sigma) and was confirmed in all ATP-depleted samples to be <100 nM.

RNA unwinding - Standard unwinding assays were performed as previously described (8). The zero time-point samples were treated with Proteinase K and stop buffer just prior to the addition of radiolabeled siRNA. The amount of double-stranded and single-stranded siRNA
detected at time = 0 was set to 100% and 0%, respectively.

RESULTS

Dcr-2 and R2D2 comprise R1 and can initiate RISC assembly - RISC assembly in Drosophila proceeds in a stepwise manner and is first detected when proteins bind siRNA within the R1 complex (14). To examine whether R1 contains any factors other than R2D2 and Dcr-2, we incubated radiolabeled siRNA with purified recombinant R2D2/Dcr-2 heterodimer (rR2D2/Dcr-2) and compared it to R1 complex formed in embryo lysates. When analyzed by gel filtration (Fig. 1A) and native gel electrophoresis (Fig. 1B), the recombinant R2D2/Dcr-2/siRNA complex was indistinguishable from R1. These results indicate that R1 is not likely to contain any additional factors, so we now refer to it as the R2D2/Dcr-2 Initiator (RDI) complex to distinguish it from the unbound Dcr-2/R2D2 heterodimer.

If R2D2 and Dcr-2 are the only protein components of the RDI, then the recombinant R2D2/Dcr-2 heterodimer should suffice for initiating RISC assembly. To test this, we performed a pulse-chase experiment with preformed RDI prepared from rR2D2/Dcr-2 (Fig. 1C). Radiolabeled siRNAs were incubated with rR2D2/Dcr-2 and allowed to form the RDI complex. An excess of unlabeled siRNA competitor was then added and the mixture was “chased” with wild-type embryo extract. The radiolabeled siRNA in the RDI complex was rapidly conveyed into R2 and later into holo-RISC, indicating that rR2D2/Dcr-2, alone, can initiate RISC assembly (Fig. 1C, lanes 2-7).

Argonaute2 affects RISC assembly at the R2 to holo-RISC transition - Argonaute2 (Ago2) is a core RISC constituent (5) that acts at several stages in the RNAi pathway, both early and late, to promote siRNA unwinding (21) and, ultimately, to cleave targeted mRNAs as the “slicer” endonuclease (22-25). Holo-RISC formation requires Ago2 because the assembly pathway stalls at the RLC in ago2 null lysate (21). To determine whether the RDI or R2 complexes are functionally similar to the RLC, we used the native polyacrylamide gel system to examine the complexes that form on radiolabeled siRNA incubated in an ago2 null lysate (ago2<sup>214</sup>) (21). In this lysate, RISC assembly arrested at R2 (Fig. 2, lane 1), but proceeded to holo-RISC both in wild-type lysates and those prepared from ago2 null embryos transformed with an AGO2 transgene (Fig. 2, lanes 2 and 3). These results indicate that R2 may be similar to the RLC (see below). Furthermore, since Ago2 is required for siRNA unwinding (21), these data indicate that R2 contains double-stranded siRNA, an indication that is consistent with our previous observation that R2 does not require ATP to form (14).

The 5’ ends of siRNA duplexes are monitored at multiple stages of RISC assembly - siRNAs that lack 5’- phospho groups on both strands are unable to enter the RISC assembly pathway (8,14,15), obscuring the potential 5’ recognition events that may occur at later stages of assembly and catalysis. To address these possible recognition events, we examined the native gel complexes that formed on siRNA that lacked a 5’ phosphate on one of the two strands. The radiolabeled, 5’-phosphorylated strand was annealed to an unlabeled, cognate strand bearing either a 5’-hydroxyl or 5’-phosphate terminus (Fig. 3A). When incubated in embryo lysate, the singly phosphorylated siRNA showed reduced levels of holo-RISC compared to the doubly phosphorylated control (Fig. 3B, lanes 1 & 2), even though there is a kinase in the lysate that phosphorylates most of the siRNA (8). Our observations suggest that a fraction of the siRNA rapidly enters the RISC assembly pathway, escaping the kinase activity. These siRNAs appear to be shielded (at least partially) from the kinase but are defective at some stage of RISC assembly, presumably because they remain singly phosphorylated. Similar effects were observed with three other siRNA sequences (Supplemental Fig. 1). As an initial test to identify the source of the defect, we incubated the singly and doubly phosphorylated siRNAs in ago2 null lysate and found that they formed comparable levels of the RDI and R2 complexes (Fig. 3B, lanes 3 & 4). Since singly phosphorylated siRNA can assemble into these early complexes, but not into holo-RISC, these data indicate that a second 5’-recognition event occurs after formation of both the RDI and R2 complexes.

We performed a more rigorous analysis to test our initial observations, examining the
formation of each complex in the assembly pathway. To assess how singly phosphorylated siRNA fare in RDI formation, we incubated siRNA with partially purified R2D2/Dcr-2 fractions depleted of ATP. These fractions are capable of forming the RDI, but cannot form R2. Furthermore, ATP depletion prevents phosphorylation of the siRNA 5' end. Under these conditions, the singly and doubly phosphorylated siRNAs formed comparable levels of RDI (Fig. 3C), indicating that singly phosphorylated siRNAs can initiate RISC assembly. Similar results were obtained when the siRNAs were incubated with R2D2/Dcr-2 instead of partially purified R2D2/Dcr-2 fractions (data not shown).

To see how well singly phosphorylated siRNAs assemble into R2, we incubated siRNA with ago2 null lysate depleted of ATP. When incubated in this lysate, the singly and doubly phosphorylated siRNAs formed similar levels of R2 (Fig. 3D), consistent with our initial observations (Fig. 3D).

We next examined the requirement for 5' phosphates in holo-RISC formation. To do this we prepared Pp-luc siRNA bearing a 5'-methoxy modification on the non-radiolabeled strand. This was necessary because, unlike both the RDI and R2 complexes, holo-RISC requires ATP to form (14,15). Therefore, we could not use 5'-hydroxyl siRNAs in extracts depleted of ATP for our analysis. The 5'-methoxy-modified RNA is commercially available only for 2'-deoxythymidine nucleotides (dT-OCH3). To control for the 2'-deoxyribose modification, we prepared an analogous siRNA bearing a 5'-phosphate group (dT-PO4). We also prepared an siRNA bearing a terminal 5'-phosphorylated ribouridine (rU-PO4) as an unmodified control. When incubated in embryo lysate and analyzed by native gel electrophoresis, the rU-PO4 siRNA formed holo-RISC as expected (Fig. 3E, lane 1). However, the 5'-phosphorylated, 2'-deoxythymidine control siRNA (dT-PO4) formed unexpectedly low levels of holo-RISC relative to the rU-PO4 siRNA control (Fig. 3E, lane 2). This defect was also observed with an siRNA containing a 5'-terminal 2'-deoxyuridine nucleotide (dU-PO4), indicating that the 2'-deoxyribose, not the thymine base, was responsible for the defect (data not shown). The singly phosphorylated dT-OCH3 siRNA formed little (if any) holo-RISC (Fig. 3E, lane 3), confirming our earlier observations (Fig. 3B). Similar patterns of holo-RISC formation were observed using different siRNA sequences (Supplemental Fig. 2), at levels that were consistent with the prevailing asymmetry rules (17,18). Interestingly, we observed elevated levels of both the RDI and R2 complexes with the singly phosphorylated dT-OCH3 siRNA (Fig. 3E, lane 3). Since these early complexes contain duplex siRNA, the singly phosphorylated siRNAs may be inhibited from unwinding.

The siRNA 5’ termini regulate siRNA unwinding - To test this, we assayed the unwinding of Pp-luc siRNAs that were chemically modified on the non-radiolabeled strand. When we examined the rate and extent of single-stranded siRNA production, significant levels of unwound siRNA accumulated for the rU-PO4 Pp-luc siRNA, but not for the analogous Pp-luc siRNAs that were modified on the non-radiolabeled strand (Fig. 3F), consistent with the levels of holo-RISC formed on these siRNAs (Fig. 3E). When we quantitated the data as the fraction of double-stranded siRNA that persisted with time, approximately half of the rU-PO4 Pp-luc siRNA remained after 2 hours (Fig. 3G). The discrepancy between the amount of single-stranded siRNA produced (~10%, Fig. 3F) and the amount of double-stranded siRNA lost over time (~45%, Fig. 3G) is likely due to degradation of the radiolabeled strand, which in this case is thermodynamically disfavored for entry into the RISC and, relative to the unlabeled strand, more frequently destroyed. In contrast, the level of duplex dT-OCH3 siRNA remained relatively unchanged (Fig. 3G). Strikingly, the level of duplex dT-PO4 siRNA dropped precipitously after just 30 minutes (Fig. 3G) even though we observed no accumulation of single-stranded RNA (Fig. 3F). Similar patterns of unwinding were observed for a different siRNA duplex (Supplemental Fig. 3). Together, these data indicate that siRNAs must be doubly phosphorylated to efficiently unwind. Furthermore, an siRNA strand bearing a 5'-terminal 2'-deoxyribose modification somehow inhibits the cognate strand from entering the RISC.

We wondered if the deoxyribose-modified strand, itself, could enter the RISC. To address this, we radiolabeled the 2'-deoxyribose-modified Pp-luc siRNA strand, annealed it to the unlabeled,
unmodified, cognate RNA and tracked its progress through the RISC assembly pathway. The modified strand (dT) assembled into holo-RISC at higher levels than the unmodified control (rU) (Fig. 4, lanes 3 & 4). One possible explanation for our observations is that the 5'-terminal 2'-deoxyribose modification destabilizes the RNA helix at the modified end. This would shift the siRNA asymmetry, resulting in enhanced selection of the modified strand and reduced selection of the unmodified strand, as we observe. We consider this scenario unlikely because thermal melting analysis revealed no significant differences in the melting temperatures and free energies of the rU-PO4 and dT-PO4 siRNAs, which differ only in the presence or absence of the 2'-hydroxyl group (Supplemental Fig. 4). This result indicates that the terminal 2'-deoxyribose modification does not significantly destabilize the RNA duplex.

To directly address how the modification affects RISC activity, we examined the cleavage of both sense and antisense Pp-luc target mRNAs guided by the Pp-luc siRNA (Fig. 5A). The control rU-PO4 siRNA was able to guide cleavage of both the sense and antisense Pp-luc mRNAs, as expected (Figs. 5B & 5C, lanes 4). In contrast, the singly phosphorylated dT-OCH3 siRNA was unable to guide significant cleavage of either the sense or the antisense Pp-luc mRNAs (Fig. 5B & 5C, lanes 6), consistent with its inability to unwind (Figs. 3B & 3C). When we assayed cleavage guided by the siRNA bearing a single 2'-deoxyribose modification on the antisense strand (dT-PO4), we observed distinct activity with respect to the two target mRNAs. The unmodified sense siRNA strand did not promote significant RISC-mediated cleavage (Fig. 5B, lane 5). In contrast, the 2'-deoxyribose-modified antisense strand generated robust cleavage of the sense mRNA (Fig. 5C, lane 5). When we performed the experiment as a time course, the dT-PO4-modified siRNA clearly cleaved more sense mRNA than the rU-PO4 control (Fig. 5D). These results confirm that a 5'-terminal 2'-deoxyribose modification enhances the RNAi activity of the modified strand while simultaneously suppressing the activity of cognate strand. Interestingly, mRNA cleavage guided by single-stranded siRNA is also enhanced by a 5'-terminal 2'-deoxyribose modification (Supplemental Fig. 5).

R2 is similar to the RLC - We have shown that the R2 complex does not require ATP to form (14) and, further, that it can assemble on singly phosphorylated siRNA (Fig. 3E). It is therefore unlike the RLC, which reportedly depends on ATP as well as both 5' phosphates (15). Although the two complexes differ in these regards, they share common features. They both directly precede holo-RISC in the assembly pathway and they both accumulate in the absence of Ago2 (Fig. 2) (16,21). To see if these complexes are truly distinct, we compared them directly. 5'-radiolabeled Pp-luc siRNA was incubated in embryo lysates that were either depleted of ATP or supplemented with ATP. These reaction mixtures were split and loaded onto either a polyacrylamide or an agarose native gel to detect R2 and the RLC, respectively. Surprisingly, in our hands the RLC formed in extracts depleted of ATP, albeit at lower levels than the ATP-supplemented control (Fig. 6A, lanes 3 & 4). ATP depletion was successful because the ATP-dependent RISC complex did not form in either gel system after depletion (Fig. 6A, lanes 1 & 3). Furthermore, the residual ATP concentration after depletion was <100 nM (data not shown), a level previously reported to block RLC formation (15).

To test the 5' phosphate requirements for RLC formation, we examined the complexes that formed on singly phosphorylated Pp-luc siRNA that were modified on one strand with a 5'-methoxy group. Unexpectedly, the singly phosphorylated siRNA assembled into the RLC but did not proceed to the RISC in the agarose native gel (Fig. 6B, lane 3). We observed similar results using the 3'-cordycepin-labeled duplexes previously employed (15) (data not shown). There was some signal in the agarose gel that co-migrated with RISC when we used the dT-PO4 siRNA (Fig. 6B, lane 2). Since the radiolabeled, unmodified strand of this siRNA can neither assemble into RISC (Fig. 3E, lane 2) nor cleave targeted mRNAs (Fig. 5B, lane 5), some of the signal in this region of the gel is not RISC or represents a nonfunctional form of RISC. Based on these results, we conclude that R2 and the RLC are similar, probably identical, complexes.
DISCUSSION

To act as silencing triggers, double-stranded siRNAs must be channeled through an ordered RISC assembly pathway that results in the selection of one strand and the destruction of the other. Our results indicate that siRNA ends are recognized at multiple steps in the pathway and that these recognition events determine whether the siRNAs will become incorporated into the RISC or blocked from further assembly.

The first recognition event occurs at initiation, when Dcr-2 and R2D2 bind the siRNA to form the RDI complex. RDI formation requires 5’-phosphorylated siRNA; however, the siRNA need not be phosphorylated on both strands. Nykänen et al. (8) previously showed that siRNAs bearing a single 5’-phosphate group are ineffective at guiding mRNA cleavage. This was true for siRNAs phosphorylated only on the guiding strand as well as those phosphorylated only on the non-guiding strand. To explain these observations, Tomari et al. invoke a model in which R2D2 acts as a sensor for siRNA asymmetry (16). They suggest that a 5’ phosphate on the thermodynamically disfavored “passenger” strand is required for stable R2D2 binding, facilitating siRNA incorporation into the RLC and the RISC. Since the non-guiding strand of the siRNA used by Nykänen et al. lacked this phosphate, they suggest that it was excluded from the RISC at the level of R2D2 binding. Consequently, they propose that R2D2 is a licensing factor, allowing only authentic siRNAs to enter the RNAi pathway (16). If the basic features of their model are correct, then our data indicate that R2D2 does not act alone in distinguishing authentic from inauthentic (non-phosphorylated) siRNA.

When only one of two siRNA strands is phosphorylated, R2D2 and Dcr-2 can still avidly engage the siRNA, probably with R2D2 located at the phosphorylated end as predicted (16). Despite this, singly phosphorylated siRNA duplexes are still ineffective silencing triggers. Our data indicate that a second 5’-recognition event occurs after R2D2 binding. RNA unwinding and activation can proceed, but only if the siRNA bears the second 5’ phosphate at the end not occupied by R2D2. Since Ago2 is required both for siRNA unwinding and target mRNA cleavage, it may be the factor that recognizes the second 5’ phosphate. Two groups have recently shown that an archaeabacterial PIWI protein has conserved residues that interact with the 5’-phosphate group of siRNA-like duplexes (26,27). Furthermore, when analogous residues were mutated in the human Ago2 PIWI domain, target mRNA cleavage was compromised (26). In light of our own observations, these results seem to implicate Ago2 in the second 5’ recognition event. Alternatively, Ago2 may not engage the 5’ phosphate until later in the assembly pathway. In this case, some other factor may interact with the second 5’ phosphate upstream of Ago2, causing the RISC assembly defect that we observe with singly phosphorylated siRNA duplexes.

Like the 5’-phosphate group, the 2’ hydroxyl of the 5’-terminal nucleotide can influence RISC assembly. A 5’-terminal 2’-deoxyribose modification on one siRNA strand inhibits the cognate strand from entering the RISC. Since the unmodified, cognate strand does not accumulate in an unwinding assay, it is probably degraded. In contrast, the modified strand has enhanced activity and is apparently favored for entry into the RISC. There are at least two (non-mutually exclusive) explanations for these observations. The 2’-deoxyribose may be a negative determinant for R2D2 binding. In support of this possibility, the dT-PO₄-modified Pp-luc-switch siRNA forms very little of the RDI and R2 complexes (Supplemental Fig. 2, lane 2), even though it has a relatively stable and phosphorylated terminus that R2D2 would be expected to bind. Decreased R2D2 binding would bias the “sensor” of siRNA asymmetry (16), leading to increased selection of the modified strand and increased degradation of the unmodified strand, as we observe. Alternatively, the 2’-deoxyribose-modified strand may have an increased affinity for some downstream RNAi factor, thereby enhancing its entry into the RISC. This may also explain why a single-stranded dT-PO₄-modified siRNA is better at guiding target mRNA cleavage than the analogous rU-PO₄ control (Supplemental Fig. 5). In either case, our observations are surprising because the RNAi apparatus generally tolerates limited modifications at the 2’ position of siRNA duplexes, both on the sense and antisense strands (28-30). However, completely substituting siRNAs with 2’-deoxy modifications on either the sense or antisense
strand abolishes their ability to guide target mRNA cleavage (31), perhaps by altering the helical geometry of the duplex. Since there does not seem to be a general requirement for 2'-hydroxyls along the length of the siRNA non-guiding strand, the defect we observe for 5'-terminal 2'-deoxyribose modifications could reflect a specific recognition event that we cannot yet fully define.

Since a 5'-terminal 2'-deoxyribose modification on one siRNA strand can inhibit the activity of the cognate strand, it should reduce the off-target effects that can complicate RNAi-mediated gene silencing. Thus, this simple and inexpensive modification should be considered in the design of effective siRNA reagents for targeted gene knock-down.

Finally, we have made progress in our goal of developing a more unified understanding of RISC assembly in Drosophila (Fig. 6C). We have shown that the R2D2/Dcr-2 heterodimer, alone, can initiate RISC assembly by binding siRNAs to form the RDI complex. Like the RDI, Complex B has been proposed to act at initiation (15). Since this complex can form in the absence of R2D2 (15), it is upstream of the RDI and may play an auxiliary role in RISC assembly. Complex B may transfer bound siRNA directly to the RDI in addition to (or instead of) the RLC. Continuing investigation will be required to resolve this issue.

Our results suggest that the RLC and R2 complexes have more in common than previously thought. Based on our side-by-side comparisons, both complexes can form in extracts that lack Ago2 and in extracts depleted of ATP. Furthermore, both complexes can assemble on siRNAs that lack a phosphate on one of the two siRNA strands. The ATP and 5'-phosphate requirements that we observe for the RLC are distinct from those reported previously (15). We do not know the reason for these discrepancies. Based on our observations, R2 and the RLC are probably identical complexes, and we now refer to R2 as the RLC.

REFERENCES

1. Fire, A., Xu, S., Montgomery, M. K., Costas, S. A., Driver, S. E., and Mello, C. C. (1998) Nature 391, 806-811
2. Zamore, P. D., Tuschl, T., Sharp, P. A., and Bartel, D. P. (2000) Cell 101, 25-33
3. Hamilton, A. J., and Baulcombe, D. C. (1999) Science 286, 950-952
4. Hammond, S. M., Bernstein, E., Beach, D., and Hannon, G. J. (2000) Nature 404, 293-296
5. Hammond, S. M., Boettcher, S., Caudy, A. A., Kobayashi, R., and Hannon, G. J. (2001) Science 293, 1146-1150
6. Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. (2001) Nature 411, 494-498
7. Elbashir, S. M., Lendeckel, W., and Tuschl, T. (2001) Genes Dev. 15, 188-200
8. Nykänen, A., Haley, B., and Zamore, P. D. (2001) Cell 107, 309-321
9. Hutvagner, G., and Zamore, P. D. (2002) Science 297, 2056-2060
10. Martinez, J., Patkaniowska, A., Urlaub, H., Luhrmann, R., and Tuschl, T. (2002) Cell 110, 563-574
11. Orban, T. I., and Izaurralde, E. (2005) RNA 11, 459-469
12. Souret, F. F., Kastenmayer, J. P., and Green, P. J. (2004) Mol. Cell 15, 173-183
13. Gazzani, S., Lawrenson, T., Woodward, C., Headon, D., and Sablowski, R. (2004) Science 306, 1046-1048
14. Pham, J. W., Pellino, J. L., Lee, Y. S., Carthew, R. W., and Sontheimer, E. J. (2004) Cell 117, 83-94
15. Tomari, Y., Du, T., Haley, B., Schwarz, D. S., Bennett, R., Cook, H. A., Koppetsch, B. S., Theurkauf, W. E., and Zamore, P. D. (2004) Cell 116, 831-841
16. Tomari, Y., Matranga, C., Haley, B., Martinez, N., and Zamore, P. D. (2004) Science 306, 1377-1380
17. Khvorova, A., Reynolds, A., and Jayasena, S. D. (2003) Cell 115, 209-216
18. Schwarz, D. S., Hutvagner, G., Du, T., Xu, Z., Aronin, N., and Zamore, P. D. (2003) Cell 115, 199-208
19. Liu, Q., Rand, T. A., Kalidas, S., Du, F., Kim, H.-E., Smith, D. P., and Wang, X. (2003) Science 301, 1921-1925
20. Tuschl, T., Zamore, P. D., Lehmann, R., Bartel, D. P., and Sharp, P. A. (1999) Genes Dev. 13, 3191-3197
21. Okamura, K., Ishizuka, A., Siomi, H., and Siomi, M. C. (2004) Genes Dev. 18, 1655-1666
22. Song, J. J., Smith, S. K., Hannon, G. J., and Joshua-Tor, L. (2004) Science 305, 1434-1437
23. Parker, J. S., Roe, S. M., and Barford, D. (2004) EMBO J. 23, 4727-4737
24. Rand, T. A., Ginalsly, K., Grishin, N. V., and Wang, X. (2004) Proc. Natl. Acad. Sci. USA 101, 14385-14389
25. Liu, J., Carmell, M. A., Rivas, F. V., Marsden, C. G., Thomson, J. M., Song, J. J., Hammond, S. M., Joshua-Tor, L., and Hannon, G. J. (2004) Science 305, 1437-1441
26. Ma, J. B., Yuan, Y. R., Meister, G., Pei, Y., Tuschi, T., and Patel, D. J. (2005) Nature 434, 666-670
27. Parker, J. S., Roe, S. M., and Barford, D. (2005) Nature 434, 663-666
28. Parrish, S., Fleenor, J., Xu, S., Mello, C., and Fire, A. (2000) Mol. Cell 6, 1077-1087
29. Chiu, Y. L., and Rana, T. M. (2003) RNA 9, 1034-1048
30. Amarzguioui, M., Holen, T., Babaie, E., and Prydz, H. (2003) Nucleic Acids Res. 31, 589-595
31. Elbashir, S. M., Martinez, J., Patkanowska, A., Lendeckel, W., and Tuschl, T. (2001) EMBO J. 20, 6877-6888

FOOTNOTES

*We thank Y. Tomari and P. Zamore for generously providing purified, recombinant R2D2/Dcr-2 heterodimer. We also thank J. Preall, J. Pellino, P. Bellare, Z. He, J. Gorra, A. Bonde, and R. Carthew for their advice, feedback, and comments on the manuscript. This work was supported by a Presidential Fellowship to JWP from Northwestern University and by grants to EJS from the March of Dimes Birth Defects Foundation (#1-FY03-96) and the NIH (GM072830).

The abbreviations used are: RISC, RNA-induced silencing complex; siRNA, short interfering RNA; RLC, RISC-loading complex; RDI, R2D2-Dcr2 initiator; RNAi, RNA interference; dsRNA, double-stranded RNA

FIGURE LEGENDS

**FIG. 1. R2D2 and Dcr-2 comprise R1 and can initiate RISC assembly.** A. $^{32}$P-radiolabeled $Pp$-luc siRNAs were incubated with embryo lysate (triangles) or purified, recombinant R2D2/Dcr-2 heterodimer (squares) and fractionated through a Superdex-200 gel filtration column. B. $^{32}$P-radiolabeled $Pp$-luc siRNAs were incubated as described in A and analyzed by native polyacrylamide gel electrophoresis. C. $^{32}$P-radiolabeled siRNAs were incubated with purified, recombinant R2D2/Dcr-2 heterodimer, then mixed with a 10,000-fold excess of unlabeled competitor siRNA. The reaction mixture was then split into aliquots and chased with Drosophila embryo lysate. The lysate was first added to the 60-minute sample and the experiment was performed as a reverse timecourse. Reactions were quenched with heparin on ice and analyzed by native gel electrophoresis. In two control experiments, the R2D2/Dcr-2 siRNP was chased with buffer instead of lysate (lane 1) and, in lane 8, the excess unlabeled competitor was added at the outset.
FIG. 2. The R2 complex accumulates in the absence of Ago2. $^{32}$P-radiolabeled $Pp$-luc siRNA was incubated with lysates from embryos as shown, quenched with heparin, and analyzed by native gel electrophoresis.

FIG. 3. Singly phosphorylated siRNAs can assemble into the RDI and R2 complexes, but can neither unwind nor enter holo-RISC. A, radiolabeled siRNA duplex used in panels B through D. The $^{32}$P-radiolabel is in red, the radiolabeled strand is black, and the unlabeled strand is purple. The unlabeled strand was prepared with either a 5’-terminal 5’ hydroxyl or 5’ phosphate, shown in parentheses. B, the siRNA from A was incubated with wild-type (WT) or ago2 null (ago2 414) lysates for 40 minutes, quenched with heparin, and analyzed by native gel. C, the radiolabeled siRNA shown in A bearing a 5’ phosphate (magenta) or 5’ hydroxyl (blue) on the non-radiolabeled strand was incubated with partially purified R2D2/Dcr-2 fractions depleted of ATP. At time points, aliquots were removed, quenched with heparin and analyzed by native gel. D, the timecourse experiment was performed as in C, but the siRNAs were incubated in ago2414 extract depleted of ATP instead of R2D2/Dcr-2 fractions. E, $Pp$-luc siRNA was modified on the non-radiolabeled strand as shown, incubated in embryo lysate, and analyzed by native gel electrophoresis. The radiolabel is shown as a red asterisk. The modified nucleotide is denoted with an X. The blue box indicates the more thermodynamically stable of the two siRNA ends. F, the $Pp$-luc siRNA shown in A was incubated in embryo lysate. At various time points aliquots were removed, deproteinized, and precipitated with ethanol. The recovered RNAs were separated on a 15% non-denaturing gel and quantified with a Phosphorimager. The results are plotted as the percentage of the total RNA that was single-stranded. G, as in F, but the results are plotted as the fraction of double-stranded siRNA remaining (relative to the amount of ds-siRNA at time=0).

FIG. 4. An siRNA strand modified with a 5’-terminal 2’-deoxyribose assembles into the RISC more efficiently than the cognate strand. Duplex $Pp$-luc siRNAs bearing 5’-terminal ribose (rU) or 2’-deoxyribose (dT) modifications on the antisense strand were prepared. The siRNAs were labeled either on the sense strand (black) or on the modified antisense strand (purple). The 5’-modified nucleotides are denoted with an X and the radiolabel is shown as a red asterisk. The radiolabeled siRNAs were incubated in embryo extract and the mixture was analyzed by native gel.

FIG. 5. The effect of siRNA 5’- terminal modifications on target mRNA cleavage. A, representation of the $Pp$-luc siRNA used in the experiments shown in panels B through D. The modified nucleotide is denoted X and the blue box indicates the more thermodynamically stable of the two siRNA ends. B, target cleavage was assayed using cap-labeled anti-sense $Pp$-luc mRNA and the siRNA shown in A. C, target cleavage was performed as in B but with radiolabeled sense $Pp$-luc mRNA target. D, as in lanes 4 & 5 of C but performed as a time course.

FIG. 6. The R2 complex is similar to the RLC. A, 5’-radiolabeled Pp-luc siRNA was incubated in embryo lysates that were either depleted of ATP or supplemented with ATP. After 40 minutes, the reactions were adjusted to 6% (v/v) glycerol and loaded on either polyacrylamide or agarose native gels. The samples loaded onto polyacrylamide were quenched with heparin before loading. B, chemically modified siRNAs were used in standard RNAi reactions that were supplemented with ATP as described in A and analyzed by agarose gel electrophoresis. C, integrated pathway for RISC assembly based on current data. Complex B is not directly required for siRNA binding in the RDI, but may channel siRNAs into the RDI, the RLC, or both.
Figure 1  Pham and Sontheimer
Figure 2
Pham and Sontheimer
Figure 3  Pham and Sontheimer
| 5′-p* | Xp -5′ | 5′-p | Xp* -5′ |
|-------|--------|-------|--------|
| rUdT  |        | rUdT  | = X    |

**Figure 4**  Pham and Sontheimer
Figure 6  Pham and Sontheimer
Supplemental Material

Molecular Requirements for RNA-Induced Silencing Complex Assembly in the *Drosophila* RNA Interference Pathway

John W. Pham and Erik J. Sontheimer

**SUPPLEMENTAL FIGURE LEGENDS**

**SUPPLEMENTAL FIG. 1.** Additional examples of singly phosphorylated siRNAs that are inhibited downstream of R2. A, RNA duplexes used in B. The radiolabeled strand is in black and the non-radiolabeled strand is in purple. The 5'-terminus of the non-radiolabeled strand is indicated in parenthesis. B, the siRNAs in A were incubated in embryo lysate and analyzed by native gel electrophoresis, as in Fig. 3B.

**SUPPLEMENTAL FIG. 2.** Additional examples of siRNAs that require both 5' phosphates for holo-RISC assembly. The experiment was performed as in Fig. 4A, except with different siRNA duplexes. Two additional duplexes were tested. “Pp-luc-switch” is the Pp-luc siRNA with a G:U wobble pair at the 5' end of the labeled strand. The G:U wobble is denoted with a broken line. This G:U wobble switches the siRNA asymmetry (1). “Sym-1U” is the siRNA shown in Figure 3A, modified to accommodate a ribouridine (rU) or a 2'-deoxythymidine (dT) modification at the 5' end of the non-radiolabeled strand (see below for the sequence). The radiolabeled strand is shown in black. The unlabeled strand is shown in purple. The 5'-terminal nucleotide on the non-radiolabeled strand is denoted X. Blue boxes indicate the more thermodynamically stable of the two siRNA ends. For the Sym-1U siRNA, the two blue boxes indicate that the siRNA is functionally symmetric. The sequence of the sense Pp-luc-switch siRNA was 5'-pUGU ACG CGG AAU ACU UCG AUU-3'. The sequence of the antisense Pp-luc-switch siRNA was identical to the standard antisense Pp-luc siRNA strand (5'-UCG AAG UAU UCC GCG UAC GUG). The sequence of the SYM-1U siRNA was: (top) 5'-pAUA ACA UAU GAC UUU CCU ACU-3' and (bottom) 5'-pUAG GAA AGU CCAU AUG UUA UGG-3'. The RDI and R2 complexes did not accumulate on the Pp-luc-switch siRNA that was modified on one strand with a 5'-terminal methoxy modification (lane 3), even though they accumulated on other similarly modified siRNAs (lane 6 and Fig. 4A, lane 3). This may be due to poor R2D2 binding to the dT-OCH3-modified Pp-luc-switch siRNA, which lacks the stable, 5'-phosphorylated termini that R2D2 prefers to bind.

**SUPPLEMENTAL FIG. 3.** Unwinding kinetics of a Pp-luc siRNA with inverted symmetry. A, graphical representation of the Pp-luc-switch siRNA used in the experiment shown in panels B and C. The siRNA is the same as the one used in Figs. 3F and 3G, except that it contains a G:U wobble pair that switches the siRNA asymmetry. The G:U wobble is denoted by a broken line. The radiolabeled strand is shown in black and the unlabeled strand is shown in purple. The 5'-terminal nucleotide on the unlabeled strand is denoted X. The blue box indicates the more thermodynamically stable of the two siRNA ends. B, same as Fig. 3F but with the siRNA shown in A. The extent of unwinding paralleled the levels of holo-RISC formation observed with the same siRNA duplexes (Supplemental Fig. 2, lanes 1-3). C, as in Fig. 3G, but with the siRNA shown in A.

**SUPPLEMENTAL FIG. 4.** A 5'-terminal 2'-deoxyribose modification does not destabilize an siRNA duplex. A, graphical representation of the Pp-luc-switch siRNA used for thermal melting analysis. B, table of thermodynamic parameters extracted from equilibrium melting curves. ΔG° was calculated at 70°C because the values for ΔG° are most accurate near the Tm of the duplex (2). The standard errors for ΔG°, ΔH°, and ΔS° are 5%, 7%, and 9%, respectively (3, 4). For the experiments, siRNAs were...
suspended in buffer (1.5 µM in 100 mM NaCl, 10 mM PIPES [pH 7.0], 0.5 mM EDTA) and annealed by heat treating (95°C, 2 minutes) and slow cooling to room temperature. Melting data were collected by measuring A_{260} while heating the samples at a rate of 0.75°C/min. The melting experiments were performed in triplicate in a double-beam spectrophotometer (Cary 500). Tₘ values were calculated from a plot of α versus T (the fraction of duplex at temperature, T). Values for ΔH° and ΔS° were calculated as previously described (5). These values were used to determine ΔG°ₗ₀ (6).

SUPPLEMENTAL FIG. 5. Target mRNA cleavage guided by single-stranded siRNA is enhanced by a 5’-terminal 2’-deoxyribose modification. Target cleavage was assayed with single-stranded antisense Pp-luc siRNA and radiolabeled, sense Pp-luc mRNA. The antisense siRNA is shown at the top and the modified nucleotide is denoted with an X. The inability of the 5'-methoxy-modified single-stranded siRNA to direct cleavage has been noted previously (7).

SUPPLEMENTAL FIGURE REFERENCES

1. Schwarz, D. S., Hutvagner, G., Du, T., Xu, Z., Aronin, N. & Zamore, P. D. (2003) Cell 115, 199-208
2. SantaLucia, J., Jr. & Turner, D. H. (1997) Biopolymers 44, 309-319
3. Allawi, H. T. & SantaLucia, J., Jr. (1997) Biochemistry 36, 10581-10594
4. SantaLucia, J., Jr., Kierzek, R. & Turner, D. H. (1992) Science 256, 217-219
5. Marky, L. A. & Breslauer, K. J. (1987) Biopolymers 26, 1601-1620
6. Plum, G. E., Breslauer, K. J. & Roberts, R. W. (1999) Thermodynamics and kinetics of nucleic acid association/dissociation and folding processes. (Elsevier Science Ltd., Oxford)
7. Schwarz, D., Hutvagner, G., Haley, G. & Zamore, P. D. (2002) Mol. Cell 10, 537-548
a

5′-Radiolabeled Strand-3′
3′-Unlabeled Strand-5′

1) 5′-pUCGAAGUAAUCCCGCUACGUG-3′
    3′-UUAGCUUCAUAAGCGCAUGC-(OH/p)-5′

2) 5′-pUGAGCUUGGGCAUGUGACUU-3′
    3′-UUAUCUGAACCCGUUACACUG-(OH/p)-5′

3) 5′-pAAUUAGAGCCAGCAACCGU-3′
    3′-UGUUAUUCUCGAGUUGCGUUGG-(OH/p)-5′

b

| siRNA | 1       | 2       | 3       |
|-------|---------|---------|---------|
| Unlabeled strand 5′-end | OH PO₄ | OH PO₄ | OH PO₄ |

Pham & Sontheimer
Supplemental Figure 1
|                  | Pp-luc-switch | Sym-1U |
|------------------|--------------|--------|
| \(5'-\text{p}^*\) | \(X-5'\)     | \(X-5'\) |

| \(X = \)       | rU-PO\(^4\) | dT-PO\(^4\) | dT-OCH\(_3\) | rU-PO\(^4\) | dT-PO\(^4\) | dT-OCH\(_3\) |
|------------------|--------------|--------------|--------------|--------------|--------------|--------------|

**Pham & Sontheimer**

**Supplemental Figure 2**
### Table: Thermodynamic Parameters for X-5' Phosphates

|       | $\Delta H^\circ$ (kcal/mol)* | $\Delta S^\circ$ (cal/mol*K)* | $\Delta G^\circ_{70}$ (kcal/mol)* | $T_m$ (°C) |
|-------|-------------------------------|-------------------------------|----------------------------------|-------------|
| $X = \text{rU-PO}_4$ | -98.2                         | -255.6                        | -10.4                            | 70.9        |
| $X = \text{dT-PO}_4$ | -147.1                        | -398.1                        | -10.5                            | 71.1        |

---

Pham & Sontheimer

Supplemental Figure 4
| Input | No Lysate | No siRNA |
|-------|-----------|----------|
|       | rU-PO₄    | dT-PO₄   | dT-OCH₃ |
|       | X         |          |         |

Pham & Sontheimer
Supplemental Figure 5
