A Dicer-2-Dependent 80S Complex Cleaves Targeted mRNAs during RNAi in Drosophila

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Summary

We use native gel electrophoresis to characterize complexes that mediate RNA interference (RNAi) in Drosophila. Our data reveal three distinct complexes (R1, R2, and R3) that assemble on short interfering RNAs (siRNAs) in vitro. To form, all three complexes require Dicer-2 (Dcr-2), which directly contacts siRNAs in the ATP-independent R1 complex. R1 serves as a precursor to both the R2 and R3 complexes. R3 is a large (80S), ATP-enhanced complex that contains unwound siRNAs, cofractionates with known RNAi factors, and binds and cleaves targeted mRNAs in a cognate-siRNA-dependent manner. Our results establish an ordered biochemical pathway for RISC assembly and indicate that siRNAs must first interact with Dcr-2 to reach the R3 “holo-RISC” complex. Dcr-2 does not similarly transfer siRNAs to a distinct effector complex, but rather assembles into RISC along with the siRNAs, indicating that its role extends beyond the initiation phase of RNAi.

Introduction

In response to invasive double-stranded RNAs (dsRNAs), like those produced by viruses and transposons, the cells of many organisms mount a defense now known as RNA interference (RNAi) (for review, see Denli and Hannon, 2003). In the first phase of this defense, the enzyme Dicer cleaves the dsRNAs into short interfering RNAs (siRNAs), roughly 21 to 25 nucleotides in length (Hamilton and Baulcombe, 1999; Zamore et al., 2000; Bernstein et al., 2001). To act, the nascent siRNAs must be incorporated into the RNA-induced silencing complex (RISC) (Hammond et al., 2000) where they are unwound and activated in an ATP-dependent manner (Nykanen et al., 2001). Only then, as single-stranded species, are they competent to bind complementary mRNAs for RISC-mediated destruction (Martinez et al., 2002).

Key aspects of RNAi have been elucidated and important proteins continue to emerge. In Drosophila, proteins like VIG, dFXR, TSN, and Ago2 have been identified as RISC-associated components (Hammond et al., 2001; Caudy et al., 2002, 2003; Ishizuka et al., 2002), although the precise roles they play remain unclear. Additionally, Dcr-2 (one of two Dicer isoforms in Drosophila) has been implicated in dsRNA processing, but is not known to act later in the RNAi pathway. Its associated factor, R2D2, apparently acts as an intermediary, linking the initiation and effector phases of RNAi (Liu et al., 2003). Despite our expanding knowledge of RNAi, the RISC assembly pathway and the full composition of the silencing complex remain unknown.

In their attempts to resolve these outstanding questions, several groups have identified active RISCs of varying sizes. Hammond et al. (2001) isolated a ~500 kDa complex from Drosophila S2 cell extracts and, using extracts prepared from Drosophila embryos, Nykaän (2001) identified a smaller ~232 kDa silencing complex. Interestingly, a still smaller (~160 kDa) silencing complex was isolated from HeLa cell extracts (Martinez et al., 2002). All three complexes can cleave targeted mRNAs, but it is unclear how they are related to one another. Furthermore, we do not know whether these complexes represent the full array of RISC-associated factors or if they represent core species that retain RNAi activity. To complicate matters further, the ~550 kDa microRNA (miRNA) complex known as the miRNP (Mourelatos et al., 2002) shows striking similarities to RISC. When endogenous miRNAs are presented with a perfectly complementary target, they can act as cleavage determinants for miRNP-mediated target destruction, like their siRNA cousins in RISC (Hutvagner and Zamore, 2002). Conversely, when siRNA-programmed RISCs are presented with transcripts containing imperfect binding sites, they can act as translational repressors, like their miRNA cousins in the miRNP (Doench et al., 2003; Zeng et al., 2003). Clearly there is significant overlap between the two silencing pathways, and the corresponding complexes are apparently flexible in their function. But what, if anything, distinguishes one complex from the other remains a mystery.

In this study, we use native gel electrophoresis to elucidate the pathway that assembles RISC and related siRNA-associated ribonucleoproteins (siRNPs). We identify a Dcr-2/siRNA initiator complex that cannot cleave targeted mRNAs. The initiator complex serves as a direct precursor to a “holo-RISC” that is much larger than those previously described and has all of the characteristics of an active RNAi effector. It harbors known RISC components and, surprisingly, also contains two components of the dsRNA processing machinery, Dcr-2 and R2D2.

Results

Identification of Silencing Complexes

To examine RISC and related silencing complexes, we used native gel electrophoresis to assay complex formation. When we incubated radionabeled siRNAs in Drosophila embryo extract and analyzed the mixture by native gel electrophoresis, three distinct complexes appeared that we called R1, R2, and R3 (Figure 1). Additionally, some of the labeled siRNAs appeared at the top of the lane, as commonly observed when large nucleoprotein complexes are analyzed by native gel (e.g., Konarska and Sharp, 1986; Shaw et al., 1989; Nadano et al., 2001); we
bryo extracts, Nykanen et al. (2001) observed two siRNA binding complexes. The smaller of these two complexes (<232 kDa) coeluted with mRNA cleavage activity whereas the larger one (~360 kDa) did not. They also detected significant mRNA cleavage activity along with unwound siRNAs in the void fractions, presumably from complexes too large to enter the gel matrix. To examine how the native gel complexes compare with the previously identified siRNA binding complexes, we performed a similar fractionation. Extracts treated with radiolabeled Pp-luc siRNAs were chromatographed on a Superdex-200 column. Fractions were collected and assayed both for their ability to form complexes on a native gel and to cleave targeted Pp-luc mRNAs. Of the three complexes, only R1 eluted as a discrete peak, migrating between the 440 kDa and 232 kDa size markers (Figure 2A, fractions 18–30). We did not detect the R2 complex. However, we did observe R3, which eluted in the void (Figure 2A, fractions 4 and 6). When we assayed the fractions for mRNA cleavage activity, most of it was also in the void (Figure 2B). We observed neither discrete complexes nor significant cleavage activity in the ~232 kDa fractions as previously observed, perhaps due to the milder conditions (lower flow rate and temperature) used during our fractionation. Based on these results, we conclude that R1 corresponds to the ~360 kDa complex identified by Nykanen et al. (2001).

To determine whether any of the native gel complexes exist as pre-formed apo-complexes in the absence of siRNAs, we fractionated a naive extract (i.e., one which had not been preincubated with exogenous siRNA duplexes) using the same gel filtration procedure (Figure 2C). The fractions were monitored for complex formation by incubation with radiolabeled siRNA duplexes, followed by native gel analysis. Only R1 formed, indicating that its siRNA binding activity remains intact in the absence of siRNA duplexes (Figure 2C, fractions 18–32). In contrast, we did not observe the R3 complex, indicating that R3 is formed by factors that are separated or lost during chromatography.

R1 Is an RNAi Initiator Complex that Contains Dcr-2

Since the factors required to form R1 remained intact after gel filtration, we combined the R1-forming gel filtration fractions (Figure 2C, fractions 18–32) and further purified them by MonoQ ion exchange chromatography. The MonoQ fractions were incubated with radiolabeled siRNAs to monitor R1 formation, yielding a discrete R1 peak (Figure 3A). Since R1 formation requires Dcr-2, we reasoned that it may contain Dcr-2 and perform functions attributed to Dcr-2, such as dsRNA processing. Indeed, when the MonoQ fractions were treated with labeled dsRNAs, only those that could form R1 (Figure 3A) were active for dsRNA processing (Figure 3B).

Extracts that lack Dcr-2 are deficient in target mRNA cleavage, even when pre-cleaved siRNAs are used as the silencing trigger (Lee et al., 2004). To determine whether the R1 complex might be required for the effector phase of RNAi, we tested whether the R1-forming MonoQ fractions (Figure 3A) could rescue the mRNA cleavage defect in a dcr-2 mutant extract. Neither the MonoQ fractions nor the dcr-2 extract, alone, could direct mRNA cleavage (Figure 3C, lanes 5–12 and lane

**Figure 1. Drosophila siRNP Complexes Can Be Resolved by Native Gel Electrophoresis**

Radiolabeled siRNAs were incubated with lysate from wild-type Drosophila embryos (lanes 3–6) or dcr-2 null mutant embryos (lanes 1–2), treated with heparin, and analyzed by native gel electrophoresis. The siRNA 5' termini are given at the top of each lane. The samples shown in lanes 1, 2, 5, and 6 contained ATP and an ATP-regenerating system, whereas the samples shown in lanes 3 and 4 were depleted of ATP with hexokinase and glucose. The origin of electrophoresis is shown on the left; siRNAs present at the top of the lane are denoted by an asterisk on the right. R1, R2, and R3 siRNP complexes and unbound siRNA are indicated on the right.
Figure 2. Target mRNA Cleavage Activity Correlates with R3 but Not R1

(A) Wild-type Drosophila embryo lysate was incubated with radiolabeled Pp-luc siRNA and then fractionated in a Superdex-200 gel filtration column. Even-numbered fractions were analyzed by native gel electrophoresis. “Lysate” denotes the unfraccionated mixture. The elution profile of molecular weight standards is given at the bottom (V0, void volume; 669 kDa, thyroglobulin; 440 kDa, ferritin; 232 kDa, catalase). Complexes and unbound siRNA are indicated on the right.

(B) Aliquots of the fractions used in (A) were tested for Pp-luc mRNA cleavage activity; RNAs from each reaction were then separated in a 6% denaturing polyacrylamide gel. Control samples include unreacted Pp-luc mRNA target (“Input”), reaction with lysis buffer in place of lysate (“No lysate”), reaction with unfraccionated lysate in the absence of siRNA (“No siRNA”), and reaction with unfraccionated lysate in the presence of Pp-luc siRNA (“Complete”). The mobilities of 5' end-labeled uncleaved Pp-luc mRNA target and cleaved product are shown on the right.

(C) As in (A), except that chromatography was performed on extract in the absence of siRNA. Radiolabeled Pp-luc siRNA was then added to each fraction and complexes were visualized by native gel electrophoresis.

13), even when triggered by a synthetic 21 nt siRNA duplex that does not require Dcr-2-mediated dsRNA processing. However, when the MonoQ fractions and dcr-2 extract were combined, mRNA cleavage activity was reconstituted (Figure 3C, lanes 16 and 17). The data suggest that later events in the RNAi pathway, including mRNA cleavage, are at least indirectly dependent upon the R1 complex.

We next performed a timecourse experiment to see how quickly the complexes form relative to one another (Figure 3D). R1 forms rapidly, reaching a maximum after about five minutes. R2 forms almost as quickly, but R3 lags significantly behind. This experiment, combined with our previous data, suggests that the complexes might be linked within a common pathway. In parallel timecourses, R3 complex assembly precedes target mRNA cleavage, as expected of an active effector complex (Supplemental Figure S1 at http://www.cell.com/cgi/content/full/117/1/83/DC1).

To address the potential precursor-product relationship between R1 and the other complexes, we performed a pulse-chase experiment that was monitored by native gel electrophoresis (Figure 3E). To do this, we formed R1 by incubating trace amounts of radiolabeled siRNA duplexes with an R1-forming MonoQ fraction (Figure 3A, fraction 8). We then added excess, unlabeled siRNA duplexes followed by whole, wild-type extract. Aliquots were removed at specific time points and loaded onto a running, native gel. As shown in Figure 3E, R1 quickly gives rise to R2 (lane 1), and later to R3 (lane 7). Between 30 and 60 min, R2 disappears and R3 arises (Figure 3E, lanes 6 and 7), suggesting that R2 may convert to R3 during this interval; however, these results do not exclude the possibility that R3 might form directly from R1, with R2 representing an unstable off-pathway complex. Both R2 and R3 were derived from R1 because when preformed R1 was simultaneously treated with both labeled and unlabeled siRNAs and then incubated with whole extract, no complexes formed (Figure 3E, lane 9), demonstrating that the amount of unlabeled siRNA added was sufficient to block de novo complex formation on labeled siRNA duplexes. As a negative control, we performed the chase with buffer rather than extract and observed no higher order complex formation (Figure 3E, lane 8), demonstrating that R1 has no intrinsic ability to convert to R2 or R3. The delayed kinetics of R3 formation in this experiment (relative to the standard timecourse in Figure 3D) is possibly due to the presence...
Figure 3. Partially Purified R1 Complex Is a Precursor in a RISC Assembly Pathway

(A) Fractions 18–32 from the gel filtration column used in Figure 2C were pooled and further purified by ion exchange chromatography in a MonoQ column. Aliquots from desalted fractions 3–12 were assayed for their ability to form R1 complex on radiolabeled Pp-luc siRNA. Lane 1 shows complex formation with unfractionated lysate. Complexes and unbound siRNA are indicated on the right.

(B) MonoQ fractions were incubated with radiolabeled Pp-luc dsRNA in the presence of ATP. RNAs from each reaction were then separated in a denaturing 15% polyacrylamide gel. Control samples include a 22 nt radiolabeled RNA marker ("Marker"), unreacted dsRNA ("Input"), reaction with lysis buffer in place of lysate ("No lysate"), and reaction with unfractionated lysate ("Crude lysate"). The mobilities of unreacted Pp-luc dsRNA and product siRNA are given on the right.

(C) MonoQ fractions 3–10 were incubated with unlabeled Pp-luc siRNA and radiolabeled Pp-luc mRNA target, with (lanes 14–21) or without (lanes 5–12) lysate from dcr-2 mutant embryos. RNAs from each reaction were then separated in a denaturing 6% polyacrylamide gel. Control
Figure 4. Dcr-2 Interacts with siRNAs in the R1 Complex

(A) Radiolabeled, 5'-phosphorylated Pp-luc siRNA duplex was incubated with wild-type extract (lane 1) or dcr-2 mutant extract (lane 2) and irradiated with 254 nm light. Samples were denatured in SDS sample buffer and loaded onto a 6% polyacrylamide SDS gel, and crosslinked proteins were detected by autoradiography. For immunoprecipitation experiments ("IP," lanes 4–7), extracts were treated as in lane 1, but were additionally incubated with protein A sepharose beads conjugated to antibodies or to preimmune serum (as indicated at the top of each lane). The "Nonspecific" antibody was directed against protein kinase C. "Total" sample was as in lane 1. The mobilities of Dcr-1 and Dcr-2 crosslinked bands are indicated on the right.

(B) Two siRNP assembly/UV crosslinking reactions as in lane 5 of (D) were loaded onto two separate lanes of a 4% polyacrylamide native gel. One lane was dried and subjected to autoradiography (top); the other was embedded within the stacking portion of an 8% polyacrylamide SDS gel and subjected to 2nd-dimension electrophoresis (bottom). For the native gel, complexes and unbound siRNAs are indicated at the top. For the denaturing gel, the mobilities of the Dcr-1 and Dcr-2 crosslinked bands are given on the left.

(C) Crosslinking reactions were performed with siRNAs bearing 5'-phosphate or 5'-hydroxyl termini, in the presence or absence of heparin and ATP (as indicated at the top of each lane). Products were analyzed by electrophoresis in an 8% polyacrylamide-SDS gel.

(D) Radiolabeled Pp-luc siRNAs were incubated in wild-type extract, treated with heparin, and loaded onto a native gel. The siRNA 5'-termini are given at the top of each lane; reactions in lanes 2, 4, 6, and 8 were ATP depleted. In lanes 5–8, incubated samples were placed on ice and irradiated with 254 nm light before heparin addition; control samples in lanes 1–4 were held on ice without UV irradiation before heparin addition. Complexes and unbound siRNA are indicated on the right.

of a large excess of unlabeled competitor siRNAs. Taken together, these results indicate that the R1 complex is a precursor to the R2 and R3 complexes in an ordered assembly pathway.

Dcr-2 Interacts with siRNAs in the Native Gel Complexes

To see if Dcr-2 is closely associated with siRNAs within the R1 complex, we performed UV crosslinking experiments. We first characterized protein/siRNA crosslinks by incubating radiolabeled siRNA duplexes in both wild-type and dcr-2 mutant lysates. The reactions were then irradiated with UV light and analyzed by SDS-PAGE. In

the wild-type lysate, a number of proteins crosslinked to the siRNAs, including a ~200 kDa protein roughly the size of Dcr-2 and a ~250 kDa protein the size of Dcr-1 (Figure 4A, lane 1). In the dcr-2 lysate, the ~200 kDa protein was undetectable, suggesting that it is, in fact, Dcr-2 (Figure 4A, lane 2). In support of this result, antibodies directed against R2D2, a known Dcr-2-associated protein (Liu et al., 2003), specifically coimmunoprecipitated the ~200 kDa band (Figure 4A, lane 6). To confirm the identity of the presumptive Dcr-1 band, we performed an immunoprecipitation experiment with antibodies directed against Dcr-1 (Bernstein et al., 2001). These antibodies specifically immunoprecipitated the


~250 kDa band from UV-treated extracts, indicating that siRNAs can crosslink to Dcr-1 (Figure 4A, lane 4); other bands present in the Dcr-1 immunoprecipitates also appeared in the negative control samples, indicating that they were nonspecific.

We next examined whether Dcr-2 and siRNAs closely interact within the R1 complex by performing a two-dimensional gel electrophoresis experiment on UV-irradiated extracts. To do this, wild-type embryo extracts were incubated with radiolabeled siRNAs, subjected to UV irradiation, treated with heparin, and analyzed by native gel electrophoresis. The entire native gel lane was then excised, layered horizontally on an SDS-polyacrylamide gel, and subjected to electrophoresis in the second dimension. When visualized in the second dimension, R1 resolves into several bands, one of which is Dcr-2 (Figure 4B). Interestingly, the Dcr-2 crosslink trails into the R2 and R3 regions of the gel. Based on these data, we conclude that R1 harbors Dcr-2 in intimate association with siRNAs. This association persists in higher order siRNPs, on a pathway leading from the R1 complex to R3.

In addition to Dcr-2, we observed a crosslinked species in R1 and higher order siRNPs corresponding to Dcr-1 (Figure 4B). Since R1 formation absolutely requires Dcr-2 (Figure 1), this result suggests that Dcr-1 may require Dcr-2 to bind siRNAs. Alternatively, Dcr-1 may associate with siRNAs independently of Dcr-2 to form an R1-like complex that is normally unstable during native gel analysis. We favor this latter possibility for two reasons. First, Dcr-1 crosslinks to siRNAs in the absence of Dcr-2 (Figure 4A). Second, Dcr-1 does not efficiently crosslink to siRNAs in the presence of heparin (Figure 4C, lane 1), which is routinely added to binding reactions before native gel electrophoresis. The decreased Dcr-1 crosslinking in the presence of heparin indicates that it likely represents a weak interaction. The Dcr-2 crosslink, on the other hand, survives the heparin challenge, suggesting that the Dcr-2/siRNA association is more stable (Figure 4C, lane 2).

Dcr-2 Transiently Associates with siRNAs Bearing 5'-Hydroxyl Groups

While examining the presence of Dcr-2 in R1, we also tested the biochemical requirements for Dcr-2/siRNA crosslinking. UV crosslinking experiments were performed either with extracts containing ATP or those depleted of ATP, using siRNA substrates bearing 5'-hydroxyl or 5'-phosphate groups. Crosslinking reactions were then analyzed both by SDS-PAGE (Figure 4C, lanes 3–6) and native gel electrophoresis (Figure 4D). The Dcr-2/siRNA crosslink did not require ATP and surprisingly formed regardless of the 5'-phosphorylation status of the siRNAs (Figure 4C), although ATP did slightly enhance the amount of crosslinked product observed on the 5'-hydroxyl siRNA (Figure 4C). In contrast, the Dcr-1/siRNA crosslink was significantly enhanced by ATP on both 5'-hydroxyl and 5'-phosphate siRNAs (Figure 4C). When the same crosslinking reactions were analyzed by native gel, we observed complexes assembled on 5'-hydroxyl siRNA duplexes in the absence of ATP, but only when the samples had been UV irradiated (Figure 4D, compare lanes 4 and 8). These results indicate that Dcr-2 can interact transiently with 5'-hydroxyl siRNAs within R1. This interaction can be trapped by UV crosslinking but is otherwise unsustainable in the absence of a 5'-phosphate. Since both functional (5'-phosphate) and nonfunctional (5'-hydroxyl) siRNAs are able to interact with Dcr-2 within the R1 initiator complex, the rejection of 5'-hydroxyl siRNAs occurs at some point in the RNAi pathway after initial binding to Dcr-2.

R3 Is an RNAi Effector Complex

As noted previously, both mRNA cleavage activity and the R3 complex fractionate in the void by Superdex-200 chromatography (Figures 2A and 2B), hinting at a possible role for R3 in target cleavage. However, we were unable to correlate the R3 complex with mRNA cleavage activity because both elute in the void volume and not as distinct peaks. To isolate the R3 complex and to determine whether it can cleave targeted mRNAs, we incubated radiolabeled Pp-luc siRNA duplexes with extract and fractionated the mixture through a Superdex-200 column. The void volume was collected and centrifuged through a 20%–50% sucrose gradient to separate high molecular weight complexes. Fractions were then tested both for the presence of the R3 complex and for their ability to cleave targeted Pp-luc mRNAs. Native gel analysis revealed that the R3 complex sedimented as a distinct 80S peak (Figure 5A, fractions 24–30). In addition, some of the siRNAs in these fractions were present in a band that slightly entered the native gel (see below). Strikingly, mRNA cleavage activity co-sedimented precisely with R3 (Figure 5B). The 80S peak fractions harbor predominantly single-stranded siRNAs, indicating that siRNPs in these fractions have been activated for target mRNA recognition and cleavage (Figure 5C). R3’s sedimentation profile naturally raises the possibility that it is a ribosome bound complex. To address this, we resolved complexes that were formed from total extract on a native gel and probed a blot of the gel for rRNAs (Supplemental Figure S2 on the Cell website). rRNAs from both small and large ribosome subunits comigrated with R3 complexes in the native gel, consistent with the notion that R3 physically associates with ribosomes.

To determine whether these activated complexes co-sediment with known RISC-associated factors, we analyzed the sucrose gradient fractions by Western blot with antibodies directed against the proteins VIG, TSN, Ago2, and dFXR (Hammond et al., 2001; Caudy et al., 2002, 2003; Ishizuka et al., 2002) (Figure 5D). All four RISC-associated factors were present in the R3 peak fractions (Figure 5D), consistent with a role for R3 as an RNAi effector complex. Strikingly, Dcr-2 and R2D2, components of the dsRNA processing machinery (Liu et al., 2003), also co-sedimented in the same 80S fractions (Figure 5D, left panel). Dcr-1 was present as well. These data correlate well with our UV crosslinking results showing that Dcr-1 and Dcr-2 are intimately associated with siRNAs in the R2 and R3 regions of a native gel (Figure 4B). The immunoblot profiles were not identical for all proteins; for example, dFXR trails into the faster-sedimenting fractions, consistent with its ability to associate with polysomes (Jin and Warren, 2003). Some het-
Figure 5. R3 Is an 80S Silencing Complex that Cofractionates with mRNA Target Cleavage Activity and with Known RNAi Factors

(A) R3-containing Superdex-200 void fractions were pooled and centrifuged on a 20%–50% sucrose gradient. Fractions were analyzed by native gel electrophoresis. The rightmost lane (“Lysate”) shows siRNP complexes formed in unfractionated extract. Sedimentation markers are indicated on the bottom; the 80S peak was defined by rRNA detection in samples from the same gradient fractions. The 30S, 50S, and 70S peaks were from E. coli ribosomes and ribosomal subunits sedimented separately in an identical gradient. Complexes and unbound siRNA are indicated on the right.

(B) Gradient fractions shown in (A) were used in an mRNA target cleavage assay. Control samples are input Pp-luc mRNA (“Input”), unfractionated lysate in the absence of siRNA (“No siRNA”), and unfractionated lysate in the presence of Pp-luc siRNA (“Complete”). The mobilities of 5'-H11032 end-labeled uncleaved Pp-luc mRNA target and cleaved product are shown on the right.

(C) Sucrose gradient fractions were deproteinized, precipitated, and loaded onto a 15% native polyacrylamide gel as described by Nykanen et al. (2001). Annealed siRNAs (“duplex”) and heat-denatured siRNAs (“duplex/heat”) were used both as markers and as controls to ensure that the procedure did not result in spurious denaturation or renaturation during handling. The siRNA duplex and the unwound 3'-labeled antisense strand are given on the right. The asterisk denotes the 3'-end label.

(D) In the panel on the left, unfractionated lysate (“Total”) and sucrose gradient fractions from (A) were separated in SDS polyacrylamide gels, blotted onto nitrocellulose, and subjected to Western analysis using antibodies against VIG, Ago2, dFXR, TSN, Dcr-1, Dcr-2, and R2D2, as indicated. The 80S sedimentation marker is shown at the bottom and was defined by rRNA detection in samples from the same gradient fractions. The panel on the right shows Western analysis of sucrose gradient fractions from siRNA-free lysate.

Eroogeneity may exist among the ~80S siRNA-containing complexes.

When we treated naïve extracts with the same chromatographic and centrifugation procedures, we did not detect Dcr-2 or R2D2 in comparable fractions (Figure 5D, right panel), even though the signal intensities of the two blots can be directly compared (see Experimental Procedures). VIG, TSN, Ago2, and dFXR, however, sediment in the 80S region of the gradient in the absence of siRNAs, possibly due to their presence in endogenous miRNA-programmed RISCs (Hutvagner et al., 2001). Consistent with this possibility, low levels of Dcr-1 (which is required for the miRNA pathway; Lee et al., 2004) were detected in fraction 30 from naïve extract (Figure 5D, right panel). Together, these results indicate that R3 may be an effector complex that is linked, functionally and physically, to the Dcr-2/R2D2 RNAi initiator complex. The siRNA/Dcr-2 interaction formed in the initiator complex appears to persist in the effector, even after siRNA unwinding. If R3 is, indeed, an effector complex, then it should be able to bind targeted miRNAs in a cognate-siRNA-dependent manner. To test this, we used a radiolabeled, 2'-O-methyl target that cannot be cleaved by RISC (Schwarz et al., 2003). The target was incubated in extracts containing either unlabeled cognate or noncognate siRNA duplexes and analyzed by native gel electrophoresis. In the presence of the cognate—but not the noncognate—siRNA duplexes, the target analog was bound by complexes that migrated identically with R3 (Figure 6, compare lanes 4 and 5). When this experiment was performed with dcr-2 mutant lysate, the 2'-O-
require ATP to form and contains Dcr-2, which directly characterize this material. At least some of it forms inde-
(Figure 7). the gel, a nonspecific complex, or some combination
Dicer, ultimately leading to target mRNA destruction R1, R2, and R3 complexes that has difficulty entering
sequentially, in a pathway that begins and ends with represent a distinct functional complex, a portion of the
consistent with a model in which the complexes form et al., 1989) and is typically left uncharacterized. It may
act at defined stages in the RNAi pathway. Our data are 2001), and transcription factor complexes (e.g., Shaw
and have further demonstrated that these complexes ska and Sharp, 1986), ribosomes (e.g., Nadano et al.,
plexes can be resolved by native gel electrophoresis cleoprotein complexes like spliceosomes (e.g., Konar-
We have shown that Drosophila
labeled siRNA (lanes 4 and 7), or in the presence of unlabeled cognate mRNA analog was shown in lane 1. Samples were
then treated with heparin and loaded onto a native gel. Unbound radiolabeled Pp-luc siRNA (lanes 5 and 8). Samples were
radiolabeled Pp-luc 2′-O-methyl mRNA analog was shown in lane 1. Wild-type lysate incubated with radiolabeled Pp-luc siRNA (lane 2)
serves as a marker for complexes that are indicated on the right. In addition, a nonspecific (i.e., Dcr-2- and siRNA-independent) com-
plex that forms on the mRNA target is denoted with an arrow.

Discussion

A Model for Silencing Complex Assembly and Function in RNAi

We have shown that Drosophila RNA silencing complexes can be resolved by native gel electrophoresis and have further demonstrated that these complexes act at defined stages in the RNAi pathway. Our data are consistent with a model in which the complexes form sequentially, in a pathway that begins and ends with Dicer, ultimately leading to target mRNA destruction (Figure 7).

Of these complexes, R1 forms the earliest. It does not require ATP to form and contains Dcr-2, which directly binds siRNAs. R1 corresponds to a previously identified ~360 kDa siRNP complex that contains duplex siRNAs and is inactive for targeted mRNA cleavage (Nykänen et al., 2001). It is also probably equivalent to a dsRNA-processing complex isolated from Drosophila S2 cells that contains Dcr-2 and R2D2 (Liu et al., 2003). R1 shares many features with this complex and can also form in extracts prepared from S2 cells (data not shown). Based on our results, as well as those of Liu et al. (2003), we conclude that the factors that form R1 process dsRNA and initiate RISC assembly. Furthermore, our results indicate that siRNAs must interact with Dcr-2 in order to reach RNAi effector complexes and that Dcr-2/siRNA association within the R1 complex is the first detectable step in the RISC-assembly pathway. Intriguingly, UV crosslinking results indicate that Dcr-2 can weakly or transiently bind 5′-hydroxyl-bearing siRNAs in the R1 complex. Nykänen et al. (2001) proposed that the siRNA 5′-phosphate serves a “licensing” function in the RNAi pathway, allowing the silencing machinery to recognize bona fide siRNAs; if this is true, our results indicate that siRNA 5′-phosphorylation status is monitored within the R1 complex after the initial Dcr-2/siRNA interaction has occurred. This could be achieved by fast dissociation of 5′-hydroxyl siRNAs from the Dcr-2 complex.

We have not yet characterized the R2 complex in detail because it neither survives gel filtration chromatography nor sediments cleanly in a sucrose gradient (data not shown). For now, we can only speculate about its composition and role in RNAi. Based on its kinetics and behavior in a chase experiment, R2 may be an intermediate that links R1 and R3, although we cannot be certain that it is “on pathway.” Since R2 forms so rapidly in this experiment, it might arise from R1 binding to a set of pre-associated factors (or to a single factor). This binding may then trigger the recruitment of still more factors, possibly accompanied by conformational re-arrangements, because relative to the other complexes, R2’s mobility in a native gel decreases slightly with time (Figures 3D and 3E). siRNA unwinding may occur during this process, leading to formation of R3.

The R3 complex is significantly enhanced by incubation with ATP, a known requirement for activated RISC formation. R3 is very large (80S) and cofractionates with the known RISC-associated components VIG, TSN, Ago2, and DxFR. The siRNAs associated with R3 are single stranded, and the complex co-sediments precisely with mRNA target cleavage activity. Finally, R3 can bind a cognate mRNA target. Based on these results, we conclude that R3 is an RNAi effector complex that is significantly larger than those previously described.

Throughout the course of this work, we frequently observed radiolabeled RNA at the top of the native gel lanes, which we labeled with an asterisk. This kind of signal did not comigrate with R3 (Figure 6, lanes 6–8). Because the dcr-2 mutant extract is incapable of forming the R3 complex (Figure 1), this result indicates that the target binding activity is, in fact, R3. Together, the data demonstrate that R3 is an RNAi effector complex that can bind and cleave targeted mRNAs.
Figure 7. Models for siRNP Assembly and RNAi in *Drosophila*

The dsRNA or siRNA silencing trigger is shown in red. R1 complex can arise either from Dcr-2 activity on long dsRNA triggers or from exogenous pre-cleaved siRNAs. R1 has already been shown to contain duplex siRNAs, and the ATP independence of R2 suggests that its formation also precedes siRNA unwinding. Both R2 and R3 complexes form from R1. We cannot formally exclude the possibility that R2 is an off-pathway dead-end complex; accordingly, two possible pathways are shown that differ in the placement of R2, either on (A) or off (B) the productive RNAi pathway. Additional complexes may be involved (see Discussion). The siRNA is unwound in the R3 complex. Known RNAi factors are indicated, and the presumably numerous as-yet-unidentified RNAi factors are shown as question marks. See text for references.

its role as an RNAi effector. On the other hand, at least some of it co-sediments with R3 and (in the experiment shown in Figure 5A) appears to slightly enter the gel, consistent with the possibility that it is a discrete silencing complex. We cannot conclusively rule out the possibility that siRNAs present at the top of the native gel lanes are associated with distinct functional complexes. However, our analyses point to R3 as an especially compelling candidate for the RNAi effector.

Our experiments suggest that R3 is a ribosome bound silencing complex. Indeed, several lines of evidence link RNAi to translation and therefore support this possibility. In *Drosophila* oocytes, untranslated mRNAs are refractory to RNAi, whereas those actively undergoing translation are not (Kennerdell et al., 2002), indicating that mRNAs most susceptible to RNAi are those that can also interact with the translational machinery. MicroRNAs have been identified within RISC (Hutvagner and Zamore, 2002) and can also affect protein synthesis from target messages (Olsen and Ambros, 1999), suggesting further functional links between RISC and ribosomes. In S2 cells, *Drosophila* RISC components pellet with ribosomes and other large complexes after high-speed centrifugation (Hammond et al., 2001; Caudy et al., 2002, 2003). Two of these components, Ago2 and dFXR, are found in complexes that include 5S rRNA as well as two ribosomal proteins, L5 and L11 (Ishizuka et al., 2002). siRNAs have also been found in association with the translational machinery, interacting with polyribosomes in the protozoan *Trypanosoma brucei* (Djikeng et al., 2003).

If R3 is a ribosome bound complex, then it is likely that RISC binds during or after siRNA unwinding and activation. We base this assertion both on the apparent ATP dependence of R3 formation and on the preponderance of single-stranded siRNAs within the complex. Zamore and coworkers previously showed that siRNA unwinding requires ATP (Nykänen et al., 2001). Although...
we cannot rule out the possibility that a potential RISC/ribosome interaction requires ATP for unrelated reasons, our data indicate that siRNA unwinding is one source of that requirement. Once formed, the R3 complex may be biochemically separable since smaller, active RISCs have been identified (Hammond et al., 2001; Nykänen et al., 2001; Martinez et al., 2002). If so, then R3 may represent a holo-silencing complex that includes factors not absolutely required for mRNA cleavage activity in vitro. These factors may include regulatory elements or those involved in the miRNA silencing pathway.

### On the Roles of Dcr-1, Dcr-2, and R2D2 in RNA Silencing

Because dsRNA processing and mRNA target cleavage activities are biochemically separable, Dicer is generally thought to release siRNAs to RISC, playing no apparent role in the effector phase of RNAi. However, the presence of Dcr-2 and R2D2 in R3-containing sucrose gradient fractions raises interesting questions about their role in RNA silencing, especially since they are not detectable in the corresponding fractions collected from naïve extracts. Liu et al. (2003) have argued that the Dcr-2-associated protein R2D2 conveys siRNAs from the RNAi initiator complex to the effector complex. Our data instead indicate that the Dcr-2/R2D2 initiator complex, itself, is incorporated into the effector in a RISC assembly pathway.

Our sucrose gradient experiments indicate that RISC components may associate with each other even in the absence of exogenous siRNAs. This association is not likely to be an apo-complex because fractionated naïve extracts cannot form R3 when they are incubated with siRNA duplexes after fractionation. Rather, it may represent endogenous microRNA-programmed RISC complexes or perhaps a partially preformed “holo-RISC” that lacks essential assembly factors (such as Dcr-2 and R2D2). Since the silencing activities of RISC and the miRNA are apparently governed by the degree of complementarity between trigger and target, and not by the identity of the silencing complex (Hutvagner and Zamore, 2002; Doench et al., 2003; Zeng et al., 2003), R3 may be able to form on both siRNAs and miRNAs. If so, then there may be distinct mechanisms for incorporating siRNA and miRNA triggers into the silencing complex. siRNA incorporation into RISC is likely to proceed on a Dcr-2/R2D2-dependent pathway since Dcr-2 and R2D2 can process dsRNAs (Liu et al., 2003) but are not detectable in sucrose gradient fractions collected from naïve extracts. On the other hand, miRNA incorporation may proceed on a different pathway to the silencing complex, possibly relying on Dcr-1. There is support for this model in the apparent division of labor between Dcr-1 and Dcr-2 in processing miRNA and dsRNA triggers (Lee et al., 2004). If this model is correct, then the observed size of R3 may reflect the diverse roles it may play, from mRNA cleavage to translational control.

### Experimental Procedures

#### General Methods

*Drosophila* embryo lysates were prepared from Canton S or dcr-2<sup>211fsX</sup> mutant flies (Lee et al., 2004) as previously described (Tuschl et al., 1999). Similarly, the miRNA and dsRNA cleavage reactions were performed in standard RNAi reaction conditions as previously described (Tuschl et al., 1999), but with slight modifications. GTP, CTP, UTP, and amino acids were omitted from both miRNA and dsRNA cleavage reactions; furthermore, cleavage reactions with dsRNA substrates were supplemented with magnesium chloride (2.5 mM final concentration).

**Pp-luc** miRNAs were transcribed with T7 RNA polymerase to generate a 465 nucleotide (nt) product. The transcripts were then 7-methylguanosine-capped using guanylyl transferase (Ambion) and <sup>32</sup>P-GTP (ICN) according to the manufacturer’s instructions. In our initial experiments, we used guanylyl transferase that was kindly provided by P. Zamore. Pp-luc dsRNAs were prepared by transcribing sense and antisense strands using T7 and SP6 RNA polymerase, respectively. Transcription reactions were performed in the presence of <sup>32</sup>P-GTP (ICN) to give internally radiolabeled products, 465 nt (sense) or 460 nt (antisense) long. The products were mixed, 1:1, in annealing buffer (30 mM HEPES [pH 7.5], 100 mM potassium acetate, 2 mM magnesium acetate), heated at 95°C for 2 min, and annealed overnight at 37°C to give duplex RNA. The synthetic **Pp-luc** siRNAs used in target mRNA cleavage, UV crosslinking, and native gel experiments were identical to those described previously (Nykänen et al., 2001). The antisense RNA was 3’-end labeled with <sup>32</sup>P-end labeling kit (Ambion) and then annealed to the corresponding fractions collected from naïve reactions, the siRNAs were used unlabeled. Duplex siRNAs were prepared by annealing the single-stranded RNAs in annealing buffer for 1 hr at 37°C after a 2 min incubation at 95°C.

#### Native Gel Electrophoresis

<sup>32</sup>P-labeled siRNA duplexes were incubated either in a 10 µl standard RNAi reaction mixture or one depleted of ATP using hexokinase and glucose, as previously described (Zamore et al., 2000). In both cases, the mixtures were supplemented with glycerol (10% v/v final concentration), incubated for 30 min, and quenched with 2 µl of a heparin mix (60 mM potassium phosphate, 3 mM magnesium chloride, 5% PEG<sub>2000</sub>, 8% glycerol, 4 mg/ml heparin), yielding a final heparin concentration of 0.67 mg/ml. The samples were then loaded onto a pre-chilled, 4% native gel (40:1 acrylamide:bisacrylamide) native gel and run at 4°C in 2x TBE at 10W. For timecourse experiments, the zero time point was pre-quenched with heparin before extract was added.

For target binding experiments (Figure 6C), we used a Pp-luc, 2’-O-methyl target analog with the sequence 5’-AUCAGUACGCC GAUAUCUUGGAA-A3’ (18S) and 5’-GCTTAATTTCGGTGATCGGACGAGA-3’ (18S). This target was 5’-end labeled with γ<sup>32</sup>P-ATP using T4 polynucleotide kinase (New England Biolabs). The labeled target analog was incubated in a standard RNAi reaction mixture (containing wild-type or dcr-2 mutant lysate) supplemented with 10% v/v glycerol in the presence of an unlabeled cognate (Pp-luc) or a noncognate siRNA duplex. After 1 hr, the reactions were quenched with heparin, loaded onto a 4% (40:1) native polyacrylamide gel, and run as described above.

For rRNA Northern blots, standard RNAi reaction mixtures were incubated at 25°C for 30 min with or without 50 nM unlabeled siRNAs and then loaded and run on a 4% (40:1) native gel. RNA from the gel was transferred to a Zeta-Probe GT membrane (Bio-Rad) and probed as previously described (Konarska and Sharp, 1988). The sequences of the oligonucleotide probes complementary to *Drosophila* rRNAs were 5’-AAGCACAGTGCCTACAAATTGCTAGT-3’ (18S) and 5’-GCTTAATTTCGGTGATCGGACGAGA-3’ (5S).

#### Chromatography

Gel filtration chromatography was performed as previously described (Nykänen et al., 2001), with slight modification. Samples were chromatographed at 4°C with a flow rate of 0.45 ml/min on an AKTA FPLC system. For anion exchange chromatography, naïve extracts were chromatographed first by gel filtration as described above. Aliquots of the fractions were incubated with <sup>32</sup>P-labeled siRNAs for 30 min at 25°C in a standard RNAi reaction containing glycerol (10% v/v total). Reactions were then treated with heparin (see above) and assayed for complex formation on a 4% (40:1) native polyacrylamide gel at 4°C. The remainder of the R1-forming fractions were pooled, supplemented with carrier insulin (final concentration 0.25 mg/ml) (Sigma), concentrated (Microcon YM-30, Millipore), and...
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The samples were precipitated with ethanol, resuspended (10 μl, 3% w/v Ficoll-400, 0.04% Bromophenol Blue, and 2 mM Tris-HCl [pH 7.4]), and run on a 15% native polyacrylamide gel at 4°C as previously described (Nykänen et al., 2001).

For Western analysis on both naïve and siRNA-programmed extracts (Figure 5D), 20 μl of each even-numbered fraction between 18 and 36 was loaded and run on SDS-polyacrylamide gels. Identical amounts of unfraccionated extract were loaded into the “total” lane of gels from both naïve and siRNA-programmed extracts to allow for direct comparison of signal intensities. The proteins were transferred to nitrocellulose membranes (Protran, Schleicher & Schuell), probed with primary, then secondary antibody (HRP-conjugated goat anti-rabbit, Pierce), incubated in a peroxide/enhancer solution (SuperSignal West Femto, Pierce), and visualized by autoradiography. Ago-2 antibodies were provided by E. Izaurralde; Dcr-2 and R2D2 antibodies were provided by Q. Liu and X. Wang; and the VIG, TSN, dfXR, and Dcr-1 antibodies were provided by A. Caudy and G. Hannon.

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