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RNAi: The Nuts and Bolts of the RISC Machine

Minireview

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Research on RNA interference and microRNA pathways continues to accelerate. Combinations of biochemical, genetic, and structural approaches are providing new insights into the mechanism by which small, 21 nt long RNAs find their way into the RISC effector complexes and how the RISCs execute their mission of RNA-guided posttranscriptional gene silencing.

Like the discovery of splicing three decades ago, the unearthing of RNA interference (RNAi) and microRNAs (miRNAs) once again has everyone's attention focused on posttranscriptional phenomena. During RNAi, dsRNA formed in cells by DNA- or RNA-dependent synthesis is processed to ~20 bp small interfering RNAs (siRNAs) containing 2 nt 3′ overhangs. These siRNAs are then incorporated into an *R*NA-*i*nduced *s*ilencing *c*omplex (RISC), which mediates the degradation of mRNAs with high sequence complementarity to the siRNA. MiRNAs are ~21 nt regulatory RNAs excised from genome-encoded precursors folding into dsRNA-like hairpins. With few exceptions, animal miRNAs base pair imperfectly to the 3′-untranslated region of target mRNAs and inhibit protein accumulation by an unknown mechanism, while plant miRNAs show nearly precise complementarity to their targets and trigger mRNA degradation. This miRNA dualism and observations that miRNAs can act under some circumstances as siRNAs, and vice versa, highlight similarities between the siRNA and miRNA pathways. Indeed, maturation of both RNA classes involves Dicer proteins, and, like siRNAs, miRNAs function as RNP particles, miRNPs or miRISCs, whose composition and, probably, assembly are related to those of the RISC (Sontheimer [2005]; Tomari and Zamore [2005]; consult these reviews when discussed findings are not specifically referenced).

Main Players in the Assembly and Function of the RISC

Many different proteins have been identified as essential for RNAi or as components of the RISC, but only a few have been functionally characterized at the molecular level. To date, Dicers and Argonautes have received the most attention. Dicers, ~200 kDa proteins, generally contain ATPase/RNA helicase and PAZ domains, two catalytic RNase III domains, and a C-terminal dsRNA binding domain (dsRBD). Their primary role is to process precursor molecules into siRNAs and miRNAs, but the enzymes also function in downstream steps of RNAi. Vertebrates and *Caenorhabditis elegans* contain single Dicer genes, while *Drosophila* and some other organisms express more Dicers with specialized functions. Of the two *Drosophila* Dicers, Dcr-1 functions mainly in the processing of miRNA precursors, while Dcr-2 is required for RNAi (Lee et al., 2004). The ~100 kDa Argonautes, with characteristic PAZ and PIWI domains, are the only proteins consistently found in all RISC and miRNP complexes and fall into two subfamilies, Ago and Piwi. The Ago group, which functions in RNAi and miRNA pathways, includes four ubiquitously expressed proteins, Ago1–4, in mammals and two, Ago1 and Ago2, in *Drosophila*. Like Dicers, individual Ago proteins are dedicated to different pathways: *Drosophila* Ago1 and Ago2, for example, function primarily in the miRNA and RNAi pathways, respectively. Among other relevant RNAi factors are small dsRBD-protein partners of Dicers, such as R2D2 and Loqs of *Drosophila*, and RDE-4 of *C. elegans* (Förstemann et al. [2005], Saito et al. [2005], and references therein), proteins with RNA helicase/ATPase domains, and a Tudor staphylococcal nuclease, Tudor-SN. Tudor-SN, a component of RISC, was recently shown to bind and possibly degrade dsRNAs hyperedited by adenosine deaminases (ADARs), pointing to an intimate connection between editing and RNAi pathways (Scadden, 2005). Other conserved RISC components are Fragile X mental retardation (FMRP) and related proteins, and VIG, but their precise function in RNA silencing is unknown.

A physiological inducer of RNAi in cells is long dsRNA, but most mechanistic studies of RNAi have utilized siRNAs as effector molecules. SiRNAs are double-stranded molecules, of which only one strand, referred to as a guide, is incorporated into the RISC, while the other—a passenger strand—is discarded. How are the duplexes converted to single-chain forms and a desired strand selected to act as a guide? The incorporated strand is generally the one whose 5′ terminus is at the thermodynamically less stable end of the duplex. In *Drosophila*, a heterodimer of one of its Dicers, Dcr-2, and R2D2 senses the differential stability of the duplex ends and determines which strand will enter the RISC. Photocrosslinking to siRNAs containing 5-iodouracils revealed that Dicer binds to the less stable and R2D2 to the more stable siRNA end (Tomari et al., 2004b). Since the siRNA asymmetry rules are quite general, R2D2-related proteins are likely involved in the definition of siRNA ends in other organisms also.

The Dcr-2-R2D2-siRNA ternary complex is the best-characterized assembly in the RISC formation pathway. The complex can be formed effectively with purified recombinant Dcr-2 and R2D2, in the absence of ATP. However, the following step, during which the siRNA duplex undergoes unwinding and the Dcr-2/R2D2 heterodimer is gradually displaced by Ago2, requires additional proteins and ATP. SiRNA unwinding occurs in a complex known as the RISC loading complex (RLC) (Tomari et al., 2004a, 2004b), which is similar to the

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complex R2 identified by Pham et al. (2004). The Dcr-2-R2D2-siRNA and RLC complexes are true assembly intermediates, since both can be chased into the catalytically competent RISC (Pham et al., 2004; Tomari et al., 2004a, 2004b). Functional RISCs able to cleave mRNA targets have been isolated in distinct forms varying in protein composition. "Minimal" active RISCs of ∼150 kDa may contain only Ago proteins associated with the siRNA guide strand, consistent with Argonautes catalyzing the mRNA cleavage (see below). The largest complex, a holo-RISC identified in *Drosophila*, fractionates at ∼80S (Pham et al., 2004) and likely represents smaller RISC forms associated with the ribosomes.

Within the RISC, mRNA cleavage occurs between residues base paired to nucleotides 10 and 11 of the siRNA, and the cleavage itself does not require ATP. The guide siRNA remains associated with the complex, allowing it to carry out multiple rounds of RNA cleavage. The turnover of the enzyme is dependent on ATP, suggesting that release of the cleaved mRNA halves involves an RNA helicase, and several proteins implicated in RNAi in *Drosophila* and other organisms contain RNA helicase/ATPase domains. However, which of the proteins participate in specific steps of RNAi is not yet established. Armitage, identified in *Drosophila*, is required for conversion of the RLC to an active RISC. Not clear is whether Armitage functions in siRNA unwinding, since it is also needed when RISC assembly is programmed with the single-stranded siRNA (Tomari et al., 2004b).

What is the sequence of events when long dsRNA or miRNA precursors, rather than processed ∼20 bp duplexes, initiate the reaction? Dicer appears to preferentially excise siRNAs from dsRNA ends, with the dsRNA (or pre-miRNA) terminus likely recognized by the PAZ domain of Dicer and the catalytic domains cutting the substrate ∼20 bp away (Zhang et al., 2004). Clearly, at the dicing step, Dicer has no opportunity to perceive the differential stability of the two ends of the reaction product. Is, then, the siRNA released from the enzyme after cleavage to be rebound by the Dcr-2/R2D2 complex, following the stability rules? Alternatively, could Dicer remain associated with the siRNA product and escort it directly to the RISC, a situation that would entail periodic violation of asymmetry principles? Numerous arguments support the former possibility (Tomari and Zamore, 2005; Sontheimer, 2005), though some data are also consistent with the latter (Sontheimer, 2005; Zhang et al., 2004). Irrespective of the scenario, the Dicer PAZ is a candidate domain to mediate recognition of the siRNA end also in the Dcr-2/R2D2/siRNA complex. However, the siRNA-Dicer interaction in this complex should differ from that occurring immediately after the cleavage reaction since one of the siRNA ends would be needed for contacting R2D2. Details of siRNA recognition by R2D2 in the Dcr-2/R2D2/siRNA complex are unknown, except that this interaction, in contrast to the Dcr-2-siRNA interaction, is enhanced by the presence of the 5′ phosphate (Tomari et al., 2004b).

Illuminating Impact of Ago Structures

Recent structural studies of Ago proteins and their subdomains have opened a new chapter in RNAi research. X-ray and NMR studies of Argonaute PAZ domains, both free and complexed with RNA, have revealed that the domain specifically recognizes the 2 nt 3′ overhang of the duplex or the 3′-OH end of a single-stranded RNA (Lingel et al., 2004; Ma et al., 2004). In the structure of the human Ago1 PAZ, bound to an siRNA mimic (Ma et al., 2004), the 2 nt overhang is inserted into a pocket lined up with conserved aromatic and hydrophobic residues. In the adjacent A-form duplex, only the strand with an anchored 3′ end is in contact with basic amino acids, suggesting that this strand will be retained upon siRNA unfolding.

The structures of full-length eukaryotic Ago proteins or PIWI domains are not yet available. However, three groups have succeeded in crystallizing prokaryotic Ago-like proteins, either alone or in a complex with siRNA mimics. The Ago-like proteins are encoded in the genomes of only a few archaea and eubacteria, and their origin and function remain a mystery. Nevertheless, the structural properties of these proteins are very informative. Song et al. (2004), followed by Parker et al. (2004), reported the structures of two archaeal proteins, PfAgo and AfPiwi, originating from *Pyrococcus furiosus* and *Achaeoglobus fulgidus*, respectively. The ∼85 kDa PfAgo includes both PAZ and PIWI domains. AfPiwi is approximately half the size of PfAgo and structurally corresponds to PfAgo middle and PIWI domains; in the AfPiwi structure, they are referred to as domains A and B and jointly as the PIWI fold. Startlingly, the PIWI domain of PfAgo and AfPiwi has a fold similar to RNase H, an enzyme that cleaves the RNA strand in DNA-RNA hybrids, immediately suggesting that PIWI represents the "Slicer," responsible for mRNA cleavage in RISCs. RNase H contains a triad of acidic amino acids, DDE, involved in the catalysis. A related set of amino acids, DDH, is conserved in PfAgo and eukaryotic Argonautes able, like Ago2, to support mRNA degradation within RISCs. Mutagenesis of human Ago2 demonstrated that all three DDH amino acids are essential for mRNA cleavage (Liu et al., 2004; Rivas et al., 2005). That the Argonaute is indeed a catalytic engine of the RISC was unequivocally proven by Rivas et al. (2005), who found that an active RISC can be reconstituted from bacterially expressed human Ago2 and a single-stranded siRNA.

The structure of AfPiwi complexed to siRNA mimics was solved recently by Parker et al. (2005) and Ma et al. (2005). The structures are very illuminating. (1) The phosphorylated 5′-terminal nucleotide of the guide strand is anchored within the highly conserved basic pocket. The 5′ phosphate interacts with four invariant residues and a bound divalent metal ion. The 5′ base stacks on the aromatic ring of an invariant tyrosine, further stabilizing the binding. Importantly, mutation of equivalent conserved amino acids in the human Ago2 attenuated its mRNA cleavage activity, arguing for a functional relevance of the interaction (Ma et al., 2005). (2) In the structure, the anchored 5′ nucleotide is not base paired to the complementary strand, in contrast to the downstream nucleotides, which are engaged in an A-form helix positioned in the basic channel at the A-B domain interphase. Unavailability of the 5′ nucleotide for base pairing rationalizes experimental and bioinformatic data indicating that the miRNA and siRNA

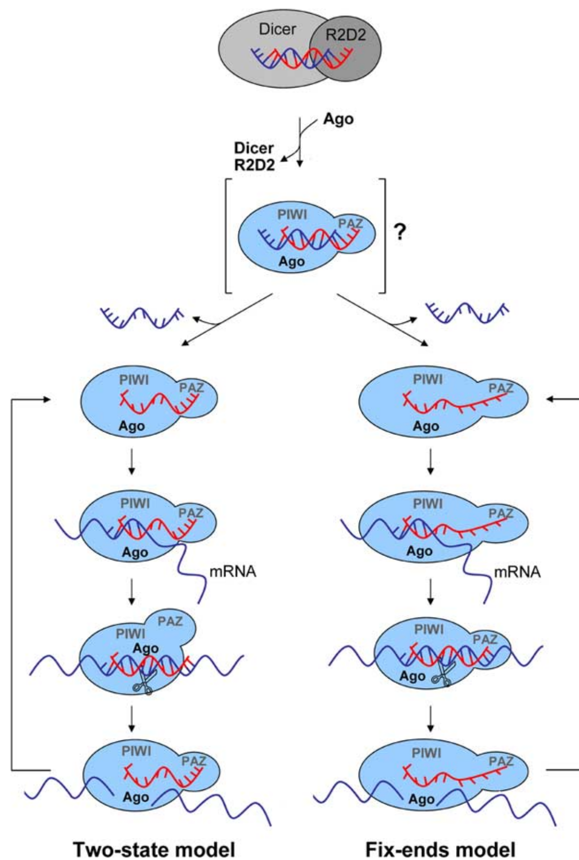

Figure 1. Models of the Assembly and Function of the Minimal Ago-Containing RISC

5′ nucleotide does not necessarily pair with the mRNA target. (3) Most of the additional identified siRNA-PIWI contacts involve the sugar phosphate backbone of four 5′-proximal nucleotides (positions 2–5) of the guide strand. This is in line with a largely sequence-independent loading of siRNAs to the RISC and with the prediction that the PIWI domain should interact stably with the guide but not the passenger (or the target mRNA) strand of the duplex. Ordered binding of the guide nucleotides 2–5, presented on the PIWI surface in a quasihelical form suitable for base pairing, is also consistent with the finding that, in both miRNAs and siRNAs, the 5′ end (e.g., the nucleotide 2–8 "seed" in miRNAs) represents a nucleation region for pairing with target mRNAs. (4) Finally, modeling of longer A-form helices into AfPiwi structures placed the mRNA target scissile phosphate in proximity to the proposed catalytic region. This reinforces an idea that the mRNA cleavage site is determined by measuring the fixed distance from the anchored siRNA 5′ end.

The RISC Cycle Revisited

AfPiwi is only a distant cousin of eukaryotic Argonautes and, rather puzzlingly, binds single- and double-stranded DNA better than RNA (Ma et al., 2005). Despite these limitations, the derived structures, as discussed above, rationalize many observations from biochemical and bioinformatic work. They also provide material for discussing models of RISC assembly and function (Figure 1). Clearly, the key initial step is the displacement of the Dcr-2-R2D2 heterodimer (or its postulated orthologs) by the incoming Ago. The details of this act are unknown, but all evidence indicates that, in the resulting Ago-siRNA complex, the 5′-phosphorylated guide strand end will be docked at the PIWI pocket, and the opposite 3′ end will contact the PAZ domain. The siRNA bound to Ago may initially be double stranded. Although reconstitution of active RISC from purified Ago and the siRNA duplex has not yet been achieved (Liu et al., 2004; Rivas et al., 2005), the archaeal AfPiwi binds single- and double-standed siRNA mimics with equal affinity (Parker et al., 2004; Ma et al., 2005). Nevertheless, discharge of the passenger strand of the potential duplex would need to follow rapidly, since Ago complexes generally contain siRNAs in a single-stranded form (Tomari et al. [2004b] and references therein). In contrast to RISC formation with double-stranded siRNA in cells or cell extracts, assembly with a single-stranded siRNA does not absolutely require 5′ end phosphorylation (although phosphorylation stabilizes the siRNA binding in the recombinant RISC and possibly increases its cleavage fidelity; Rivas et al. [2005]). Hence, the 5′ phosphate, by contributing to maintenance of the guide 5′ nucleotide in the flipped conformation, is likely more important for the unwinding and release of the passenger strand than for subsequent steps of the RISC cycle.

Two models of Argonaute function in the RISC can be envisaged (Figure 1). One is a slightly refined version of the "two-state" model proposed by Tomari and Zamore (2005). Following rejection of the passenger strand, with the 5′ end of the guide siRNA tethered to the PIWI and the 3′ end to the PAZ domain of Ago, the "double-anchor state" complex would be ready to engage in the initial interaction with the target mRNA, using the guide 5′-proximal seed nucleotides. To progress to a more stable RISC-mRNA complex involving the downstream base pairing, the interaction of siRNA with the PAZ domain would need to break down. In addition to making the guide 3′ region available for base pairing, the transition to a "single-anchor state" would also eliminate the topological constraints, which might in fact represent a factor limiting the seed interaction to the siRNA nucleotides 2–8. With the resulting guide-target duplex extending now over two A-form helical turns, cleavage of the target would occur, followed by release of the processed target halves. Reanchoring of the guide 3′ end, possibly coupled with ejection of the cleaved product, would complete the cycle.

The second, "fix-ends" model does not require recurrent disruption of the guide-PAZ contact. In this model, release of the passenger strand would be accompanied by a change to an alternative Ago conformation that sets the PIWI and PAZ domains further apart and consequently stretches, with some unstacking, the guide strand, particularly its 3′ region. The 5′-proximal seed nucleotides, still presented on the PIWI surface in a quasihelical form, would initiate the interaction with the mRNA target. The ensuing energetically favorable propagation of the helix toward the 3′ region would bring back the PIWI and PAZ domains to the more compact conformation similar to that postulated for the complex of Ago with the double-stranded siRNA.

Cleavage of the target, followed by release of processed mRNA halves, would be accompanied by return of the Ago protein and the guide siRNA to extended conformations. Importantly, structural studies by Ma et al. (2004) indicate that persistent interaction of the two 3′-terminal nucleotides of the guide with PAZ should not affect formation of the siRNA-mRNA helix.

Experiments with PAZ domain mutants or tests of siRNA 3′ end accessibility to chemical or enzymatic modifications during the RISC cycle are required to evaluate the two models. Recently, Rivas et al. (2005) found that recombinant active RISC can be formed even with 17 nt long siRNA. Since the RNA seemed to be properly anchored at the 5′ end, its 3′ end is unlikely to have reached the PAZ domain. At first glance, this result could be viewed as an argument against the fix-ends model. However, the recombinant RISC does not turn over, and we do not know if the 17 nt RNA can support multiple cleavage rounds. If it can, a third model of the RISC function should also be envisaged, in which the siRNA-PAZ interaction would occur at the RISC assembly but not later steps.

All four mammalian Ago proteins, Ago1–4, associate with siRNAs, but only complexes incorporating Ago2 can cleave mRNA targets. Consistently, mouse Ago2 knockout cells are resistant to induction of the RNAi response. The inability of mammalian Ago1, Ago3, and Ago4 to act as a Slicer is readily explained by substitutions of some of the DDH triad and other amino acids. (Liu et al., 2004; Meister et al., 2004; Rivas et al., 2005). On the other hand, all mammalian Ago proteins associate with endogenous miRNAs and appear to function in translational repression (Liu et al., 2004; Meister et al., 2004). The latter statement is supported by the finding that the repressive effect of miRNAs on protein accumulation can be mimicked in HeLa cells by an miRNA-independent tethering of Ago2, Ago3, and Ago4 to the mRNA reporter (Pillai et al., 2004).

In contrast to the assembly of the mRNA-cleaving RISC, little is known about the assembly of functional miRNPs or miRISCs. However, the positioning of miRNA within the mature complex must be similar to that of the siRNA guide strand, since endogenous miRNPs can cleave RNA when presented with highly complementary targets. What factors decide whether mRNA will be cleaved or translationally repressed when bound by miRNA or an siRNA RISC? How can Ago2 induce either of the two outcomes? All available evidence indicates that perfect complementarity in the central part of the siRNA-mRNA duplex, allowing A-form helix formation in the region facing the DDH triad, is mandatory for the cleavage. The presence of bulges or other irregularities in this region, effectively interfering with A helix formation, will prevent the cleavage, despite association of the si- or miRISC complex with mRNA. The emerging picture is that a default effect of depositing Ago proteins, including the slicing-competent Ago2, on mRNA is translational repression, with cleavage by Ago2 requiring proper base pairing of guide and target RNAs.

Structural information on RISCs at different assembly and functional steps is now required. In light of recent progress in molecular understanding of the ribosome cycle, the RISC data should follow soon. Establishing the function of numerous additional proteins not discussed in this review, which cofractionate with RISCs and miRNPs, will also yield useful data. Since RNA silencing affects so many genes and cellular pathways, the process is likely to be subject to highly interesting modes of regulation.

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