Molecular Mechanism of Target RNA Transcript Recognition by Argonaute-Guide Complexes

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Argonaute proteins participate in conferring all known functions of RNA-mediated gene silencing phenomena. However, prior to structural investigations of this evolutionarily conserved family of proteins, there was little information concerning their mechanisms of action. Here, we describe our crystallographic analysis of the PIWI domain of an archaeal Argonaute homolog, *A/Piwi*. Our structural analysis revealed that the Argonaute PIWI fold incorporates both an RNase-H-like catalytic domain and an anchor site for the obligatory 5' phosphate of the RNA guide strand. RNA-*A/Piwi* binding assays combined with crystallographic studies demonstrated that *A/Piwi* interacts with RNA via a conserved region centered on the carboxyl terminus of the protein, utilizing a novel metal-binding site. A model of the PIWI domain of Argonaute in complex with a small interfering RNA (siRNA)-like duplex is consistent with much of the existing biochemical and genetic data, explaining the specificity of the RNA-directed RNA endonuclease reaction and the importance of the 5' region of microRNAs (miRNAs) (the "seed") to nucleate target RNA recognition and provide high-affinity guide-target interactions.

Argonautes are the central component of RNA-induced silencing complex (RISC)-like ribonucleoprotein (RNP) complexes that mediate all known effector functions associated with RNA interference (RNAi) (Hammond et al. 2001). Target RNA transcripts are selected by a guide strand-Argonaute complex. Depending on the degree of complementarity between guide and target strands, the subclass of Argonaute protein, and the origin of the guide RNA, the RNA-programmed RISC-like complex mediates either cleavage of the target, translation repression, or heterochromatin remodeling. Moreover, in addition to conferring these RNAi-based effector functions, Argonautes participate in selecting the single-stranded RNA guide from the double-stranded siRNA/miRNA duplex. Findings that a highly purified form of *Drosophila* RISC was dmAgo2 (Rand et al. 2004) and that recombinant hAgo2 was capable of selecting and cleaving target RNA transcripts (Rivas et al. 2005) provided the crucial insights that the critical biological functions of RISC are performed by Argonaute.

Our interests have focused on understanding the molecular mechanisms of Argonaute proteins. From a combination of structural, biochemical, and biophysical approaches, we addressed the following questions: (1) How does the guide RNA interact with Argonaute? (2) How does the Argonaute-guide RNA complex recognize its complementary RNA transcript selectively? (3) What structural features of the Argonaute-guide-target RNA complex determine whether the target strand is cleaved?

Argonaute proteins, originally discovered in plants (Bohmert et al. 1998), are indigenous to the three domains of life (Carmell et al. 2002). They are characterized by an amino-terminal PAZ domain (of ~150 residues) and a carboxy-terminal PIWI domain of about 400 residues linked by a region of variable length and sequence. PAZ domains also feature in Dicer, the endonuclease that gen-

erates siRNA and miRNAs from longer double-stranded RNA precursors. In both Argonaute and Dicer, the PAZ domain interacts with the 3' end of an RNA molecule. However, intriguingly, the PIWI domain is unique to the Argonautes. An Argonaute homolog from the archaea *Archaeoglobus fulgidus* is the only known member of this family to consist solely of a PIWI domain. The protein of 427 amino acids comprises the conserved motifs that are characteristic of the PIWI domain and shares approximately 20% sequence identity with the PIWI domains of eukaryotic Argonautes. The protein therefore serves as a useful model system to understand the function of the PIWI domain within Argonautes.

PIWI IS COMPOSED OF RNASE H AND LAC REPRESSOR-LIKE DOMAINS

We determined the crystal structure of *A/Piwi* to 2.0 Å resolution, revealing important insights into the functions of the PIWI component of Argonaute (Parker et al. 2004). *A/Piwi* is organized into two major domains that we defined as A and B (Fig. 1). The combination of these two domains creates the PIWI fold that includes all conserved sequence motifs that were initially used to define the PIWI domain. The intimate association of the A and B domains also suggests that the functional and structural unit of the PIWI fold is generated from these two domains.

Domain A is formed from a simple α - β - α sandwich fold with weak structural resemblance to a subdomain of the Lac repressor. However, differences in the chain topology and structural context of this domain relative to its counterpart in the Lac repressor suggest that the two proteins share no functional similarity. The structure of domain B, on the other hand, revealing an architectural similarity to the RNase H family of endonucleases, provided significant insights into its function. Domain B

Figure 1. Ribbons representation of the crystal structure of *AfPiwi* in complex with siRNA. (Green) Guide strand; (yellow) target; (salmon) domain A (mid); (blue) domain B (PIWI); (cyan) residues of the G1-binding pocket. The 5' nucleotide (G1) of the guide strand is located within the G1-binding pocket (CR1). (M) Metal ion.

(defined as the PIWI domain by Joshua-Tor and colleagues from their crystal structure of the *Pyrococcus furiosus* Argonaute [*PfAgo*]) (Song et al. 2004) shares structural characteristics of the RNase H family members: archaeal RNase HII, *Escherichia coli* and human immunodeficiency virus (HIV) RNase HI, ASV- and HIV-encoded integrases, and *E. coli* RuvC, namely a central 5-stranded β -sheet surrounded on both sides by a pair of α -helices (Fig. 2). In RNase H enzymes, two aspartic acid residues situated on adjacent β -strands contribute to divalent metal coordination at the catalytic site. Although neither of these aspartic acids is conserved in *AfPiwi*, their presence in eukaryotic Argonautes, *PfAgo*, an archaeal Argonaute, and the eubacterial Argonaute *Aquifex aeolicus*, suggested a potential catalytic function and implicated Argonaute as the Slicer activity of RISC (Song et al. 2004). Substantial evidence now supports the notion that

Argonautes are indeed responsible for the Slicer activity of RISC. Mutation of either catalytic aspartic acid in hAgo2 abolishes Slicer activity in vivo (Liu et al. 2004) and in vitro (Rivas et al. 2005), and similarly to RNase H, Slicer is an Mg^{2+} -dependent enzyme that utilizes metal-activated water molecules to cleave its phosphodiester bond substrate, yielding 3'OH groups and 5' phosphates (Martinez and Tuschl 2004; Schwarz et al. 2004; Nowotny et al. 2005).

CONSERVED REGION 1 (CR1) DEFINES A PUTATIVE RNA-BINDING SITE

To gain insight into functional regions of the PIWI domain, we mapped regions of sequence conservation, shared between all three kingdoms of life, onto the molecular surface of *AfPiwi*. This analysis revealed three conserved regions: CR1, CR2, and CR3. All three conserved regions map to the same face of the PIWI domain surface. CR3 is unique to the eukaryotic Argonautes, as residues at this site are not conserved in eubacteria or archaea. In eukaryotes, this conserved region constitutes the "PIWI box," a region of the PIWI domain of Argonautes implicated in interactions with Dicer (Tahbaz et al. 2004). CR2, within the RNase-H-like domain B, corresponds to the RNase-H-like catalytic site, and its presence in eubacteria and archaea, in addition to eukaryotes, suggests that Argonaute-associated endonuclease activity is evolutionarily conserved. Strikingly, the region of highest structural conservation shared among all Argonaute sequences is CR1, a region at the interface of the A and B domains (Fig. 1). This interface features a deep cleft and is linked to CR2 by a channel located within domain B (see Fig. 4). The carboxy-terminal carboxylate group of the polypeptide chain, exposed on the molecular surface, defines the center of the interface that in *AfPiwi* is coordinated to a divalent cation (cadmium in our crystal structure). The aliphatic side chain of Leu-427 and preceding four amino acids are buried at the domain interface, ensuring that the carboxy-terminal carboxylate is rigidly located at the molecular surface. Metal coordination by a carboxy-terminal carboxyl group is unusual and suggests an interesting structural feature. Compared with the side chains of aspartic acid and glutamic acid residues, a carboxy-terminal carboxyl group is allowed reduced rotational freedom, and because Leu-427 is clamped at the interface of the A and B domains, it is likely that the metal ion site in *AfPiwi* is quite rigid. In our structure, the divalent cation also contacts the amide side chain of Gln-159, a residue conserved in the PIWI sub-family. The divalent cation-binding site is surrounded by four residues, contributed by domain A, almost universally invariant in Argonaute sequences (Fig. 3). These residues, two lysines, glutamine, and tyrosine, are characteristic of RNA-binding proteins and suggested the exciting possibility that CR1 may represent an RNA-binding site. Geometric arguments, based on the distance between CR1 and CR2, and the property of RISC to cleave the target strand at a defined site, namely, the phosphodiester bond bridging the two nucleotides that base-pair with the 10th and 11th nucleotides of the guide strand (Elbashir et al. 2001a,b;

Figure 2. The PIWI domain (domain B) features an RNase-H-like fold (cyan). (A) structure of RNase HII from *Methanococcus jannaschii* (Lai et al. 2000). (B) *AfPiwi* domain B. (Red) Positions of residues of the catalytic triad.

Figure 3. G1-binding pocket anchors the 5' end of guide strand (target strand omitted for clarity). Details of protein–RNA guide interactions are at the G1-binding pocket. (M) Metal ion.

discussed in Parker et al. 2004), provided a strong indication that CR1 may anchor the obligatory 5' phosphate of the guide strand (Nykänen et al. 2001; Chiu and Rana 2002; Schwarz et al. 2002), placing the scissile phosphate of the target strand within CR2. Although other studies had demonstrated that the PAZ domain of Argonaute binds RNA (Song et al. 2003; Yan et al. 2003; Lingel et al. 2004; Ma et al. 2004), it was not previously known whether PIWI acts as an RNA-binding domain. To test the possibility that *A*/Piwi interacts with RNA, in a manner that is dependent on metal binding to the carboxy-terminal carboxylate group, we performed protein–RNA-binding experiments, monitoring RNA–*A*/Piwi interactions by means of electrophoretic mobility-shift assays and UV-induced protein–RNA cross-linking. These studies, employing an siRNA-like molecule of 16 nucleotides with 5' phosphate groups and 3' dinucleotide overhangs, demonstrated that the high-affinity *A*/Piwi–RNA interactions required divalent cations and were severely diminished by addition of a glycine residue to Leu-427, a modification expected to disrupt the geometry of the divalent cation binding site of CR1 (Parker et al. 2004).

To understand the molecular mechanism of PIWI–RNA interactions, we cocrystallized *A*/Piwi with a 16-nucleotide siRNA-like duplex (Parker et al. 2005). The resultant structure represents a model of either a guide-passenger duplex or a guide-target duplex associated with the PIWI domain and provided the critical insight that CR1 functions as an anchor site for the 5' phosphate of the guide strand. The 5' end of the RNA duplex (defined as the 5' end of the guide strand), which participates in extensive interactions with the protein, is highly ordered, but the duplex becomes progressively less well ordered toward its 3' end. In our crystal structure, we observed well-resolved electron density for the nine 5' nucleotides, assigned G1–G9 for the guide strand and

T1–T9 for the complementary nucleotides of the target strand (Fig. 1). The remainder of the RNA duplex is not clearly resolved in the electron density and is assumed to be mobile.

A/Piwi binds to the 5' end of the siRNA-like duplex, engaging the RNA in an L-shaped channel (Fig. 1). Nucleotides 2–9 on each strand base-pair, adopting an A-form helix that extends from the domain interface along the channel of domain B to CR2. A striking feature of the structure is the profound distortion of the 5' end of the RNA duplex phosphodiester backbone, resulting in the unwinding of the 5' complementary nucleotides G1 (uridine) and T1 (adenosine). G1, which carries the obligatory 5' phosphate of the guide, is displaced into a pocket surrounding the carboxyl terminus of the PIWI domain, CR1, now defined as the G1-binding pocket. T1 passes through the short “exit” portion of the channel, allowing the two 3' overhanging nucleotides, T(-1) and T(-2), to fold back along the protein surface. The unpaired G1 and T1 nucleotides are physically divided by the α D helix of domain A, which contributes two aromatic residues (Phe-151 and Tyr-152) capping the first 5' base pair of the RNA helix (U2–A2), therefore substituting for the unpaired nucleotides at the extreme 5' end of the duplex (Fig. 1).

Distortion of the 5' end of the guide strand is facilitated by RNA–phosphate interactions with conserved residues of the G1-binding pocket, particularly to the divalent cation (manganese in the RNA–*A*/Piwi complex) coordinated to the carboxylate of Leu-427 (Fig. 3). Via interactions that require distortion of the 5' end of the guide, both the phosphate groups of G1 and G3 form direct contacts to the metal ion. In addition to the metal-mediated RNA contacts within the G1-binding pocket, the side chains of four invariant residues in Argonaute proteins (Tyr-123, Lys-127, Gln-137, and Lys-163) also contact the 5' phosphate group of G1. Tyr-123 further stabilizes the flipped conformation of G1 by stacking with the unpaired uracil base. The 5' phosphate group forms further contacts to the backbone amide of Phe-138, fixed in position by the adjacent Gln-137.

Interactions between *A*/Piwi and the guide are also mediated by the phosphate groups of G2–G5. A second key anchor site is the phosphate group of G3. In addition to its coordination by the metal, the phosphate interacts with Gln-159 and Arg-380, residues conserved in the PIWI and Ago subfamilies, respectively. The phosphate of G2 contacts Tyr-152, conserved as tyrosine or threonine in the Ago subfamily, and the main-chain amide of Arg-140. The phosphate of G4 also contacts Arg-380, and the phosphate of G5 interacts with Arg-385, via a water molecule. The only protein–base contact occurs between Asn-155, conserved in the Ago subfamily, and the 2' oxygen of the G2 uracil. Hydrogen bond acceptors occupy equivalent positions in cytosine and purines. Therefore, as anticipated, none of the interactions provide RNA sequence specificity. Interactions with G1–G5 account for all conserved residues of CR1, strongly indicating a conserved mode of RNA 5'-end binding within the Argonaute family. In contrast to the extensive interactions between PIWI and the guide, few contacts are formed to the target strand, consistent with its requirement to dissociate after mRNA cleavage.

Figure 4. Model of a 19-nucleotide siRNA-*A/Piwi* structure. Guide strand nucleotides G1–G9 (and complementary target nucleotides and 3' dinucleotide overhang) determined from the crystal structure are in light green and yellow, respectively. The modeled regions of both strands are in darker green and yellow. (Red) Positions of catalytic triad residues, and the scissile bond of the target strand.

MODEL FOR TARGET STRAND RECOGNITION AND CLEAVAGE

Nucleotides G10–G16 of the guide and T10–T14 of the target are less clearly defined in the electron density maps. To visualize the trajectory of a full-length (19-nucleotide) siRNA guide bound to an mRNA target, we modeled the remaining nucleotides, assuming an A-form helix contiguous with the visible duplex (Fig. 4). The weaker electron density for nucleotides G10 and G11 is compatible with this conformation. This model of a PIWI domain–siRNA complex provided significant insights into mechanisms of target strand recognition and mechanisms of target strand cleavage.

Distortion and displacement of G1 into the conserved binding pocket result in the unwinding of the cognate G1–T1 base pair, whereas the bases of nucleotides G2 to G8 are ordered and exposed to solvent. Bases 3' of G9 are turned in toward the molecular surface of the protein and are therefore less accessible for base-pairing (Fig. 4). The nucleotides with exposed bases, accessible for base-pairing with a target transcript, as revealed by our crystal structure, correlate with the definition of a “seed” sequence identified in animal miRNAs. Bioinformatics analysis of the complementarity between miRNAs and their target recognition sequences within the 3'UTRs (untranslated regions) of RNA transcripts revealed that nucleotides 2–7 of the miRNA were complementary to the target sequence. The 5' nucleotide and nucleotides 3' of the seed region shared little to no sequence homology with their target. This implies that the seed sequence is sufficient for target recognition, suggesting that regions of the miRNA outside of the seed region would not base-pair with their target sequence (Lewis et al. 2003, 2005; Stark et al. 2003). The notion that a seed sequence dominates miRNA–target RNA interactions was strongly supported by empirical data of Doench and Sharp (2004).

Introducing mismatches between an miRNA and its target within the seed region profoundly reduced miRNA-mediated repression of translation, whereas mismatches 3' to the seed region had little to no effect on translation repression. It is likely that an analogous “seed” region may operate for siRNAs because in RISC-mediated mRNA cleavage, bases at the 5' end of the siRNA guide dominate its affinity for target RNA (Haley and Zamore 2004).

The model of the extended siRNA in complex with *A/Piwi* provides insights into the catalytic mechanism of Argonaute. Toward the middle of the guide RNA, the trajectory of the RNA directs the target strand into the RNase H catalytic site, placing the scissile phosphate, linking T10 and T11 (which base-pair with G10 and G11, respectively) in close proximity to the putative catalytic site. Modeling the position of the RNase H catalytic aspartic acid residues into the *A/Piwi*–siRNA complex provides a plausible explanation for the specificity of target RNA cleavage. With the 5' phosphate of the guide strand anchored at the G1-binding pocket, an RNA duplex with complementary guide and target strands forming an A-form RNA duplex will adopt the correct geometry to position the target strand within the catalytic site, resulting in cleavage between the 10th and 11th nucleotides. Since an A-form RNA duplex is the preferred substrate of RISC (Chiu and Rana 2003; Haley and Zamore 2004), this could explain why some animal miRNAs containing mismatches between guide and target strand within the central region do not direct target strand cleavage, whereas plant miRNAs, which are normally homologous to their targets over their entire length, direct target strand cleavage. Argonaute proteins lacking either of the catalytic aspartic acids (e.g., human Ago4 and Hiwi2) would be predicted to lack the capacity to cleave RNA. In addition to the aspartic acid diad, a third catalytic residue (histidine in human Ago2), located on the α H helix, most likely contributes to metal binding at the catalytic site (Nowotny et al. 2005; Rivas et al. 2005).

MODEL FOR GUIDE STRAND SELECTION

The *A/Piwi*–RNA complex potentially also represents the initial phase of RISC association with an siRNA duplex; here, the target strand would represent the passenger strand. Argonautes are required for efficient unwinding of the siRNA duplex (Okamura et al. 2004). The relative stabilities of the ends of an siRNA duplex determine strand fate: The strand whose 5' end is located at the less stable end becomes the guide, the other (passenger) strand is discarded (Khvorova et al. 2003; Schwarz et al. 2003). Specific recognition of the siRNA-like 5' end, and partial unwinding of the duplex revealed from the *A/Piwi*–RNA complex, suggests that binding to PIWI may be the first step in siRNA duplex unwinding in RISC; PIWI could therefore participate in strand selection.

The guide–passenger duplex bound to *A/Piwi* is the exact structural analogy of the guide–target complex, predicting that the passenger strand would enter the RNase-H-like catalytic site, reminiscent with that of a target

strand and hence subject to cleavage. Consistent with this notion, studies from Zamore (Matranga et al. 2005) and Wang (Rand et al. 2005) have recently revealed that passenger strand cleavage is associated with RISC assembly, guide strand binding, and dissociation of the passenger strand. Similar to the situation for target strand release following cleavage, cleavage of the passenger strand near its center would significantly reduce its affinity for the guide (since nucleic acid duplex interactions are highly cooperative) promoting its dissociation. However, there is also evidence that an ATP-dependent RNA helicase also participates in passenger strand unwinding.

CONCLUSIONS

A role for the PIWI domain in mediating interactions with the 5' end of the guide RNA complements the function of the PAZ domain in recognizing RNA 3' overhangs. Thus, Argonaute functions as a scaffold protein, engaging guide RNA to optimize recognition of target RNA transcripts, particularly those that are homologous to the seed region of the guide strand. On binding the target strand, we envisage, based on the low affinity for blunt-ended strands (Ma et al. 2004), that the PAZ domain dissociates from the 3' end of the guide. The 5' anchor site of the G1-binding pocket therefore provides the fundamental interaction between Argonaute and RNA, orienting and ordering the 5' region of the guide strand for interactions with its RNA target. Our findings that both the 5' phosphate receptor site and the Slicer catalytic site are located within the PIWI domain is consistent with the result of Miyoshi et al. (2005) that the PIWI domain of DmAgo2 alone incorporates the capacity for RNA-directed RNA endonuclease activity.

The study of prokaryotic Argonautes, homologs of their eukaryotic counterparts, has provided considerable insights into molecular mechanisms of RNA silencing phenomena. Both the G1-binding pocket and RNase-H-like catalytic site are conserved throughout evolution. Data from our laboratory showed that disrupting the invariant carboxy-terminal carboxylate position of *A/Piwi* significantly diminished *A/Piwi*-RNA interactions (Parker et al. 2004), whereas Tuschl, Patel, and colleagues found that mutating residues in hAgo2 whose counterparts form the G1-binding pocket of *A/Piwi* (Parker et al. 2005) abolished hAgo2-mediated-RNA cleavage (Ma et al. 2005). However, it should be noted that prokaryotes are not known to utilize RNA silencing mechanisms, and the roles of prokaryotic Argonautes are not currently understood. Future studies will be directed at obtaining crystal structures of eukaryotic Slicer Argonautes in complex with RNA duplex substrates and in providing a thermodynamic explanation for the finding that the 5' seed region of miRNAs/siRNAs dominates interactions with the target mRNA transcript.

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