BMB
Reports

BMB Rep. 2015; 48(12): 643-644
www.bmbreports.org

Perspective

Single-molecule fluorescence measurements reveal the reaction mechanisms of the core-RISC, composed of human Argonaute 2 and a guide RNA

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In eukaryotes, small RNAs play important roles in both gene regulation and resistance to viral infection. Argonaute proteins have been identified as a key component of the effector complexes of various RNA-silencing pathways, but the mechanistic roles of Argonaute proteins in these pathways are not clearly understood. To address this question, we performed single-molecule fluorescence experiments using an RNA-induced silencing complex (core-RISC) composed of a small RNA and human Argonaute 2. We found that target binding of core-RISC starts at the seed region of the guide RNA. After target binding, four distinct reactions followed: target cleavage, transient binding, stable binding, and Argonaute unloading. Target cleavage required extensive sequence complementarity and accelerated core-RISC dissociation for recycling. In contrast, the stable binding of core-RISC to target RNAs required seed-match only, suggesting a potential explanation for the seed-match rule of microRNA (miRNA) target selection. [BMB Reports 2015; 48(12): 643-644]

Diverse small RNAs—including small interfering RNAs (siRNAs), microRNAs (miRNAs), and piwi-interacting RNAs (piRNAs)—play important roles in regulation of gene expression in eukaryotes. The biogenesis pathways and biological functions of these small RNAs are distinct. However, all small RNAs are incorporated into the Argonaute protein family to form an effector complex, such as the RNA-induced silencing complex (RISC) and RNA-induced transcriptional silencing complex (RITS). Therefore, Argonaute is a core enzyme for all small RNA-related gene-silencing pathways. To form a functional RISC, Argonaute is initially loaded with a double-stranded miRNA or siRNA, and then maturated by ejecting one strand (the passenger strand) while retaining the other strand (the guide strand). After the maturation process, RISC recognizes its target messenger RNA (mRNA) based on the guide-target sequence complementarity and the accessibility of the target site. Then, RISC regulates the translational process via either a slicer-dependent or -independent pathway. Although Argonaute proteins play critical roles in the functions of small RNAs, their activities in RISC have not been characterized, resulting in several unanswered questions on the mechanisms of gene regulation by miRNAs and siRNAs.

For instance, some members of the Argonaute family have slicer activity; i.e., they cleave and initiate efficient degradation of target RNAs. The slicer reaction of the catalytically active Argonautes requires high sequence complementarity between the guide and target strands. When target cleavage is inhibited due to limited base paring or lack of catalytic activity of Argonaute, the expression of target mRNAs is believed to be repressed via translation repression or deadenylation followed by mRNA degradation. However, this simple view that miRNA regulation occurs via slicer-dependent and -independent pathways based on the guide-target sequence complementarity and the catalytic activity of Argonaute proteins has been challenged by several lines of evidence. For instance, Tomari's group reported slicer-independent gene regulation to be a major pathway for plant miRNAs that regulate highly complementary target mRNAs despite being loaded on catalytically active Argonautes.

In target recognition by small RNAs, the base pairing of 2-7 or 2-8 nt of miRNAs is disproportionally important. High-resolution crystal structures of the Argonaute-guide binary complex showed that this region is preorganized favorably for target binding. Based on these observations, it has been proposed that this region functions as a seed for guide-target base pairing. Many miRNA targets predicted based on the seed-match rule

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http://dx.doi.org/10.5483/BMBRep.2015.48.12.235

Received 16 November 2015

Keywords: Argonaute, miRNA, siRNA, Single-molecule FRET

Abbreviations: miRNA, microRNA; siRNA, small interfering RNA; RISC, RNA-induced silencing complex; FRET, fluorescence resonance energy transfer

Perspective to: Myung Hyun Jo et al. (2015), Human Argonaute 2 Has Diverse Reaction Pathways on Target RNAs, Molecular Cell, Volume 59, Issue 1, p117-124, 2 July 2015, http://dx.doi.org/10.1016/j.molcel.2015.04.027

ISSN: 1976-670X (electronic edition)

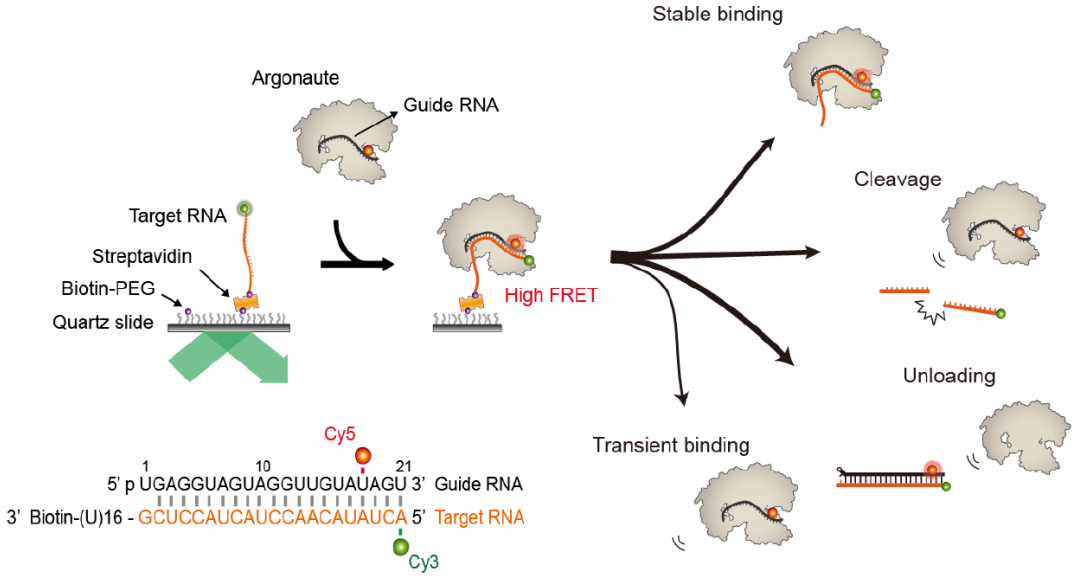

Fig. 1. Single-molecule experimental scheme and reaction pathways of core-RISC composed of human Argonaute 2 and a guide RNA.

have been validated as functional, supporting the view that miRNA targets are selected during the binding step. However, this model has not been directly tested, and how miRNA-mRNA duplexes with only 6-7 base pairs can be stable remains an open question. Furthermore, reports of noncanonical miRNA targets that do not follow the seed-match rule are abundant.

To address some of the unanswered questions related to the mechanisms of RISC reaction, we performed single-molecule fluorescence resonance energy transfer (FRET) experiments with human Argonaute 2, which allowed us to characterize the reaction mechanisms of human Argonaute 2 and their kinetic parameters (Fig. 1). First, we confirmed the previous proposal that guide-target base pairing originates at the seed region. Surprisingly, however, the reaction pathways of the core-RISC after target binding were diverse. The first type of reaction was target cleavage, followed by core-RISC recycling. In this pathway, the cleavage reaction occurred rapidly (<1 s), while dissociation of the cleaved targets was relatively slow ($\sim$17 s). Therefore, the rate-limiting step in core-RISC recycling is dissociation of the cleaved targets. The dissociation rate of the cleaved target, however, was several orders of magnitude higher than the intrinsic dissociation rate of the cleaved miRNA-mRNA duplexes, revealing that Argonaute significantly destabilizes the cleaved guide-target duplexes once the cleavage reaction occurs.

We also observed that guide-target base pairing sometimes fails to propagate over the mid-region. Crystal structures revealed that the guide strand loaded in Argonaute has kinks, suggesting that structural rearrangement of core-RISC is required for full base pairing of guide-target duplexes. We speculated that when this core-RISC structural rearrangement fails, transient binding of core-RISC occurs. The success rate of cleavage complex formation increased with Mg^{2+} concentration, revealing an additional role for Mg^{2+} ions in core-RISC, which has not been reported previously.

It has been commonly supposed that human Argonaute 2 represses the expression of the highly complementary target RNAs by cleaving the target RNAs. However, we found that a considerable portion of the core-RISC on perfect-match targets forms stable binding complexes without target cleavage. We speculated that the slicer-independent pathways could be an important mechanism of gene regulation by miRNAs, including for target mRNAs with extensive sequence complementarity. The stable core-RISC binding mode was efficiently formed on seed-only match targets, indicating that Argonaute plays an important role in stabilizing guide-target duplexes with limited base pairing. If stable binding of RISC plays important roles in the slicer-independent gene regulation by miRNAs, it is conceivable that other proteins, RNA helicases for example, regulate the function of miRNAs by destabilizing the stable RISC complexes on target RNAs. RISC in living cells, however, includes several other protein components in addition to Argonaute, which may modulate the activity of Argonaute in important ways. Human cells have Argonaute proteins other than Argonaute 2, which may have different activities. In this respect, further studies using other Argonautes and more complete RISCs are required.

ACKNOWLEDGEMENTS

This work was supported by a Creative Research Initiatives grant (Physical Genetics Laboratory, 2009-0081562) to S.H.