이학석사학위논문

Single Molecule FRET Studies on Argonaute protein

단일분자 **FRET** 방법을 이용한 **Argonaute** 단백질 연구

2014 년 8 월

서울대학교 대학원

생물물리 및 화학생물학과

신 수 철

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이 논문을 이학석사 학위논문으로 제출함

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신수철의 이학석사 학위논문을 인준함

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Master's Thesis

Single Molecule FRET Studies on Argonaute protein

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Abstract

RNA silencing processes have a variety of functions, including gene expression control. These processes, such as the RNA interference and microRNA pathways use small RNAs that play crucial roles in regulating gene expression. However, small RNAs cannot work alone. They should, with Argonaute proteins and other cofactors, form RISCs (RNA-induced silencing complex) to do their gene silencing functions. Although overall the mechanism of RNA silencing processes is well understood, many molecular details of these pathways are not. Here, by using single-molecule fluorescence resonance transfer (FRET) assays and human Argonaute 2 (hAgo2), we monitor the molecular details and kinetics of RNA silencing pathways.

Key words: Argonaute, RISC, Single Molecule FRET

Student number: 2012-23915

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1. Introduction

RNA silencing processes, which are routinely observed in eukaryotes, have a variety of functions [1-2]. For example, many eukaryotes use miRNA pathways to control gene expression on the posttranscriptional level [3-5]. In human genes, presumably 30% of them are related to this regulation process [6]. Also, siRNA pathway is widely used in biological research to silence genes of interest and is investigated as RNA therapeutics [7-8].

In these RNA silencing processes, Argonaute proteins play key roles by mediating the maturation of small RNA, followed by sequence-specific interaction with target RNA [1-5]. Targeted RNAs are silenced either by cleavage reaction catalyzed by some Argonaute proteins or by Argonaute-mediated recruitment of additional silencing factors [9].

Furthermore, biochemical, structural, and computational studies suggest that Argonaute protein divide their small RNA into several domains with distinct functions [10]. The most important domain is the seed region, which consists of guide nucleotides 2-7 (g2-7) or 2-8 (g2-8). The seed sequence is the primary determinant of target selection in the RNA silencing pathways [10-11]. Mid region, which comprises g10-14, is crucial for

target cleavage reaction [10]. Mid region should be extensively base paired to slice the target. Also, it is known that tail region (g18-21) facilitates Argonaute loading [10].

In spite of these extensive studies, detailed mechanism and function of Argonaute in miRNA or siRNA pathways at the molecular level are still elusive. Here, by using single-molecule FRET and human Argonaute 2, we characterized the molecular details of Arognaute function in RISC action.

2. Experimental Scheme

In order to observe molecular dynamics of RISCs by using single-molecule FRET (smFRET) assyas, we used Cy5 (FRET acceptor) labeled let-7 miRNA as a guide RNA. Target RNA, which has complementary sequence with the guide RNA, is labeled by Cy3 (FRET donor). The target strand has a spacer (a 16-nt uridine) between biotin and the target sequence to avoid surface hindrance. For smFRET experiments, the Cy3-labeled target strand was immobilized on a polymer-coated quart via streptavidin-biotin interaction. Then, RISCs were added into the detection chamber (Figure1). Changes in fluorescence intensities were monitored over time in a total-internal-reflection fluorescence microscope.

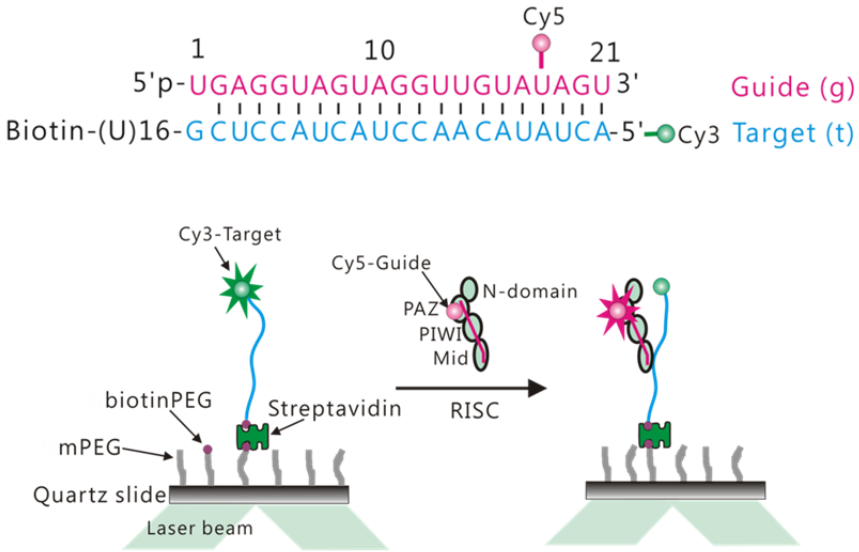

Figure 1. Single molecule FRET experimental scheme to observe Argonaute functions with guide and target RNAs. After target RNA molecules (Cy3 labeled) were immobilized, RISCs (Argonaute proteins complexed with Cy5 labeled guide RNAs) were injected to the channel.

3. Results

3.1 Acceleration of target binding by Argonaute

After injecting 2nM RISCs, we observed fast appearance of Cy5 spots and simultaneous disappearance of Cy3 spots (Figure 2a). In contrast, when the guide strand (2nM) was added, the target binding rate was much slower than that of RISC (Figure 2a), indicating that target binding is accelerated by Argonaute.

The quantitative analysis shows that the binding rate of RISC is increased by 10-fold compared with that of free guide (Figure 2b). The histogram of binding time is well fitted to single exponential function.

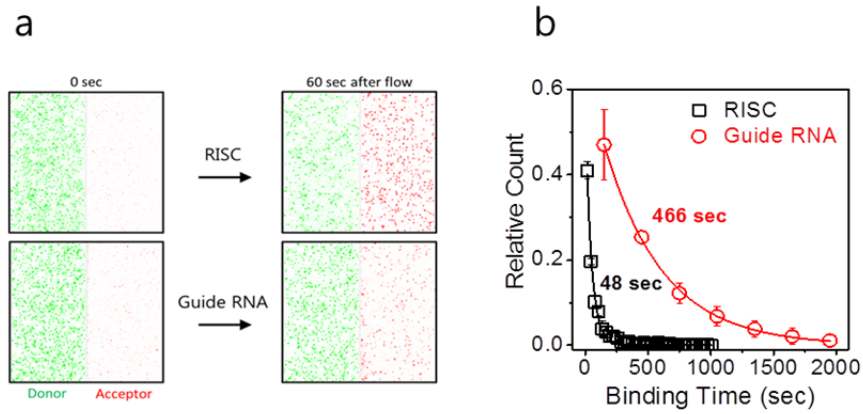

Figure 2. (a) Single molecule images of donor channel (green) and acceptor channel (red) after RISC or guide injection. (b) Binding times of RISC (black) and free guide (Red). They are well fitted to single exponential functions.

To understand how Argonaute affects the target binding, we sequentially introduced two bases mismatches to guide-target base paring (Figure 3a). Kinetics analysis of these two bases mismatch mutants shows that binding rate decreases significantly in m:2-3, m:3-4, m:4-5, m:5-6, and m:11-12(Figure 3b). This suggests that not only seed region (g2-7 nt) but g11-12 increases target binding rate substantially.

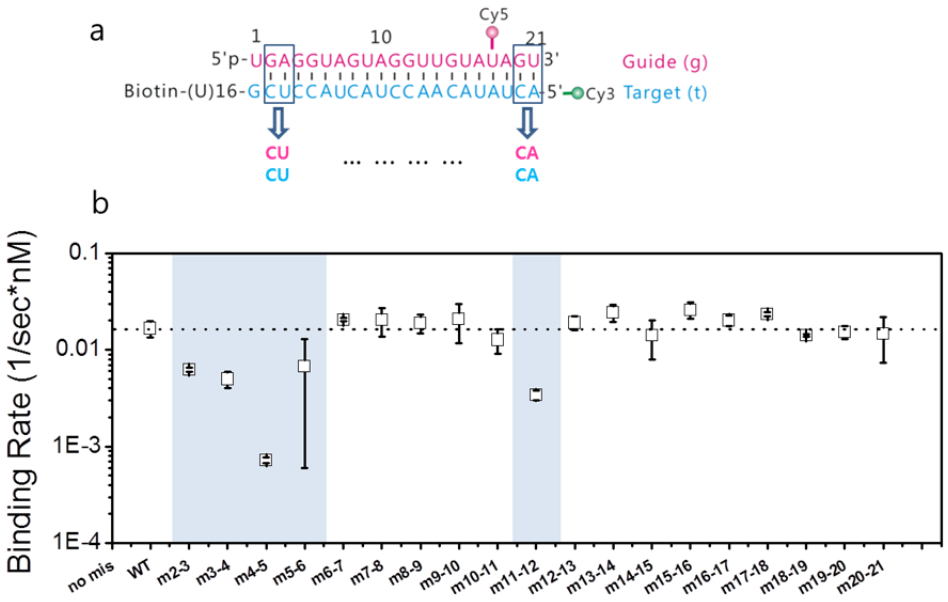

Figure 3. (a) Two bases mismatch mutants of target and guide RNA strands. (b) Binding rates of two bases mismatch mutants.

3.2 Dynamic and diverse pathways after target binding

3.2.1 Cleavage and transient binding

After target binding of RISCs, we observed the target cleavage by the RISCs in 28% of binding events. Furthermore, we could classify cleavage events into two types. In type 1, FRET signal disappeared earlier than Cy5 signal (Figure 4a). In type 2, FRET and Cy5 signal disappeared simultaneously (Figure 4b).

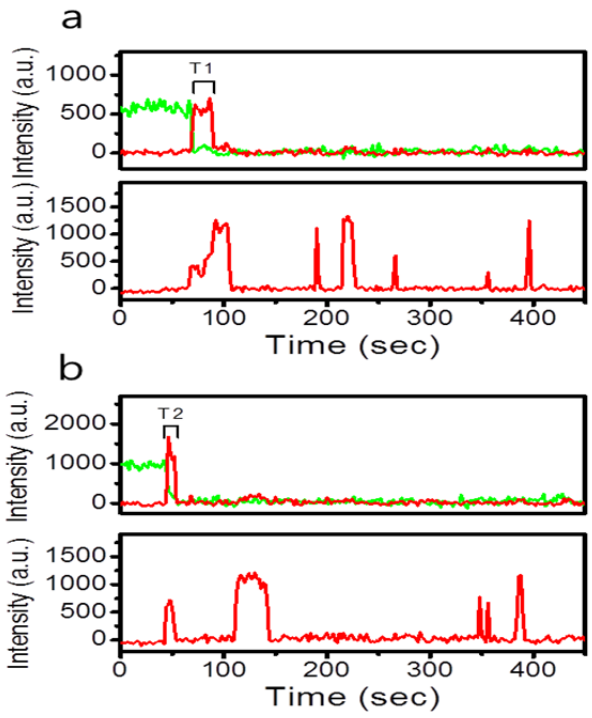

Figure 4. Two types of cleavage events. (a) FRET signal (above) disappear earlier than Cy5 signal (below). (b) FRET signal (above) and Cy5 signal (below) disappear simultaneously.

We interpret the situation of type 1 as that 5' portion of the cleaved target is dissociated earlier than 3' portion from the RISC. In contrast, type 2 could be interpreted as that 3' portion of the cleaved target is dissociated earlier than 5' portion from the RISC. We could distinguish cleavage events from photobleaching events by the repetitive Cy5 signal jumps. Cy5 signal jumps could be viewed as that, after the dissociation of RISCs from cleaved target, another RISC binds to remained 3' portion of target strand. Quantitative analysis says that type 1 is 73 % and type 2 is 27 %. Also, dwell time T1in figure 4a and dwell time T2 in figure 4b are similar (Figure 5), suggesting that these two types of reactions are branched reactions and type 1 reaction is faster than type 2.

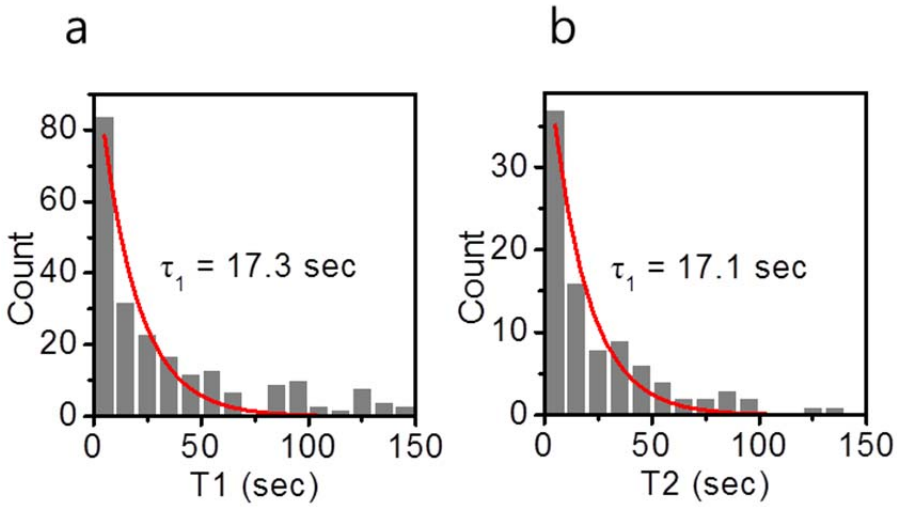

Figure 5. (a) Dwell time histogram of T1. (b) Dwell time histogram of T2. Their decay time constants are similar

Actually, the cleavage event has at least two sequential steps: target slicing and product release. Different probabilities of Type 1 and Type 2 but similar dwell time of T1 and T2, together with single exponential behavior of T1 and T2, indicates that there exist a rate limiting step in the cleavage event and actually it is the product release step.

To understand molecular details of the rate limiting step, we prepared truncated target which is exact 3' portion of the cleaved product. Dissociation rate between this truncated target and guide RNA was much longer than T1 and T2 (Figure 6). Therefore, we conclude Argonaute has an active role in the rate limiting step, which is very critical for the enzyme recycling.

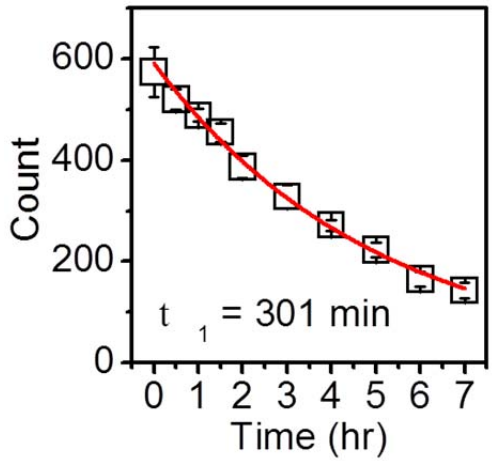

Figure 6. Binding lifetime of guide-cleaved target (3' portion of product) duplex without RISC.

In addition to cleavage events, we observed the transient binding events that RISCs were dissociated from target RNAs without target cleavage (Figure 7).

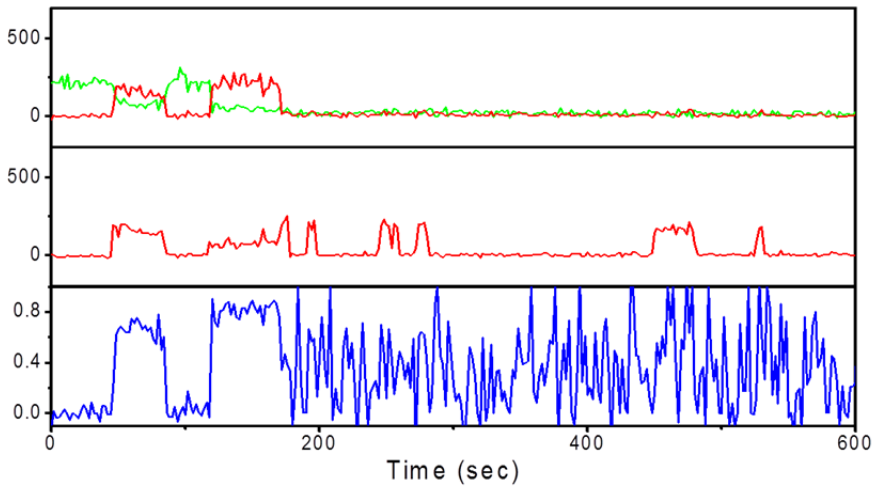

Figure 7. Representative trace showing transient binding. At first binding, RISC was dissociated from target without cleavage (transient binding). Second high FRET signal shows typical target cleavage event by another RISC.

Among all post-binding events, 10% are transient binding that has 132-s average binding lifetime. Interestingly, FRET efficiency of transient binding complex is lower than cleavage competent complex (Figure 8), indicating that in the transient binding mode, RISC and target do not form extensive base pairing in the tail region.

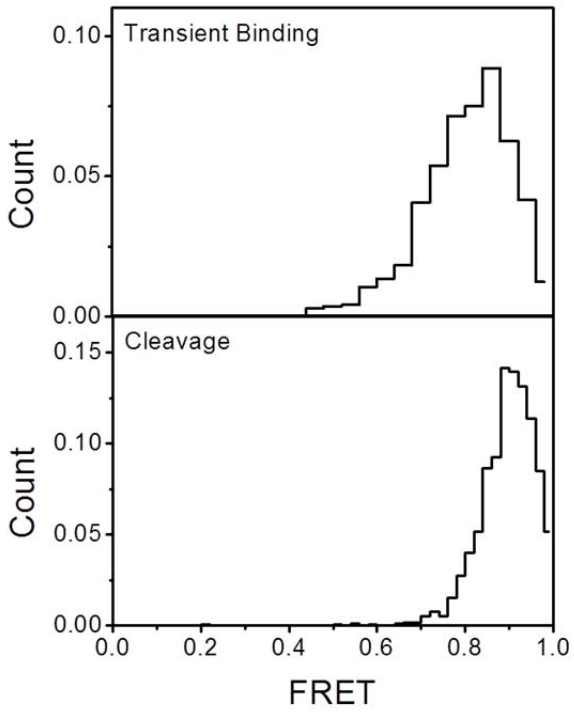

Figure 7. FRET histograms of transient binding tertiary complexes and cleavage competent tertiary complexes. FRET efficiency of transient binding complexes is lower than that of cleavage complexes.

3.2.2 Stable binding and Argonaute dissociation

We also found long binding FRET traces after target binding. Unlike cleavage or transient binding, high FRET signal maintained for a long time (Figure 8).

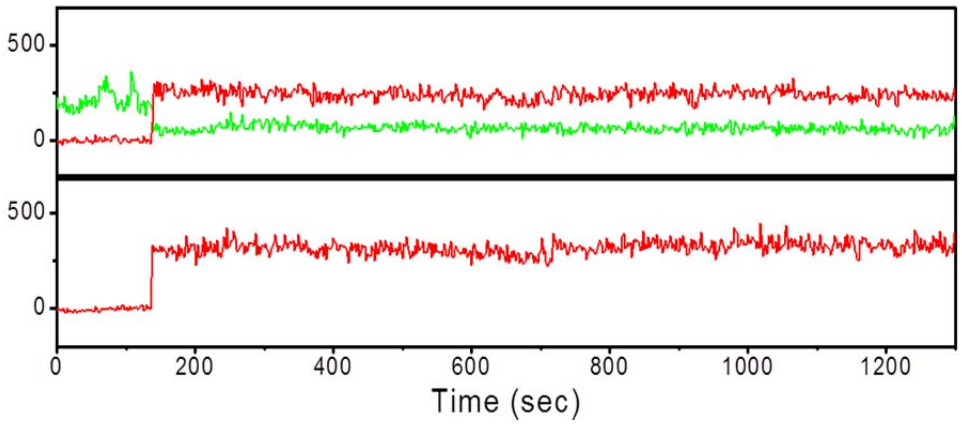

Figure 8. Representative traces of long binding complexes.

Significant portion (63%) of post-binding event was long binding event. Two possible situations can show this type of trace. First possibility is that RISC binds to the target and then only Argonaute protein dissociates, while guide strand is still base paired with immobilized target strand. This can be happened because guide-target RNA duplex is very stable. Another possibility is that RISC binds to target and remained for a long time. Then what is the actual situation? To address this question, we treated the long binding complex with RNase III, which cleaves RNA duplex efficiently (Figure 9).

Interestingly, RNase III treatment resulted in 55% disappearance of high FRET signals (Figure 9), indicating that Argonaute dissociation and stable RISC binding coexist in long binding species.

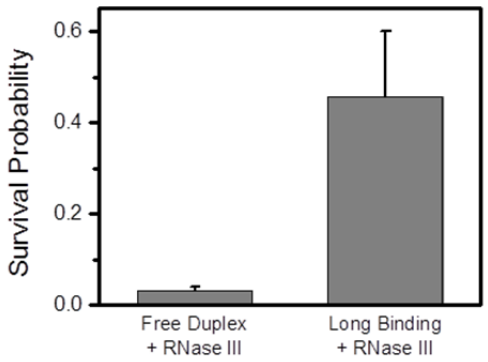

Figure 9. Survival probability of High FRET signals after RNase III treatment. While RNase III treatment to guide-target RNA duplex removes almost all high FRET signals, RNase III treatment to long binding species removes only 55% of high FRET signals.

Another question is whether the target is cleaved or not in the stable RISC binding complex. If the target is cleaved but not dissociated from RISC, this indicates extremely inefficient cleavage product release of RISC. To understand molecular details of this stable binding mode, we treated Protease K (which can digest Argonaute protein efficiently, Figure 10c) and then treated 3.5M urea (which can efficiently denature the guide-cleaved RNA duplex but cannot denature the guide-target RNA duplex, Figure

10a, b) with long binding species (Figure 10). If target RNAs are cleaved in stable binding complexes, 3.5M urea after Protease K treatment removes 45% high FRET signals at most. However, Figure 10c shows that after the Protease K treatment, 3.5M urea cannot denature the almost all RNA duplex, suggesting that intact target RNA, which is not cleaved, is base-paired with guide RNA in the stable binding mode. Therefore, we concluded that stable binding does not represent the inefficient product release but could indicate another active role of Argonaute in miRNA regulation pathways.

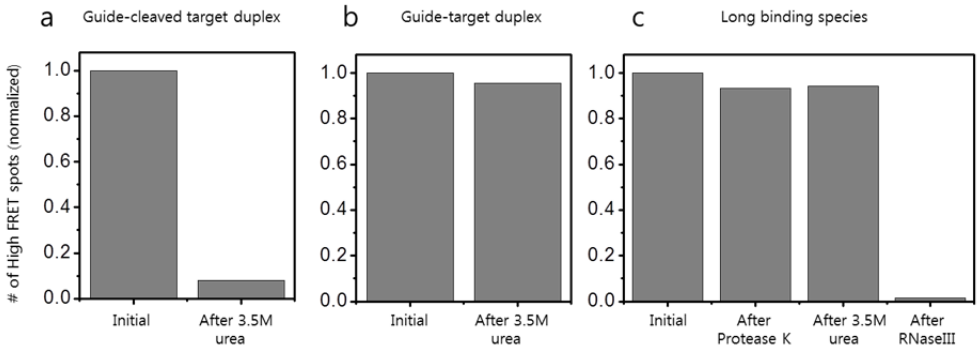

Figure 10. Normalized high FRET numbers of (a) guide-cleaved target duplex after 3.5M urea treatment, (b) guide-target duplex after 3.5M urea treatment, (c) long binding species after sequential treatment of protease K, 3.5M urea, and RNaseIII. Almost all disappearance of high FRET spots after RNaseIII guarantees that Argonautes in the stable binding complexes are completely digested by the Protease K proteins.

3.3 Roles of Magnesium Ions in RISC activities

Another finding is that magnesium ions play important roles in the RISC activities. We observed that magnesium ions promoted the cleavage reaction by two different ways. First, magnesium ions help to form cleavage competent tertiary complex. When concentration of magnesium ions in reaction pathways increased, population of cleavage complexes was increased whereas that of transient binding complexes was decreased (Figure 11a). This suggests that magnesium ions facilitate cleavage reaction via shifting the transient binding modes to cleavage modes. Second, recycling rate of cleavage reaction was increased when we increased the magnesium concentration (Figure 11b), indicating that magnesium ions help the cleavage reaction by increasing the recycling rate.

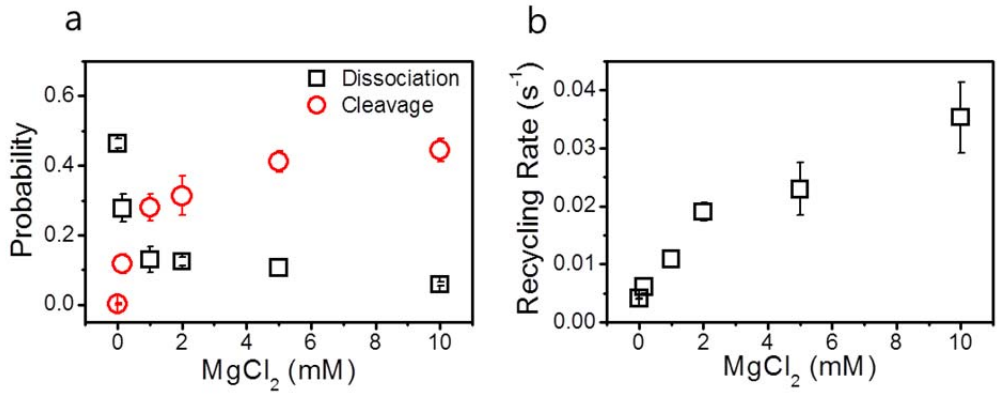

Figure 11. (a) Probabilities of target cleavage (black) and transient RISC binding (red) at varying Mg2+ concentration. (b) RISC recycling rates at varying Mg2+ concentration.

4. Conclusion

Argonaute protein has been well known as a core protein of RISC which is post-transcriptional regulator guided by small RNAs. The behavior of guide-Argonaute binary complex interacting with target RNA is analyzed at the single-molecule level by using smFRET experiments. To begin with, Argonaute facilitated the target recognition of guide RNA. Two bases mismatch effects on target binding indicate that 2-7nt and 11-12nt region of RISC play a critical role in target recognition of RISC. We think that this accelerated target binding is due to the preorganization of 2-7nt region of RISC and the exposure of 11-12nt to the solvent side in the RISC.

Also, we characterized dynamic and diverse post-binding events in RISC action. We found that there exist four distinct reaction pathways after target binding. First pathway is target cleavage reaction. We confirmed that dissociation of cleaved target is the rate limiting step in the cleavage reaction. In addition, we identified that Argonaute has an active role in this rate limiting step by accelerating product release, which is very critical for the enzyme recycling. Second pathway is the transient binding that could be interpreted as that, due to the kink of the RISC, guide-target base pairing could not be extensive at the tail region of RISC. Third pathway is Argonaute dissociation that Argonaute unloads its guide and is dissociated from guide-target duplex. This pathway may have an advantage in the reuses of Argonaute proteins in reaction pathways. Last type is the stable binding mode which can be effectively used in miRNA regulation pathways by recruiting of additional silencing factors.

Finally, we found the roles of magnesium ions in the reaction pathways. Magnesium ions increase the chance of forming cleavage competent complex and increase the recycling rate, suggesting that magnesium ions are very important factors in the cleavage reaction of RISC.

5. Materials and methods

Oligonucleotide preparation. RNA target and guide oligonucleotides were purchased from ST Pharm (South Korea). RNA target oligonucleotides labeled with Cy3 at the 5'-terminal, and their 3'-terminals were biotinylated for immobiliaztion after polyU linker to minimize the steric hinderance. The 17th or 18th base of RNA guide oligonucleotides were labeled with Cy5 via internal amino modifier containing six carbon chains. All nucleotide sequences are shown below, where mismatch bases were indicated by red letters.

Single-molecule FRET experiment. Quartz slides and coverslips were cleaned with piranha solution (mixture of 3:1 concentrated sulfuric acid:30% (v/v) hydrogen peroxide solution) for about 20 minutes, and coated with a 40:1 mixture of polyethylene glycol (m-PEG-5000; Laysan Bio, Inc.) and biotinylated PEG (biotin-PEG-5000; y6Laysan Bio, Inc.). A flow cell was assembled by sandwiching double-sided sticky tape (3M) between a quartz slide and a coverslip. Polyethylene tubing (PE50; Becton Dickinson) was connected to the flow cell for convenient buffer exchanges. For surface immobilization of target RNAs, Streptavidin (0.2 mg/ml, Invitrogen) and target RNAs were sequentially injected into the flow cell. Unconjugated target RNAs were washed out. To form core RISCs, guide RNAs (40nM) were incubated with excessive human Argonaute 2 (0.2-1 μM) at 23℃ for an hour. In this condition, no free guide was detected (Figure S9). RISC solution was diluted in an imaging buffer (10 mM Tris

(pH8.0) with KCl (135mM), MgCl2 (1 mM unless mentioned otherwise), Trolox (1 mM, Sigma), glucose oxidase (1 mg/mL, Sigma), catalase (0.04 mg/mL, Sigma), glucose (0.4% (w/v), Sigma), and RNase inhibitor (Promega)) just before experiments, and injected into a flow cell while single-molecule FRET images were taken using a prism-type total-internal-reflection-fluorescence microscope equipped with an electron-multiplying CCD camera (IXON DV597ECS-BV; Andor Technology) at a frame rate of 0.5-10 Hz (Roy et al., 2008). During experiments, the flow cell temperature was maintained at 37℃ using a commercial temperature control system (Live Cell Instruments, South Korea).

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국문 초록

RNA silencing 과정은 유전자 발현 조절을 포함한 다양한 기능을 가지고 있다. 예를 들어 RNA 간섭현상 및 micro RNA pathway 등은 작은 RNA를 사용하여 유전자 발현을 조절한다. 그러나 이러한 작은 RNA들은 혼자서 이 과정을 수행할 수 없고 Argonaute이라는 단백질 및 다른 cofactor들과 함께 RISC(RNA-induced silencing complex)라는 리보핵산 단백질을 형성하여 그 기능을 수행한다. 지금까지 RNA silencing에 대한 대략적인 메커니즘 연구는 많이 수행되었지만 분자적 레벨에서의 수준은 아직 연구되지 않았다. 본 연구에서는 단일분자 FRET을 이용하여 RNA silencing 현상에서 중요한 역할을 담당하고 있는 Argonaute 단백질의 작동 메커니즘을 밝혀내었다.

핵심어: Argonaute, RISC, 단일분자 FRET

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