論文の内容の要旨
Dissertation Abstract

論文題目
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Biochemical dissection of Argonaute domain function in RISC assembly
(RISC形成における Argonaute タンパク質の生化学的ドメイン機能解析)

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Argonaute (Ago) proteins are highly conserved among species and can be structurally characterized by their N, PAZ, MID and PIWI domains. Functionally, they effectuate RNA interference or mediate translational repression and deadenylation/decay of target mRNAs, as a component of the RNA-induced silencing complex (RISC).

Formation of mature, functional RISC requires is called RISC assembly, and entails (I) loading of a small RNA duplex into Ago and (II) unwinding of the two strands within Ago. While the ability to unwind small RNA duplexes is one of its hallmark features, how Ago enacts unwinding has remained enigmatic.

Since the roles of the PIWI, MID and PAZ domains are becoming increasingly well understood, my doctoral research focused mainly on the functionally obscure N domain of Ago. I investigated if and how the N domain contributes to RISC assembly. By selectively mutating well-conserved residues in human Ago2 and performing biochemical analyses, I have identified that the N domain of human Ago2 does not contribute to duplex loading, but is critical for duplex unwinding. In addition, I studied fly Ago2 RISC assembly. I discovered that the interdomain linker of fly Ago2 is crucial for RISC loading, while the extensive polyglutamine rich region, that takes up about a quarter of the full-length fly Ago2, is redundant.

Results

To investigate the role the N domain partakes in RISC assembly, I generated 41 N mutants for human Ago2. Human Ago2 is a slicer-competent Ago protein capable of unwinding duplexes via slicer-dependent and slicer-independent unwinding. First, I examined whether the 41 N mutants could efficiently undergo RISC assembly using siRNA duplexes. Characteristically, siRNA duplexes lack mismatches, are more rigid and stably paired than miRNA duplexes, and depend on passenger strand cleavage for efficient unwinding and mature RISC formation. RISC loading was unaffected for all 41 mutants. Strikingly, 16 out of 41 (~39%) mutants were unable to

efficiently unwind siRNA duplexes and form mature RISC. This indicated that the N domain participates in slicer-dependent, siRNA unwinding.

To better understand the duplex unwinding defect, I examined whether N mutants could cleave siRNA duplex passenger strands, a crucial step in slicer-independent unwinding. Interestingly, no N mutants could cleave passenger strands efficiently. To determine whether the unwinding defect observed for siRNA duplexes was strictly the result of inhibited passenger strand cleavage, I employed a fully complementary, nicked duplex lacking the phosphodiester bond between nucleotide 9 and 10 of the passenger strand; the putative cleavage site. Since N mutants were inept at unwinding nicked duplexes, unlike catalytic mutant, this suggests that the N domain affects not passenger cleavage itself, but an preparatory step important for duplex unwinding.

The observation that N mutants have slicer-independent unwinding defects (using nicked duplexes) was confirmed using miRNA duplexes. I found that while RISC assembly was unimpaired for all 41 mutants, 4 (~10%) mutants were unable to unwind miRNA duplexes efficiently. Interestingly, these N mutants also had unwinding defects for siRNA duplexes, suggesting that the defects seen for miRNA (unwinding) and siRNA (passenger strand cleavage and unwinding) duplexes are coupled.

Since N mutants had less unwinding defects using miRNA duplexes and such duplexes are less stable than siRNA duplexes, I examined the relationship between duplex stability and the extent of the duplex unwinding defect. I investigated if making a miRNA less stable could alleviate the unwinding defect of a strong N mutant. Introduction of three mismatches in a duplex largely rescued the unwinding defects; illustrating that more stable duplexes are more dependent on the N domain to unwind properly. Interestingly, when we rescued N mutants using a less stable miRNA duplex and performed target cleavage assay we found N mutants could competently cleave target RNA, unlike passenger strand cleavage, suggestion a differential role for the N domain during RISC assembly and RISC function.

Collectively, I discovered that the N domain of human Ago2 acts as the initiator of duplex unwinding during RISC assembly, and showed that this action is critical for bridging RISC loading and strand separation for both miRNA and siRNA duplexes. N mutations resulted in duplex unwinding defects, while RISC loading was unaffected. My results reveal RISC assembly is a tripartite process composed of: (I) RISC loading; Ago

undergoes conformational opening and loads a small RNA duplex. (II) Wedging; the end of the duplex is pried open and contorted via active wedging by the N domain, positioning the duplex for unwinding. (III) Unwinding; the passenger strand is removed via slicer-dependent or slicer-independent unwinding, forming mature RISC (**Figure 1**).

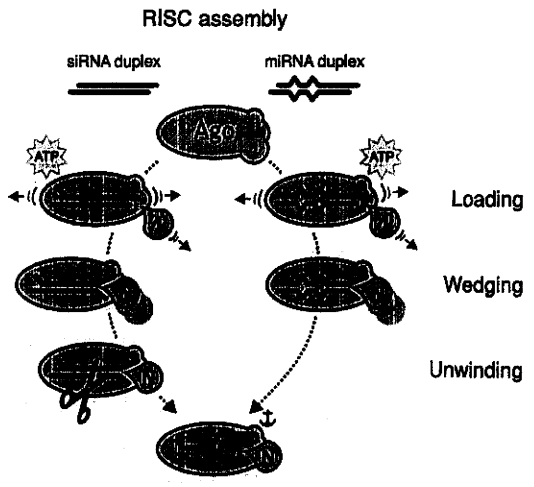

Figure 1 – The N domain in RISC assembly. I found that the N domain drives duplex unwinding for both miRNA and siRNA-like duplexes and postulate a new model for RISC assembly. Duplexes are first loaded into Ago protein, then the N domain positions the duplex appropriately for unwinding via 'wedging', i.e., prying the duplexes open at one end.

By studying fly Ago2 I found that the interdomain linker that connects the N-PAZ lobe with the MID-PIWI lobe is important for RISC loading. At first, via cleavage assays, I found that mutations in this linker nearly completely inhibit target cleavage. Further experiments revealed that duplex unwinding was strongly impaired, too. Via immunoprecipitation experiments it seemed that RISC loading was not as severely affected as duplex unwinding. However, additional studies revealed that siRNA duplexes were in fact not loaded into fly Ago2 but were indirectly bound. By performing crosslinking experiments, I found that siRNA duplexes were retained by RISC loading complex (RLC) components Dicer-2 and R2D2 and that linker mutant fly Ago2 was distinct from an siRNA binding mutant. In case of linker mutant fly Ago2, the RLC-siRNA complex cannot transfer the siRNA duplex to fly Ago2, indicating that linker mutant fly Ago2 is a RISC loading mutant stuck in a transition that is normally very transient. Additional studies, focused on the polyglutamine rich region of fly Ago2, revealed that this amino-terminal extension—that takes up about a quarter of full-length fly Ago2—is completely redundant for RISC function.