Similarities between Argonautes and the alpha-sarcin-like ribotoxins: Implications for microRNA action

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Abstract: We report structural, functional, and biochemical similarities between Argonautes, the effector proteins of RNA-induced silencing complexes (RISCs), and alpha-sarcin-like ribotoxins. At the structural level, regions of similarity in the amino acid sequence are located in protein loops both in the ribotoxins and in the Argonautes. In ribotoxins, these protein loops confer specificity for a highly conserved segment of ribosomal RNA, the Sarcin-Ricin-Loop (SRL) that undergoes cleavage by the ribotoxin ribonuclease. This leads to suppression of translation. In addition to the structural similarity with ribotoxins, the Argonaute proteins (Ago) show both functional and biochemical parallels. Like the ribotoxins, the Agos exhibit ribonuclease activity and like the ribotoxins, translational suppression mediated by miRISC-resident Ago is accompanied by intact polysomes. Furthermore, in both translationally suppressed systems, the puromycin reaction, reflecting correct translocation and peptidyl-transferase activities, is unharmed. These findings support a mechanism for Ago-miRISCs whereby regulated cleavage of ribosomal RNA leads to translational suppression.

Keywords: ribotoxins; argonaute proteins; SRL; miRNA; translational suppression; alpha-sarcin; rRNA cleavage

Introduction

Ribotoxins, alpha-sarcin representing one class and ricin another, potently suppress translation at the elongation stage by cleaving a specific site within the large 28S rRNA component of the 60S ribosomal subunit.^{1–3} The extraordinary specificity of these toxins is restricted to one cleavage site within the 7000 or so nucleotides. The alpha-sarcin-like ribotoxins cleave³ the phosphodiester bond on the 3' side of G4325 in 28S rRNA, generating a distinctive 3'

α -fragment. The ricin-like ribotoxins depurinate the A4324 residue immediately upstream of the site of α -sarcin-mediated cleavage.^{4,5} These cleavage sites are located within a ribosomal RNA loop, designated the sarcin-ricin-loop or SRL.^{3,6–9} Cleavage by alpha-sarcin-like ribotoxins or depurination by the ricin-like toxins accounts for their toxicity and ribosome-inactivating activity. The SRL includes some 20 nucleotides and is the most conserved region⁷ in both 28S rRNA and bacterial 23S rRNA, showing sequence conservation from bacteria, through worms to humans.

The Argonaute (Ago) proteins, like the ribotoxins, also inhibit protein synthesis [for recent reviews, see

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Refs. 10–12]. In contrast to the ribotoxins, they are an endogenous set of proteins and represent the principal effectors of protein synthesis inhibition mediated by both short interfering RNA (siRNA) and microRNA (miRNAs).

Intriguingly, like the ribotoxins, the Ago proteins also possess endoribonucleolytic activity. This has been extensively documented for the Ago2 protein,^{13,14} the executor component of RNA-Induced-Silencing-Complexes (RISCs), consisting of siRNAs (siRISCs). For siRISC-mediated translational inhibition, full complementarity between the siRNA and target mRNAs leads to cleavage of mRNA mediated by the Ago Slicer ribonuclease. In the absence of complete complementarity between the mRNA target and translational miRNA, suppression mediated by miRNA-containing RISCs, does not involve direct cleavage of mRNA. Although originally not considered a ribonuclease, Ago1, the critical component of miRISC-mediated translational suppression, has recently been shown to also possess ribonuclease activity.¹⁵ These findings suggest that like Ago2, the ribonucleolytic activity of Ago1 may be vital for translational suppression mediated by miRISCs. However, the mechanism of action of miRISCs in translational suppression remains enigmatic.

The puzzle in translational suppression mediated by miRISCs stems principally from the fact that despite extensive homology between the Ago1 protein of miRISCs and the Slicer Ago2 protein of siRISCs, and notwithstanding the recently documented Ago1 ribonucleolytic activity, miRISCs, unlike the siRISCs, do not directly mediate mRNA cleavage.

As for the alpha-sarcin-like ribotoxins, they potentially suppress translation and possess ribonucleolytic activity, yet the inhibition they exert is not accompanied by mRNA cleavage.

These likenesses in translational suppression mediated by alpha-sarcin-like ribotoxins and miRISCs prompted us to investigate whether there are similarities in the molecular make-up of the Ago proteins and the ribotoxins. We show here a marked similarity between these seemingly diverse groups of proteins. Intriguingly the homology is localized to regions of the ribotoxins responsible for their binding to the critical Sarcin-Ricin-Loop, subsequently cleaved by the ribotoxin. Additionally, a review of the literature reveals a paradoxically intact puromycin reaction, observed not only in ribosomes translationally suppressed by alpha-sarcin-like ribotoxins but also in ribosomes suppressed by miRISCs. Because of these common features, our findings indicate that translational suppression mediated by alpha-sarcin-like ribotoxins and the Argonaute proteins making up the miRISCs share a similar mechanism of action.

Results and Discussion

Sequence similarity between alpha-sarcin-like ribotoxins and Argonaute proteins-localization in protein loops recognizing the SRL

Ribotoxins contain motifs determining a structural core common to other ribonucleases, and especially T1-like ribonucleases. As distinct from the T1 ribonucleases, ribotoxins include extra protein domains that have been proposed to be naturally genetically engineered onto the T1 RNase skeleton.^{16,17} These domains typically form large loops numbered 1 through 5 (Figs. 1 and 2). The loop-like elements of the ribotoxins direct the RNase component of the ribotoxin to a specific region on the ribosome, the SRL (Fig. 2, right panel).

We observed similarities between restrictocin, a member of the ribotoxin family, and the Argonaute (Ago) proteins. A comparison of the restrictocin ribotoxin with bovine Ago2 is presented in Figure 1. Because of the high conservation of the amino acid sequence, similar results are obtained when the comparison is made with Agos1, 2, 3, and 4, both from murine and human origin. Moreover, other ribotoxins of the alpha-sarcin family also yield profiles similar to those of the Argonaute proteins.

Specifically, 48 amino acids (32%) of the restrictocin protein are identical with Ago2 [Fig. 1(A)]; allowing for conservative substitutions, this similarity increases to 65%. For each homologous tract, the similarity is localized predominantly in the restrictocin-specific sequences typically spanning seven or more amino acids. Examination of the ribotoxin and Argonaute proteins showed that the structure-breaking amino acids, proline and glycine, usually terminate the homologous regions [highlighted in Fig. 1(A) by double underline].

It bears mention that critical lysine residues present in both restrictocin and Argonaute proteins [Fig. 1(B)] are conserved in all human and mouse Argonaute proteins Agos1–4.

Ribotoxin loop 3 is especially pertinent to recognition of the SRL RNA structure. It includes the pentapeptide sequence **DSKKP**. This sequence is critical to the interaction of the ribotoxin¹⁶ with a bulged-G motif⁷ within the SRL RNA structure, promoting specific binding and substrate cleavage. Indeed, mutations of the lysine residues in **DSKKP** so vital for SRL recognition, ablate ribotoxin translational inhibitory activity.^{16,17} It is thus striking that this string **DSKKP** is conserved in Ago proteins [Fig. 1(A,B)] as **DGKKP** [Fig. 1(B)] and that the extended 11 amino acid ribotoxin sequence ¹¹²**YKFDSKKPKED** appears within *Pyrococcus furiosus* Argonaute as ¹⁹⁴**FEEYTKPKLD** (Fig. 2, note ribotoxin **FD** appears in the reverse order in *Pyrococcus* as **EY**).

According to the three-dimensional structure determined by X-ray crystallography,¹⁸ it is clear

A Homology of ribotoxin-restrictocin with Ago2

Restrictocin	Aspergillus restrictus AAA32707
Argonaute 2	Bos Taurus NP_991363
	<div> <div>β1</div> <div>β2</div> <div>Loop 1</div> </div>
restrictocin	ATWTCINQQLNE-----KTNKWEDKRLLYNQAKAESNSHH-APLSDG (#41)
Argonaute 2	EVVTFGHQSVRRP[62aa]HCGQMKRKYRVCMVTRRPA-SHQTFPLQQE[17aa]
	<div> <div>Loop 1</div> <div>β3</div> <div>Loop 2</div> </div>
restrictocin	KTGSSYPHWFTN--G-----YDNGKLIKGRTE-----IKFGKADCDRPPK (#80)
Argonaute 2	KLVLRYPHLPCLQVG[88aa]YGRNKAIA--TP[17aa]IKVWAIACFAPQR[46aa]
	<div> <div>Loop 2</div> <div>β4</div> <div>Loop 3</div> </div>
restrictocin	HSQNGMGKDDHYLLEFE-----TFPDG-----HDYKFDSKKP (#112)
Argonaute 2	HLKNTYAGLQLVVVILE[54aa]LLPQG[17aa]HPPAGDGKKP[9aa]
	<div> <div>Loop 3</div> <div>β5</div> <div>Loop 4</div> <div>β6</div> <div>Loop 5</div> </div>
restrictocin	KEDFG-----PARVIYT-----YPNKVFCGIVAHQR-GNQG-----DLRLCSH (#149)
Argonaute 2	DAHFN[37aa]PTRIIFY[43aa]HHTRLFCTDK-NEVRGKSG[18aa]DFYLCSH
B	
Restrictocin	<div> <div>**</div> <div>IKFGKADCDRPPK</div> <div>*</div> <div>HDYKFDSKKP</div> <div>*</div> <div>YPNKVFCGIVAHQR-GNQG</div> <div>*</div> <div>DLRLCSH</div> </div>
hArgonaute-1	IKVWAIACFAPQK HPPAGDGKKP HHTRLFCAADK-NERIGKSG DFYLCSH
hArgonaute-2	IKVWAIACFAPQR HPPAGDGKKP HHTRLFCTDK-NEVRGKSG DFYLCSH
hArgonaute-3	IKMWAIACFATQR HPPAGDGKKP HHTRLFCAADR-TERVGRSG DFYLCSH
hArgonaute-4	IKVWAVACFAPQK HPPAGDGKKP HHTRLFCAADK-TERVGRSG DFYLCSH
mArgonaute-1	IKVWAIACFAPQK HPPAGDGKKP HHTRLFCAADK-NERIGKSG DFYLCSH
mArgonaute-2	IKVWAIACFAPQR HPPAGDGKKP HHTRLFCTDK-NEVRGKSG DFYLCSH
mArgonaute-3	IKMWAIACFATQR HPPAGDGKKP HHTRLFCAADR-TERVGRSG DFYLCSH
mArgonaute-4	IKVWAVACFAPQK HPPAGDGKKP HHTRLFCAADK-MERVGRSG DFYLCSH
mArgonaute-5	IKVWAIACFAPQR YFPVGDGKKP -----CWH

Figure 1. Homology of Argonaute (Ago2) to ribotoxin (restrictocin). A: Argonaute 2 (bovine, accession number NP_991363) and the ribotoxin protein restrictocin (accession number AAA32707) were manually aligned. Location of the ribotoxin loops and beta-strands are indicated above the restrictocin sequence. Dashes in the restrictocin sequence indicate amino acid segments present in the Argonaute protein (the number of aa is indicated in square brackets in the Argonaute protein) and absent from restrictocin. B: Conservation of lysine residues in Argonaute and restrictocin.

that the *Pyrococcus* sequence **KKPK** is located strategically within a loop (Fig. 2) at the entrance to the groove, defined by the PAZ and PIWI domains, that can accommodate an RNA substrate. The **KKPK** motif present in loop 3 of the ribotoxins is essential to SRL binding by ribotoxins (Fig. 2, right panel). Another sentinel loop of the *Pyrococcus* Ago protein that guards entry into the cleft is located diagonally across the **KKPK** loop.

The extended sequence making-up this loop peptide is ⁵⁸¹**FDSQGYIRKI-VPIKIGEQRGE**, similar to the restrictocin sequence ⁶²**YDNGKLIKGRTE-PIKFGKADCD** present in a large ribotoxin loop.

In addition, the *Pyrococcus* Ago protein consists of ⁶¹⁴**FNIKLDNKK** that exhibits a marked similarity to the ribotoxin loop sequence ¹¹⁰**HDYKFDSKK** (Fig. 2). The sequence ⁶¹⁴**FNIK**... appears within a loop on the same side as ⁵⁸¹**FDSQ**.. and likely acts as an additional sentinel loop.

The most striking aspect of the protein sequence similarities between the alpha-sarcin-like ribotoxins and the Argonautes is that they are, as a rule, con-

fined to the protein loop embellishments representing ribotoxin additions to the T1 RNase skeleton (Figs. 1 and 2). These allow the ribotoxins to recognize SRL.

Intact “puromycin reactions” in translational suppression mediated by both alpha-sarcin-like ribotoxins and miRISCs

The mechanism of action mediated by miRISCs is unclear and there is a controversy as to which stage of protein synthesis, initiation¹⁹ or elongation,^{20–25} is affected in miRISC-mediated translational repression. The latter carefully controlled and independently conducted studies showed that miRNAs and their translationally suppressed target mRNAs exist in polysomal configurations and suggest that elongation may also be one of the affected stages of protein synthesis.

Polysomes engaged in correctly functioning, uninhibited protein synthesis show an intact puromycin reaction wherein puromycin leads to polysomal dissociation. The intact puromycin reaction

Figure 2. Location of Ago sentinel loops homologous to the ribotoxin-restrictocin SRL-recognition loops on the *Pyrococcus furiosus* Argonaute protein. Left panel—Sentinel loops of the Ago protein (PDB: 1Z25) showing homology to ribotoxin loops are highlighted in dark blue and superimposed on the ribbon representation of the *P. furiosus* Ago protein [color scheme as in Ref. 18, N-terminal domain (blue), the “stalk” (light blue), the PAZ domain (red), the Mid domain (green), the PIWI domain (purple), and the interdomain connector (yellow)]. Homologous segments are indicated in the insets: identical amino acids and conserved substitutions are indicated by bold and regular red fonts, respectively. Right panel—Recognition of the SRL region of ribosomal RNA by the loop structures (indicated by dotted ovals) present on the ribotoxin protein (PDB: 1JBS).¹⁷ Note, the loop-recognition elements in the ribotoxin and the corresponding loops located in the Ago proteins (left panel), are indicated by red dashed ovals.

dictates the positioning of the peptidyl tRNA in the ribosomal P site, the sole location from which, following peptidyl-transferase activity, it can form the released peptidyl-puromycin moiety. Somewhat surprisingly, puromycin treatment of polysomes translationally suppressed by miRNA results in their dissociation.^{23–25} This reflects an intact puromycin response and thus a correctly functioning translocation reaction in the miRNA-translationally suppressed system.

On the one hand, polysomal structures accompanying miRNA-mediated suppression of translation reflect a block in elongation and a defect at the elongation stage. On the other hand, the puromycin reaction seen in miRNA-mediated translational suppression intimates that elongation is, in fact, intact. This is because an intact puromycin reaction requires that both translocation and peptidyl transferase function correctly.

Translational suppression elicited by the ribotoxins exhibits the same paradox. Protein synthesizing systems inhibited by ribotoxins such as alpha sarcin²⁶ are, like miRNA translational suppression, also accompanied by polysomal structures. This too indicates a defect occurring at the elongation stage.^{1–3,9} Here, as in miRNA-mediated translational repression, the puromycin reaction²⁶ is intact, and reflects correct translocation and peptidyl-transferase activities.

Given the intact puromycin reaction, the only stage of elongation that could be affected by alpha sarcin is the binding of amino-acyl tRNA to the ribosomal A site.²⁶ Subsequent experimental studies^{6,27} supported this conclusion and showed that alpha-

sarcin cleaves a precise site within the large 28S rRNA component of the 60S in the Sarcin-Ricin-Loop. This represses translation by abrogating the binding of aminoacyl tRNA to the ribosomal A site.

Model for miRISC-mediated translational suppression

Translational suppression effected by the ribotoxin alpha-sarcin and miRNAs is extensively similar. Both undergo an intact puromycin reaction that is accompanied by blocks in elongation (described above). Moreover, the singular effector proteins of miRISCs are the Argonautes and these, like the ribotoxins, harbor protein domains with ribonuclease activity.

Furthermore, as we have shown here, the Ago proteins contain ribosomal-SRL-recognition segments similar to those appearing in ribotoxins. This suggests a similar function for these loops in directing the endoribonuclease component of the ribotoxin and the Argonaute proteins, to the SRL stem and loop structure. These findings indicate that the Argonautes may under certain conditions, such as miRISC translational suppression, mediate their inhibition by a mechanism similar to that used by ribotoxins, namely, cleavage of SRL (Fig. 3).

Cumulatively, our results suggest the following (Fig. 3): (a) the miRISC forms incompletely base-paired duplexes at 3' UTR target sequences on discrete mRNAs; (b and c) the Ago proteins in conjunction with the miRNA component recognize and bind to a critical target site on the RNA component of the large ribosomal subunit, (d) the Ago protein associated with miRISC, after verging on the rRNA target

Figure 3. Model for mechanism of action of miRNA-mediated translational suppression. (A) The miRISC composed of the Argonaute protein (green) the miRNA (red) and ancillary proteins such as GW182 forms incompletely base-paired duplexes at 3' UTR target sequences on discrete mRNAs (the curved blue line); (B and C) The Ago protein (green) in conjunction with the miRNA component (red line) recognizes and binds to a critical target site designated the SRL (enclosed by the dotted rectangle and enlarged in the inset) on the rRNA component of the large ribosome subunit. This is illustrated by the black "squiggly" line appearing on the large ribosomal subunit (pink oval). (D) The Ago protein associated with miRISC, after verging on its rRNA target site, cleaves the ribosomal RNA (cleavage indicated within the dotted red oval), thereby leading to disruption of protein synthesis: the miRISC translational suppression.

site on the ribosome, cleaves the ribosomal RNA, thereby disrupting protein synthesis—the miRISC translational suppression.

Although there is no mRNA degradation associated with miRISC suppression, endoribonucleolytic activity is retained in the Ago effector proteins of the miRISC complexes. This is probably mandatory for rRNA cleavage. In this context, it should be noted that whereas Ago proteins Ago1 through Ago4 are competent for miRNA-mediated translational suppression, mouse Ago5 does not support such suppression.²⁸ Accordingly, mAgo5 alone lacks Ago sequences²⁸ similar to those in the restrictocin loop4- β 6-loop5 [Fig. 1(B)].

Finally, localization of miRISC complexes in the 3'UTR,²⁵ and not in coding sequences directly adja-

cent to the ribosomes, allows the complex, because of spatial considerations, to flip back and bind to the most distal 3' ribosome. There the complex executes the cleavage and halts the ribosome in its progression on mRNA. This, in turn, stalls the more 5' proximal ribosomes, leading to translational suppression (Fig. 3).

Polysomes translationally suppressed by miRNAs are obviously present at levels far lower than those of translationally intact polysomes, making it unlikely that the total cellular translational apparatus would be inactivated by miRISC-targeted ribosomes. In this context, it is notable that the endoribonuclease activity of the Ago proteins leaves behind 5'-phosphate and 3'-hydroxyl termini, rendering an Ago-cleaved SRL amenable to repair at later stages

by the appropriate ligases and resulting in the restoration of a translationally competent ribosome.

In addition to the α -sarcin-like ribotoxins, the potent plant toxin ricin also represses translation by eliciting changes within the SRL site: it depurinates the A4324 residue immediately upstream of the site of α -sarcin mediated cleavage.⁵ Shiga toxins made by Shiga toxin-producing *Escherichia coli* also target the same site and suppress translation in a similar fashion to ricin.^{29,30} Nature has found it expedient in all these cases, in toxins derived from plants (ricin-like proteins), fungi (α -sarcin-like proteins), and bacteria (Shiga-like toxins), to target the exact same 28S rRNA region to suppress translation. Furthermore, translational suppression elicited by certain bacterial colicins is also mediated by specific ribosomal RNA cleavage, albeit on the 16 rRNA component.³¹ Additionally, specific cleavages on ribosomal RNA accompanying translational suppression have been documented in interferon-treated and virally infected cells.^{32,33}

In summary, our studies reveal protein sequence similarities between two apparently disparate protein families: the α -sarcin-like ribotoxins and the major constituents of RNA-induced silencing complexes, the Argonautes. The homology resides in protein loops that provide the ribotoxins with specificity for the Sarcin-Ricin-Loop, suggesting that similar segments in the Argonautes also target the SRL. Moreover, suppression of translation in systems inhibited by either miRISCs or ribotoxins is accompanied by both intact polysomes and a puromycin reaction that is uniquely unharmed. These findings support a mechanism for miRNA-mediated translational suppression whereby regulated cleavage of ribosomal RNA leads to translational suppression (Fig. 3), probably mimicking a more widespread means found in Nature.

Materials and Methods

Representatives of the α -sarcin-like ribotoxin family, restrictocin (accession number AAA32707, see Fig. 1) and α -sarcin, were subjected to a ClustalW analysis against Ago proteins from diverse organisms. These included Ago2 from *Bos taurus* (accession number NP_991363, Fig. 1), Agos1–4 from *Homo sapiens*, Agos1–5 from *Mus musculus* as well as the Argonaute protein from *Pyrococcus furiosus*. The sequences were subsequently manually aligned according to the initial ClustalW analysis. The amino acid sequences from restrictocin showing similarity to the Ago proteins were located on the Argonaute protein from *Pyrococcus furiosus*.¹⁸

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