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Fungal Ribotoxins

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Advanced article

Abstract:

Fungal ribotoxins constitute a family of extracellular ribonucleases with exquisite specificity against rRNA. They induce apoptotic death of cells after inhibiting protein translation. Ribosomes become functionally incompetent because ribotoxins cleave one single phosphodiester bond, located at a unique and universally conserved loop, needed for elongation factors function. As secreted proteins, ribotoxins need to cross the membrane of their target cells in order to exert their catalytic activity and they do it without receptor mediation. Using lipid model systems, it has been shown that they are able to enter cells with membranes enriched in acidic phospholipids. Both membrane-interacting and ribosomal-recognition activities are characterized by distinct structural features. Even though the natural function of ribotoxins is not known yet, their production by entomopathogenic fungi has suggested their insecticidal role. After decades of detailed study, the biotechnological potential of ribotoxins in pest control and as antitumor agents is becoming evident.

Key words: Antitumoral, elongation-factor, entomopathogen, fungal-toxin, immunotoxin, insecticide, ribonuclease, ribosome, ribotoxin, sarcin.

Key Concepts:

- Ribotoxins are extremely specific ribonucleases targeted against ribosomes
- Ribotoxins are produced by fungi, some of them entomopathogens.
- They show a high degree of structural conservation, including the local arrangement of the active site residues
- Cleavage of a single rRNA phosphodiester bond leads to cell death by inhibiting translation
- Ribotoxins are cyclizing RNases because they follow a general acid-base mechanism with production of a 2',3'-cyclic intermediate.

- Ribotoxins must first enter their target cells to exert their lethal action.
- Cell entrance is possible in cells with membranes enriched in acidic phospholipids and altered permeability.
- Ribotoxins are optimal candidates to be employed as pest control agents and in antitumor immunotoxins.

Introduction

Ribotoxins are a group of extracellular and highly specific ribonucleases (RNases) secreted by fungi (Lacadena *et al.*, 2007, Olombrada *et al.*, 2017a). Their name arises from the fact that they have the ability to be extremely toxic by efficiently inactivating ribosomes after cleaving a single phosphodiester bond located at a universally conserved sequence (Schindler & Davies, 1977, Endo *et al.*, 1983). This cleavage produces the inactivation of the ribosomes leading to cell death by apoptosis (Olmo *et al.*, 2001). However, and given that they are extracellular proteins, they must first enter the cells to exert their cytotoxic action. It is this entrance the rate limiting step of ribotoxins' action. There has not been found a protein receptor for ribotoxins which, therefore, take advantage of permeability membrane changes produced by tumor transformation, or virus infection, as well as their higher affinity for negatively charged phospholipids (Gasset *et al.*, 1989). This explains why α-sarcin, the most representative member of the group, was originally discovered as an antitumor agent (Olson & Goerner, 1965). Unfortunately α-sarcin was not as specific as desirable, producing unwanted side-effects. Therefore, the research in this field was eventually abandoned. It is now known, however, that ribotoxins constitute a more extended family of proteins than initially described, with more variety of fungal origins and sequences, but sharing key structural and enzymatic features which make them optimum candidates to be employed in a different biotechnological approaches like pest control and anticancer drugs development (Olombrada *et al.*, 2014, Olombrada *et al.*, 2017a).

General structural features

All ribotoxins known are rather small proteins which share at least two different elements of ordered secondary structure: A β-sheet, where the active site is located, and a short α-helix (Figure 1). Interestingly, several other non-toxic fungal extracellular RNases show identical three-dimensional arrangement including the nature and geometrical disposition of the most important active-site residues (Figure 2). This explains why ribotoxins are considered the toxic representatives of a much wider protein group, the RNase T1 family, which is one of the most deeply studied proteins in history (Yoshida, 2001). Observation of their three-dimensional structures explains their functional differences in terms of toxicity (Figure 1) since ribotoxins display long non-ordered and positively charged loops, which are much shorter and negatively charged in the non-toxic relatives. In fact, ribotoxins only share a maximum

of 20% of their sequence with the other non-toxic RNases. These loops in ribotoxins are responsible for recognizing the acid phospholipids which facilitate their cell entry and also for specifically embracing the ribosomes to produce their highly specific and lethal enzymatic cleavage (García-Ortega *et al.*, 2002, García-Mayoral *et al.*, 2005, Álvarez-García *et al.*, 2009).

Ribotoxins have been detected in many different fungi (Martínez-Ruiz *et al.*, 1999), including entomopathogenic (Herrero-Galán *et al.*, 2008, Olombrada *et al.*, 2017b) and edible (Landi *et al.*, 2017) species, but only the three-dimensional structure of three of them has been solved so far: α-sarcin (Pérez-Cañadillas *et al.*, 2000), restrictocin (Yang & Moffat, 1996) and hirsutellin A (HtA) (Viegas *et al.*, 2009). α-Sarcin and restrictocin show practically indistinguishable structures (Figure 1) as expected from their higher than 85% sequence identity. On the other hand, HtA displays unique features, starting with its size which is 20 amino acids shorter (130 against 150), though still larger than the non-toxic T1-like RNases (100-110 amino acids). Moreover, HtA shows just 25% of sequence identity with the other larger ribotoxins. Therefore, HtA structure contains non-ordered loops very different in conformation and length while keeping the common central core characteristic of this RNases family (Figure 1). Even so, it still conserves all functional features of ribotoxins. These *a priori* exceptional features of the ribotoxin HtA seem to be now more common with the recent discovery of anisoplin, a new ribotoxin, from *Metarhizium anisopliae* with 70% sequence identity to HtA (Olombrada *et al.*, 2017b).

Geometric arrangement of the active site residues

All ribotoxins show practically identical geometric disposition of their active-site residues (Figure 2). This arrangement is also coincident with the one shown by RNase T1, in good agreement with their common general acid-base catalytic mechanism (Lacadena *et al.*, 1998) (Figure 3). Accordingly, these RNases share at least four amino acids located at strategic positions: Two histidines, one glutamic acid and one arginine (His50, Glu96, His137, and Arg121, following α-sarcin numbering; Figure 2) directly involved in the catalytic steps leading to the required proton transference to cleave the bond (Figure 3) (Lacadena *et al.*, 1999). They are located in the central β-sheet (Figure 2) with their side chains pointing towards the concave face of the protein structure. This active site shows three highly representative features: (1) high density of charged residues (Pérez-Cañadillas *et al.*, 2000), (2) low surface accessibility of all these titratable atoms and, consequently, (3) unusual pK_a values of the catalytic Glu and His residues (Pérez-Cañadillas *et al.*, 1998), as well as unusual Nδ tautomeric forms of the latter ones (Pérez-Cañadillas *et al.*, 2003).

Another important residue in the active site is Tyr48 (α-sarcin numbering), conserved in most of the members of the T1 family (Figure 2) and essential for α-sarcin full enzymatic activity (Álvarez-García *et al.*, 2006). However, inspection of the arrangement of the two smaller ribotoxins known so far (HtA and anisoplin)

(Olombrada *et al.*, 2017b), both produced by entomopathogenic fungi, shows that an Asp residue appears at the equivalent position (Figure 2). Interestingly, mutagenic analysis involving this strategic position has shown how these smaller versions display a very different electrostatic arrangement (Herrero-Galán *et al.*, 2012a, Maestro-López *et al.*, 2017), representing an optimum compromise among conformational freedom, stability, specificity, and active-site plasticity. All these features together allow them to accommodate the characteristic abilities of ribotoxins into a shorter and more stable structure of intermediate size between that of the other nontoxic fungal RNases and the previously known larger ribotoxins.

Enzymatic mechanism

Ribotoxins cleave RNA following a mechanism shared by all extracellular fungal RNases characterized so far. Using dinucleosides, such as GpA, for example, it has been shown how the hydrolysis of the 3′-5′ phosphodiester bond of these substrates takes place via a 2′-3′ cyclic mononucleotide which is then converted to the corresponding 3′-monophosphate derivative as the final product of the reaction (Figure 3). Thus, ribotoxins perform a general acid–base type endonucleolytic cleavage of RNA which fits into a two-step mechanism, considered as the signature of cyclizing RNases (Lacadena *et al.*, 1998, Yoshida, 2001): A transphosphorylation reaction which is followed by the hydrolysis of the mentioned cyclic intermediate (Figure 3). **See also: Acid–Base Catalysis by Enzymes** (*DOI: 10.1002/9780470015902.a0000602.pub2*).

At least in α-sarcin, during the first step of the reaction Glu96 acts as the general base and His137 as the general acid (Figure 3). The hydrolysis of the cyclic derivative is then catalysed by the same groups, but playing opposite roles. It is now well known that these α-sarcin His137 and Glu96 are the only residues that are essential for performing the catalytic acid–base type reaction, though some other mutants have been found to be inactive against the ribosome or an isolated mimetic version of the targeted rRNA fragment, the sarcin-ricin loop or SRL (Lacadena *et al.*, 1998, Lacadena *et al.*, 1999). In fact, this Glu/His combination is the most common pair of catalytic residues found in microbial RNases (Yoshida, 2001). The other His residue, His50, is required in its protonated form to assist the electrostatic stabilization of the transition state. Finally, the role of Arg121 has been studied with its replacement by Gln or Lys. These mutations did not modify the conformation of the protein, but abolished its ribosome inactivating activity (Lacadena *et al.*, 2007). Unexpectedly, these mutants were still active against a small and nonspecific substrate such as ApA. Interestingly, the loss of the positive charge at that position produced dramatic changes in α-sarcin's ability to interact with phospholipid membranes suggesting that proteins which have evolved to interact with nucleic acids, such as RNases, would have developed structural determinants to recognize polyphosphate lattices, such as cell membranes, which certainly can be considered as two-dimensional phosphate networks.

The substrate

Ribotoxins specifically cleave a single phosphodiester bond within a universally conserved rRNA sequence located in a key ribosomal structure known as the sarcin-ricin loop (SRL) (Figure 4). This name arises from the early observation that this SRL is not only the target of fungal ribotoxins but also of the well-known family of ribosome-inactivating proteins (RIP), best represented by ricin (Stirpe, 2015). These RIP are also highly specialized toxic proteins, produced by plants and fungi that inactivate ribosomes by acting as N-glycosidases on the same unique rRNA structure as ribotoxins do (Endo & Tsurugi, 1987, Correll *et al.*, 1999). They depurinate a single nucleotide contiguous to the phosphodiester bond cleaved by ribotoxins (Figure 4), producing a very similar inactivating effect. Obviously, ribotoxins are also *ribosome-inactivating proteins*. However, there is a rather general consensus to employ this name only for the N-glycosidases while the term ribotoxins refers only to the toxic RNases of this review. **See also: Ribonucleases** (*DOI: 10.1038/npg.els.0003895*).

Cleavage of the large rRNA at the SRL leads to complete inactivation of the ribosome because this loop interacts with translation factors that bind and exert their essential function on the ribosome assisted by GTP hydrolysis (Nierhaus *et al.*, 1992). It has been precisely determined that it is elongation factor G (EF-G) binding the most perturbed event by ribotoxins cleavage (García-Ortega *et al.*, 2010). Binding is strongly impaired and consequently GTP hydrolysis and mRNA–tRNA translocation during elongation do no take place at a significant rate leading to dysfunctional ribosomes. ***See also: Elongation Factors: Bacterial*** (*DOI: 10.1038/npg.els.0003932*), ***rRNA Structure*** (*DOI: 10.1038/npg.els.0000537*), *and* **Ribosome Structure and Shape** (*DOI: 10.1038/npg.els.0000534*).

The positively charged surface of ribotoxins allows them to establish favourable electrostatic interactions between their active site residues and the rRNA, explaining their highly specific recognition of the SRL (García-Mayoral *et al.*, 2005, Korennykh *et al.*, 2006, Álvarez-García *et al.*, 2009). So far, the regions which are known to participate in this interaction are the Lys-rich region of loop 3, which would interact with a phosphodiester bond around the bulged G of the SRL, and the stretch comprising residues 51–55 of loop 2 which, altogether with some residues of loop 5, would contact the GAGA tetra-loop that is cleaved by the toxin (Figure 4) (Yang *et al.*, 2001, García-Mayoral *et al.*, 2005). Docking models suggest other α-sarcin regions recognizing more ribosomal elements (García-Mayoral *et al.*, 2005), a prediction that would justify the different affinity shown by ribotoxins against ribosomes from different species, in spite of the universal conservation of the SRL. For example, the α-sarcin 11-16 residues stretch would interact with ribosomal protein uL14, explaining why deletion of the N-terminal β-hairpin renders an active but non-specific RNase unable to unequivocally target the SRL (García-Ortega *et al.*, 2002, García-Mayoral *et al.*, 2004). In addition, some other not yet detected ribosomal regions could also participate in this specific recognition. Good candidates would be those ones involved in the

recruitment of elongation factors during translation. That could have been the case of the highly dynamic protruding structure of the ribosome that serves as an anchoring platform for elongation factors, known as the *ribosomal stalk*, which has been shown to fulfil this specific function for ricin, for example (Tumer & Li, 2012). Quite surprisingly, given that ricin and α-sarcin share identical rRNA target, ribotoxins do not seem to need to interact with the ribosomal stalk in order to reach the SRL (Olombrada *et al.*, 2014). It has to be then concluded that the search for key specific interactions established between ribotoxins and ribosome from different origins is far from being closed yet.

Crossing the membrane

As mentioned above, the toxicity of ribotoxins results from the combination of their highly specific RNase activity and their ability to cross membranes. Given that no protein receptor for ribotoxins has been found, the lipid composition of membranes plays an important role in their cytotoxic specific activity. Using lipid model systems has been shown that α-sarcin interacts with lipid vesicles enriched in acidic phospholipids, promoting vesicle aggregation. This event leads to vesicle fusion with intermixing of phospholipids and leakage of their aqueous contents (Gasset *et al.*, 1989, Gasset *et al.*, 1990) (Figure 5). Within this idea, this protein has been also proven to have that ability to translocate across a negatively charged bilayer in the absence of any other protein component (Oñaderra *et al.*, 1993). Interestingly, the outer monolayer of tumor cell membranes appears to be enriched in negative phospholipids. Quite surprisingly, however, this behaviour with model vesicles does not seem to be strictly conserved among ribotoxins. Again, HtA is the known exception because it does not promote vesicle aggregation even though it shows higher membrane-permeabilizing ability than α-sarcin in leakage experiments and is still able to penetrate its target cells with at least as much efficiency as α-sarcin (Herrero-Galán *et al.*, 2008).

The structural details of this ribotoxins-lipid interaction have also been determined to great extent in α-sarcin. In this protein, the β-sheet region comprising residues 116–139 seems to be a key element in the hydrophobic interaction with membranes (Mancheño *et al.*, 1995, Mancheño *et al.*, 1998). Loop 3 Lys residues 111 and 114 (Figure 1) would also take part in the electrostatic interactions needed to bring vesicles into contact (Castaño-Rodríguez *et al.*, 2015) (Figure 5). On the other hand, in the case of HtA a role in membrane-permeabilizing activity has been assigned to Trp 71 and 78 (Herrero-Galán *et al.*, 2012b). Trp residues in α-sarcin, although differently located in the structure, seem to play a very similar role too (De Antonio *et al.*, 2000) (Figure 5). This ability to interact with lipid membranes has also been associated with the positively charged N-terminal β-hairpin of ribotoxins because its deletion in α-sarcin yields a non-toxic but active ribonuclease with altered membrane interaction properties (García-Ortega *et al.*, 2002). Intriguingly, it is in this region where HtA shows more variability when compared to α-sarcin (Figure 1). The N-

terminal β-hairpin of HtA is much shorter, a difference which appears to be compensated by the extension of loop 5, which also exhibits a higher amount of positive charges (Herrero-Galán *et al.*, 2012a).

Biological function and biotechnological applications

It is not clear why fungi secrete ribotoxins though they should have predating and/or defensive functions. At least for *Aspergillus*, the main ribotoxin producer genus so far, they seem to be produced during conidia maturation, most probably as a defence mechanism against predators(Brandhorst *et al.*, 1996).The discovery that the entomopathogenic fungus *H. thompsonii* was synthesizing HtA (Herrero-Galán *et al.*, 2008), followed by the recent characterization of anisoplin (Olombrada *et al.*, 2017b), a new small HtA-like ribotoxin produced by other entomopathogenic fungi such as *Metarhizium anisopliae*, suggested the possibility of being insecticidal proteins. This function was then proved for α-sarcin and some other ribotoxins such as HtA (Olombrada *et al.*, 2013, Olombrada *et al.*, 2014, Olombrada *et al.*, 2017a, Olombrada *et al.*, 2017b).

These results have opened a new biotechnological venture to use ribotoxins as the base to design new and environmentally friendly bioinsecticides. In fact, resistance to pesticides has increased over the years and, simultaneously, pest diseases are the cause of up to 40% losses in agriculture production around the world. Some entomopathogenic fungi, such as the ribotoxins producers *H. thompsonii* and *M. anisopliae*, have been already commercialized as control agents to manage crop diseases (Kanga *et al.*, 2002). Accordingly, ribotoxins could be used independently or as part of biopesticide formulas, being a more controlled and reproducible product than the whole fungal extract (Olombrada *et al.*, 2013, Olombrada *et al.*, 2014, Olombrada *et al.*, 2017a). The potential toxicity of ribotoxins against vertebrates could be overcome by the design of new variants with diminished non-specific toxicity (Herrero-Galán *et al.*, 2012b). Finally, their combination with insect pathogenic viruses such as some baculoviruses represents another promising approach for biocontrol. Natural baculoviruses have been already used as effective biopesticides thanks to their specificity, but their genetic modification to deliver ribotoxins seems to be an optimum alternative for pest control (Olombrada *et al.*, 2017a).

As mentioned at the beginning of this review, ribotoxins were first discovered as antitumor agents. Unfortunately, further studies revealed an unspecific cytotoxicity against non-tumor cells which discouraged their use in anticancer therapies. Fortunately enough, the interest for ribotoxins has revived as part of antitumor immunotoxins (Tomé-Amat *et al.*, 2015a). They are chimeric molecules composed of a specific antibody fragment, responsible for targeting a specific cell surface antigen, linked to a ribotoxin moiety that promotes cell death. Immunotoxin designs based on the employment of ribotoxins have been shown to be highly effective, with the additional benefit of not showing any detectable undesirable side effect, most probably

due to the high specific antigen recognition exerted by the employed antibody (Tomé-Amat *et al.*, 2015a, Jones *et al.*, 2016, Olombrada *et al.*, 2017a). This approach has been recently improved with the incorporation of different variants such as one that is unable to cross membranes but still retains the ribonucleolytic activity (Tomé-Amat *et al.*, 2015b) or a deimmunized variant of α-sarcin showing a complete lack of T cell activation in *in vitro* assays (Jones *et al.*, 2016).

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Further Reading:

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Glossary:

Baculovirus: Family of viruses which specifically infect invertebrate animals. Some are so specific against its insect host that can be used biological agents in pest control. They are also used to produce eukaryotic proteins in heterologous systems made of insect cell lines.

Biopesticide: Pesticides derived from natural materials such as animals, plants or microorganism and usually considered more environmentally friendly than the classical pesticides of chemical synthesis origin.

Elongation-factor: Family of proteins which intervene in translational elongation through interaction with specific regions of the ribosome. They are GTPases which use the energy arising from GTP hydrolysis to facilitate the movement and turnover required to elongate the polypeptide chain.

Entomopathogen: Any agent that is pathogenic to insects.

Glycosidase: Family of enzymes that catalyze the hydrolysis of glycosidic linkages. Therefore they take part in degrading oligosaccharides and glycoconjugates.

Tautomer: Constitutional isomers of organic compounds that readily interconvert by relocation of a proton.

Figures:

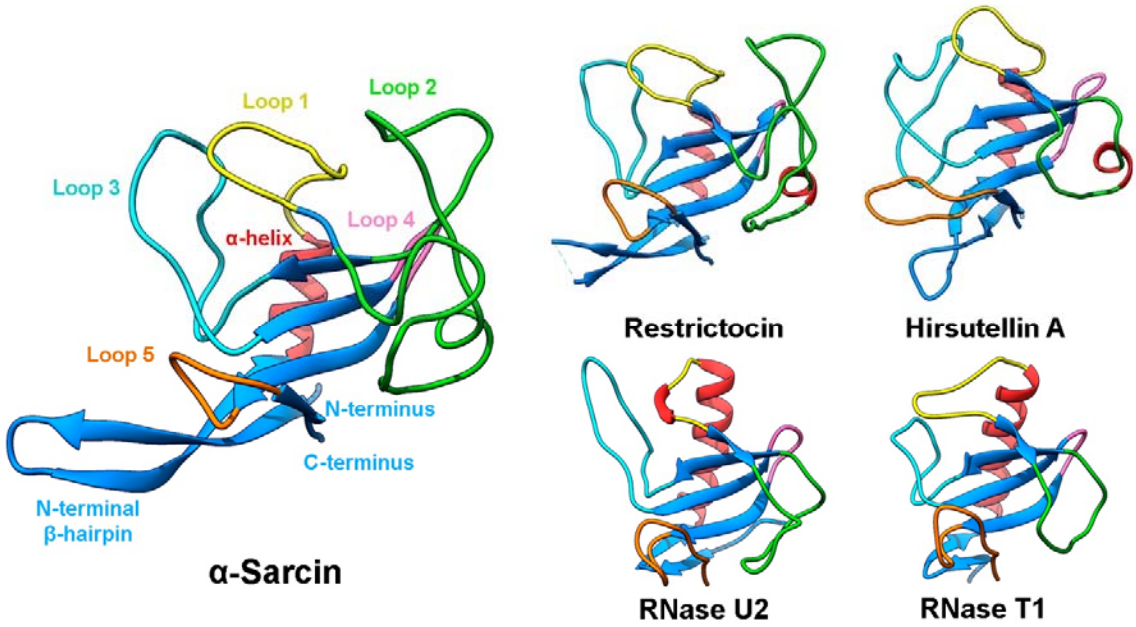


Figure 1. Representation of the three-dimensional structure of representative fungal RNases. Diagrams showing the three dimensional structure of ribotoxins α-sarcin (PDB ID: 1DE3), restrictocin (PDB ID: 1AQZ) and HtA (PDB ID: 2KAA), and two non-toxic fungal extracellular RNases from the same family: RNases T1 (PDB ID: 9RNT) and U2 (PDB ID:1RTU) Diagrams were generated using the Chimera software (Pettersen *et al.*, 2004).

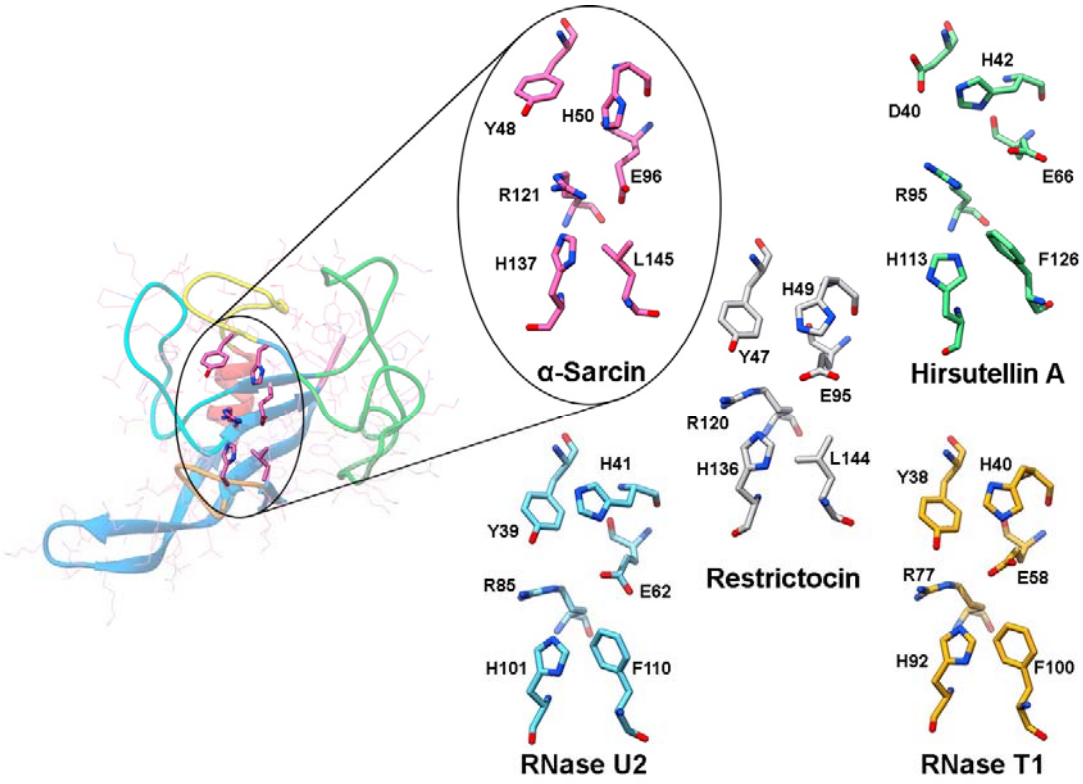
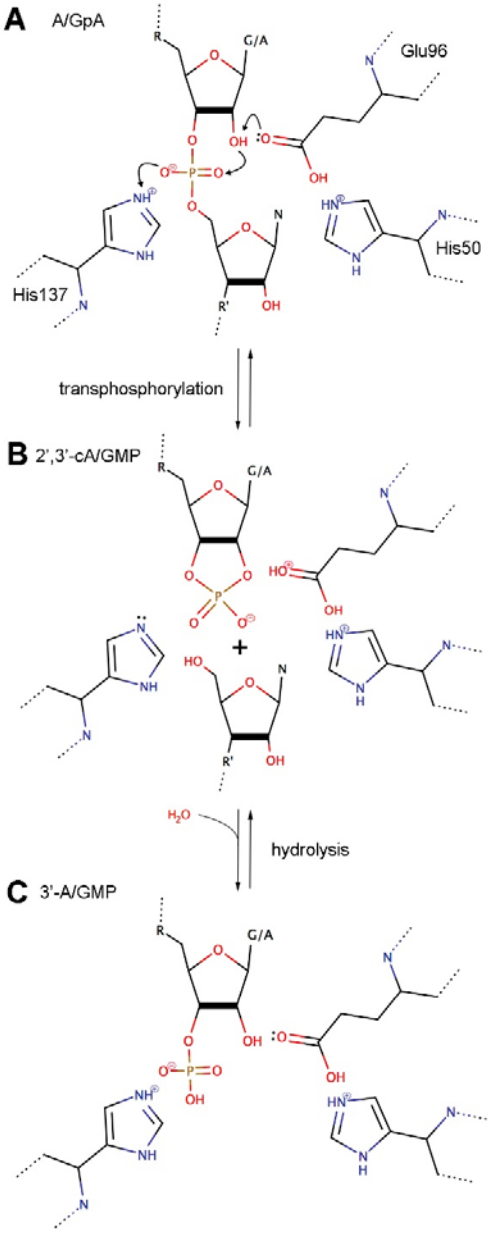


Figure 2. Representation of the active site arrangement of the most representative fungal RNases. The catalytic triad made of two His and one Glu residues is conserved in all proteins shown, as well as α-sarcin Arg121, while a fifth residue, α-sarcin Leu145, maintains its highly hydrophobic character (Phe or Leu). The position corresponding to α-sarcin Tyr48 is also conserved except for HtA and anisoplin (not shown) where the equivalent position is occupied by an Asp residue (Asp40). Diagrams were generated using the Chimera software (Pettersen *et al.*, 2004).


A A/GpA
Glu96
His137
His50
transphosphorylation
B 2',3'-cA/GMP
hydrolysis
C 3'-A/GMP

1

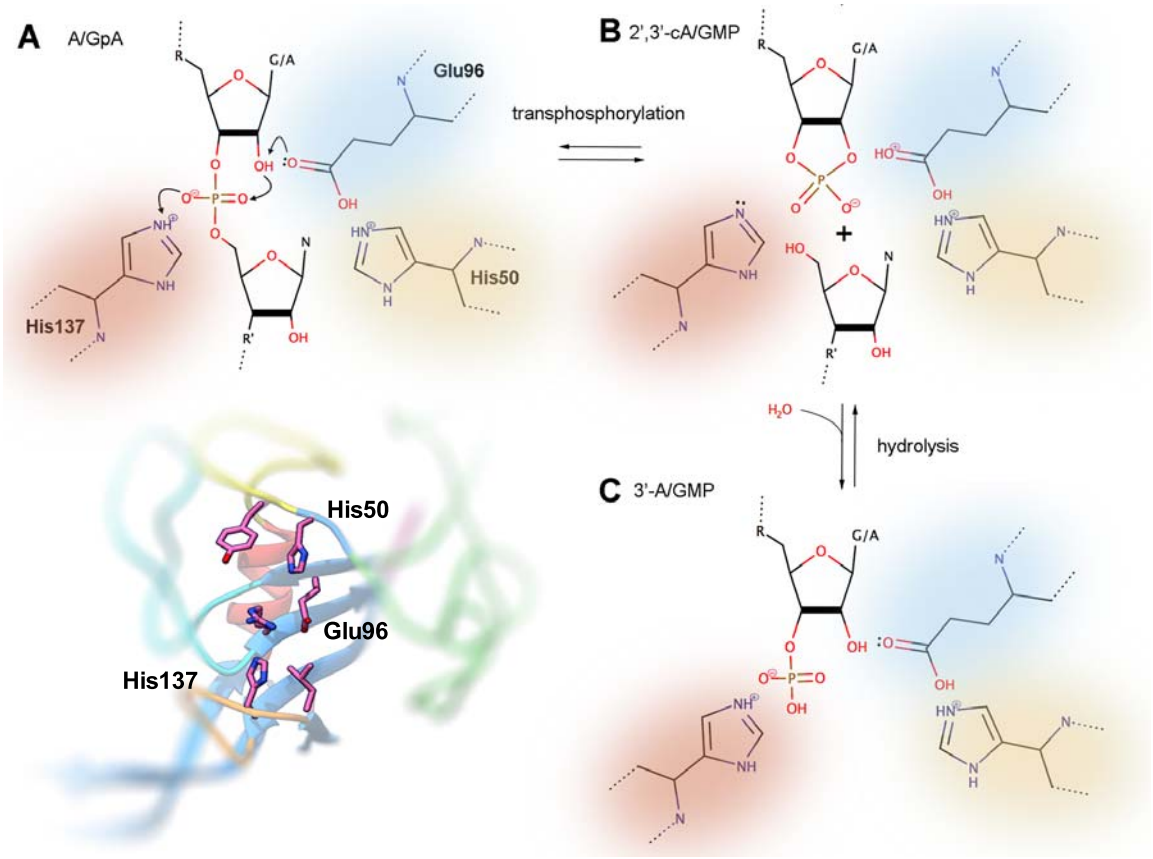


Figure 3: Catalytic mechanism of cyclizing RNases. The catalytic mechanism of cyclic RNases such as ribotoxins against a dinucleotide substrate (ApA or GpA) is shown. A transphosphorylation process (in which the corresponding 2',3' cyclic mononucleotide and adenosine are produced) is followed by hydrolysis of the cyclic nucleotide to produce the corresponding 3'-mononucleotide. Side chains of residues corresponding to α-sarcin His50, Glu96, and His137 are also shown, indicating at the bottom left corner of the figure their spatial location in the context of the whole protein three-dimensional structure.

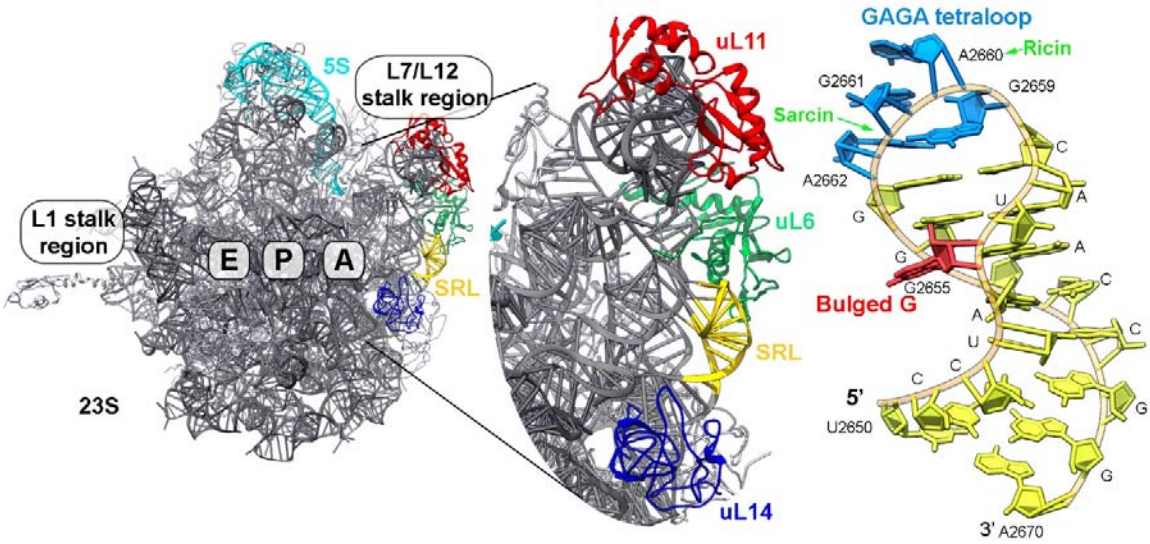


Figure 4. **The substrate of ribotoxins**. (A) Three-dimensional structure of the large ribosomal subunit of *Escherichia coli* (PDB ID: 2AW4). The location of L1 and L7/L12 stalks (absent in this crystal) and E, P and A sites are indicated. Conserved proteins around the SRL (orange) appear in different colors: uL6 (green), uL11 (red), and uL14 (blue). Other ribosomal proteins appear in light gray. 23S (dark gray) and 5S (cyan) rRNAs are also shown; (B) SRL structure. The bulged G (red), the GAGA tetraloop (blue), the bond cleaved by α-sarcin and the adenine depurinated by ricin are indicated. Diagrams were generated using the Chimera software (Pettersen *et al.*, 2004).

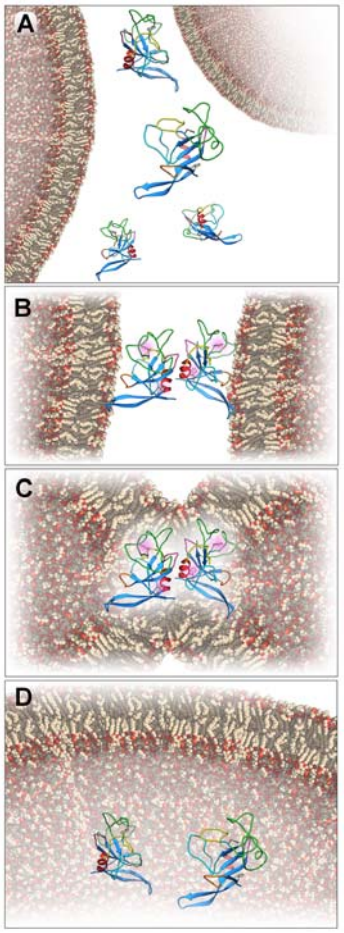


Figure 5. Schematic representation of the translocation mechanism of α-sarcin acroos the bilayer of negatively charged phospholipid vesicles. (A) Binding experiments reveal a strong ribotoxin–lipid vesicle interaction that causes vesicle aggregation (B) mediated by the formation of a vesicle dimer maintained by protein–protein associations. The N-terminal stretch as well as some of the positively charged loops play a key role at this step. (C) Then, the β-sheet region comprising residues 116–139, altogether with the Trp side-chains (in pink), establishes a destabilizing hydrophobic interaction with the membrane which leads to (D) protein internalization.