Title: **Fungal ribotoxins: structure, function and evolution**

Running title: Fungal ribotoxins

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Abstract

Ribotoxins are a family of fungal extracellular ribonucleases which inactivate ribosomes by specifically cleaving a single phosphodiester bond located at the universally conserved sarcin/ricin loop of the large rRNA. The subsequent inhibition of protein biosynthesis is followed by cell death *via* apoptosis. Ribotoxins are also able to interact with membranes containing acid phospholipids, their cytotoxicity being preferentially directed towards cells showing altered membrane permeability, e.g. transformed or virus infected cells. Many features of their cytotoxic action and their ribonucleolytic mechanism have been elucidated by comparison with other extracellular non toxic fungal RNases, best represented by RNase T1. The study of structure-function relationships in ribotoxins is of particular interest, since they are postulated as potential therapeutic agents against different human pathologies. The production of hypoallergenic variants with application in several *Aspergillus*-related allergic syndromes and the construction of immunotoxins against different carcinomas are promising examples of such potential therapeutic utilisation.

Introduction

In 1963, during a screening program started seven years earlier by the Michigan Department of Health searching for new antibiotics and antitumour agents, the culture filtrates of a mould isolated from a sample of a Michigan farm soil were found to contain a substance inhibitory to both *sarcoma 180* and *carcinoma 755* induced in mice (Jennings *et al.*, 1965; Olson and Goerner, 1965). The mould was identified as a strain of *Aspergillus giganteus* and the protein responsible for these effects was named α-sarcin (Olson and Goerner, 1965). Two other antitumour proteins with similar activities, restrictocin and mitogillin, both produced by *Aspergillus restrictus*, were later found,

and came to be part of a new family of proteins, today called ribotoxins, which would be joined much later by Asp f 1, identified three decades after as one of the major allergens of *Aspergillus fumigatus* (Arruda *et al.*, 1992). The unspecificity exhibited by these proteins in their cytotoxic action (Roga *et al.*, 1971) caused the abandon of their study until the mid-1970s, when it was demonstrated that they were capable of inhibiting protein biosynthesis by specifically cleaving a unique phosphodiester bond in the large ribosomal RNA subunit (Schindler and Davies, 1977; Endo and Wool, 1982). This bond is of particular interest because it is located at a universally conserved site with important roles in ribosome function (Wool *et al.*, 1992). This site is known as the sarcin-ricin loop (SRL), because it is the target for the toxins α-sarcin and ricin, best representatives of ribotoxins and ribosome inhibiting proteins (RIPs), respectively. Such similarity in their action is the reason why ribotoxins were suggested to be included in the RIPs family, but some authors (Nielsen and Boston, 2001; Peumans *et al.*, 2001) claim that this name should be restricted to plant N-glycosidases that depurinate a single nucleotide contiguous to the phosphodiester bond cleaved by ribotoxins.

Seven proteins produced by *Aspergillus* have been described as ribotoxins to date, together with six from *Penicillium* and two more from *Neosartorya glabra* and *Hirsutella thompsonii*, and genes have been characterised in five additional strains of the first *genus* (Jennings *et al.*, 1965; Olson and Goerner, 1965; Arruda *et al.*, 1992; Lin *et al.*, 1995; Huang *et al.*, 1997; Wirth *et al.*, 1997; Martínez-Ruiz *et al.*, 1999a; Kao *et al.*, 2001, Herrero-Galán *et al.*, 2008). α-Sarcin, restrictocin and Asp f 1 are the most exhaustively characterised members of the family. These proteins show a high degree of conservation, displaying amino acid sequence similarities above 85 per cent (Figure 1). However, hirsutellin A (HtA), an extracellular protein produced by the invertebrate fungal pathogen *Hirsutella thompsonii*, has been recently demonstrated to be a ribotoxin

(Herrero-Galán *et al.*, 2008), though it displays only about 25 per cent sequence identity with previously known members of the same family (Figure 1) (Boucias *et al.*, 1998; Martínez-Ruiz *et al.*, 1999b; Herrero-Galán *et al.*, 2008). This suggests that ribotoxins are more widely distributed among fungi than previously believed (Martínez-Ruiz *et al.*, 1999a).

Ribotoxins belong to a larger group of fungal extracellular unspecific RNases that show a high degree of sequence and structural similarity to those of the α-sarcin family but are not cytotoxic (Figures 1 and 2). RNase T1 is the best known representative of this family, as well as one of the most exhaustively characterised enzymes (Heinemann and Hahn, 1989; Gohda *et al.*, 1994; Zegers *et al.*, 1994, 1998; Steyeaert, 1997; Arni *et al.*, 1999; Loverix and Steyaert, 2001). RNase U2 from *Ustilago sphaerogena* also stands out as the unspecific fungal extracellular RNase most closely related to ribotoxins (Sacco *et al.*, 1983; Martínez del Pozo *et al.*, 1988; Martínez-Ruiz *et al.*, 1999a). Ribotoxins share with RNases of the T1 family their main structural core but contain longer and positively charged loops (Figure 2). So, these loops are supposed to be essential for their specific toxicity (Martínez del Pozo *et al.*, 1988), which gives rise to the hypothesis that a T1-like RNase could have acquired ribosome specificity and membrane interacting ability by the insertion of short recognition domains (Lamy *et al.*, 1992; Kao and Davies, 1995). Thus, the study of the evolution and mechanism of action of ribotoxins is of particular interest, as it could lead to the identification of the structural determinants that have allowed these proteins to become such efficient toxins, which would be a major step towards their biomedical utilisation as weapons against different human pathologies. Comparative structural and functional studies between ribotoxins and RNases from the T1 family are crucial in the course for achieving that goal.

Structure

Ribotoxins are basic proteins of 149-150 amino acids with a high degree of identity, including two disulfide bridges conserved along the whole family (Figure 1) (Rodríguez *et al.*, 1982; Sacco *et al.*, 1983; López-Otín *et al.*, 1984; Fernández-Luna *et al.*, 1985; Arruda *et al.*, 1990; Wirth *et al.*, 1997; Martínez-Ruiz *et al.*, 1999b). This observation includes HtA, although it is 20 residues shorter than the other known ribotoxins (Martínez-Ruiz *et al.*, 1999b). Sequence differences are mainly concentrated at the loops, where ribotoxins also differ from RNases of the T1 family (Figure 2) (Martínez-Ruiz *et al.*, 1999a).

The three dimensional structures of restrictocin (Yang and Moffat, 1996; Yang *et al.*, 2001) and α-sarcin (Pérez-Cañadillas *et al.*, 2000, 2002; García-Mayoral *et al.*, 2005a) have been elucidated. For α-sarcin, nuclear magnetic resonance and other techniques have been used to make a very detailed map of its structural and dynamic properties (Campos-Olivas *et al.*, 1996a, 1996b; Pérez-Cañadillas *et al.*, 2000, 2002; García-Mayoral *et al.*, 2005a, b). This protein folds into an α+β structure with a central five-stranded antiparallel β-sheet and an α-helix of almost three turns (Figure 2). The sheet is composed of strands β3, β4, β5, β6 and β7, arranged in a -1, -1, -1, -1 topology. It is highly twisted in a right-handed sense, defining a convex face against which the α-helix is orthogonally packed, and a concave surface that holds the active site residues: His 50, Glu 96, Arg 121 and His 137, all of them with their side chains projecting outwards from the cleft. This main structural core, including the active site, is conserved among all fungal extracellular RNases, the α-helix being longer in the T1 family. Major differences are concentrated in the loops of non periodic structure and the amino terminal region. In ribotoxins, residues 1-26 form a long β-hairpin that can be

considered as two consecutive minor β-hairpins connected by a hinge region. The first one is closer to the open end of the hairpin, whereas the second sub-β-hairpin is formed by two short strands, β1b and β2b, connected by a type I β-turn. This last part of the N-terminal hairpin is exposed to the solvent and shows a high mobility (Pérez-Cañadillas *et al.*, 2002). The secondary structure elements are connected by large loops of non periodic structure but with very well defined conformations, maintained by a complex network of intraloop and interloop interactions, including hydrogen bonds, hydrophobic interactions and salt bridges (Yang and Moffat, 1996; Pérez-Cañadillas *et al.*, 2000). From a dynamic point of view, NMR studies have shown that these loops undergo fast internal motions, ranging from picoseconds to nanoseconds (Pérez-Cañadillas *et al.*, 2002). Loop 2 of α-sarcin, one of the regions exhibiting more differences with RNases from the T1 family, deserves special attention because of its functional implications. It is rich in Gly and Lys residues, largely solvent exposed and highly mobile, though very well defined. In this loop, the stretch comprising residues 52-54 is essentially frozen within the molecular framework and includes Asn 54, a conserved residue among fungal extracellular RNases (Mancheño *et al.*, 1995a) that establishes a hydrogen bond between its amide side chain proton and the carbonyl group of Ile 69. This interaction is also conserved in RNases from the T1 family (Sevcik *et al.*, 1991; Pfeiffer *et al.*, 1997; Hebert *et al.*, 1998) and it has been suggested that this region could form the substrate recognition pocket in restrictocin (Yang and Moffat, 1996), a hypothesis that was later confirmed by the results obtained with α-sarcin Asn 54 mutants, located in the equivalent region of this protein (53-56).

Thermal denaturation studies, by both differential scanning calorimetry (DSC) and circular dichroism (CD) measurements, have certified the high stability of ribotoxins (Gasset *et al.*, 1995a). The presence of eight Tyr and two Trp residues has allowed a

great variety of spectroscopical studies, some of them leading to the determination of the pK_a values corresponding to pH-induced conformational transitions (Martínez del Pozo *et al.*, 1988; De Antonio *et al.*, 2000). These results were completed later by the assignment, thanks to NMR measurements and predictions, of the pK_a values of all titratable residues in the molecule (Pérez-Cañadillas *et al.*, 1998; García-Mayoral *et al.*, 2003). Such a detailed structural characterisation has culminated with the determination of the different tautomeric states of every histidine residue in the protein (Pérez-Cañadillas *et al.*, 2003).

Finally, as far as the active site is concerned, it is important to mention that it is composed of, at least, Tyr 48, His 50, Glu 96, His 137, Arg 121 and Leu 145, although only three of them (His 50, Glu 96 and His 137) are directly involved in proton transfer during the catalysis (Lacadena *et al.*, 1999; Martínez-Ruiz *et al.*, 2001). These three residues present unusual pK_a values, and the two histidines adopt unusual tautomeric forms, which is a common feature of microbial RNases. In addition, His 137 establishes an important hydrogen bond with a backbone oxygen in loop 5. This loop is in part responsible for the low surface accesibility of all titrable atoms, which translates into important restrictions for the substrate, as will be discussed below (Pérez-Cañadillas *et al.*, 1998, 2000, 2003).

Function

Enzymatic activity

Both ribotoxins and T1-like RNases act as acid cyclising RNases, following a two-step mechanism (Figure 3) (Lacadena *et al.*, 1998). This mechanism, as well as the roles of most of the residues forming its active site, have been clearly established for RNase T1 (Steyeaert, 1997; Loverix and Steyaert, 2001; Yoshida, 2001). In the first step, a

transphosphorylation reaction occurs to form a 2',3'-cyclic phosphate intermediate. Secondly, this intermediate is hydrolysed to the corresponding 3'-phosphate (Figure 3). This mechanism is shared by most RNases and is based on the general acid-base type endonucleolytic cleavage of RNA. However, the type of substrate (single or double stranded RNA), the specificity, the catalytic residues and the parameters defining the enzyme vary depending on the family. RNases from the T1 family can hydrolyse single stranded RNA, acting specifically on 3'-GpN-5' sequences. RNase U2 is an exception, cleaving additionally 3'-ApN-5' bonds (Egami *et al.*, 1980). RNase T1 optimum activity occurs at pH values around neutrality, whereas RNase U2 reaches its highest efficiency at acid pH (Arima *et al.*, 1968a, b; Uchida and Egami, 1971). Analysis of the cleavage reactions performed by α-sarcin against different dinucleoside monophosphates proved that this protein is also a cyclising RNase with an optimum pH of 5.0 (Lacadena *et al.*, 1998, 1999; Pérez-Cañadillas *et al.*, 1998). However, ribotoxins are much more exquisite enzymes, their specificity going further than a single preference for a type of nucleotide. As it has been already mentioned, ribotoxins exert their ribonucleolytic action on a single phosphodiester bond at the SRL, the one between G4325 and A4326 in 28S rRNA (rat ribosome numbering; G2661-A2662 in *E. coli*), releasing the so-called α fragment (488 bp in the rat ribosome) (Schindler and Davies, 1977; Endo and Wool, 1982; Endo *et al.*, 1983, 1988). This single cut is enough to inhibit protein biosynthesis, as it interferes with elongation factors function (Brigotti *et al.*, 1989; Wool *et al.*, 1992). Finally, cell death by apoptosis occurs (Olmo *et al.*, 2001).

There is evidence that all ribotoxins isolated to homogeneity conserve their specificity in the nanomolar range. However, at micromolar concentrations they are capable of hydrolising RNA exhaustively, exhibiting preference for guanine at the 3'-end (Endo *et*

al., 1983). This loss of specificity is shown by the hydrolysis of different substrate analogues, as it is the case of homopolymers such as poly(A), poly(G) or poly(I) (Endo *et al.*, 1983). Activity has been detected even on dinucleoside monophosphates, which can be considered as their minimum substrate, though the way they are recognised by the enzyme seem to differ from the natural substrate. Despite of this, the advantage in this case is that the products, substrates and intermediates of the reaction can be separated and quantified by HPLC, providing information about the different steps of the catalysis (Lacadena *et al.*, 1998). This kind of studies have allowed the establishment of the mechanism followed by ribotoxins when performing their ribonucleolytic action, the non-cytotoxic microbial RNases T1 and U2 being of great help as reference models. Accordingly, α-sarcin behaves as a cyclising RNase, following the same general reaction scheme as the other members of the RNase T1 family (Figure 3). The production and characterisation of many site-directed and randomly produced mutants have allowed the determination of not only the ribotoxin residues involved in the catalytic reaction, but also their different roles during the cleavage of a phosphosiester bond. All the identified α-sarcin's active site residues have their corresponding counterparts in RNase T1 (Figures 2 and 3). Residues His 137 and Glu 96 are essential for the catalytic reaction, acting as a general acid and a general base, respectively, during the first step and reversing their roles during the subsequent hydrolysis of the cyclic derivative (Brandhorst *et al.*, 1994; Kao and Davies, 1995, 1999; Lacadena *et al.*, 1995, 1999; Sylvester *et al.*, 1997; Kao *et al.*, 1998). His 50 would also contribute to the stabilisation of the transition state but, in this case, would not be able to substitute for Glu 96 as the general base in the E96Q mutant (Lacadena *et al.*, 1999), as it is the case for the equivalent RNase T1 His residue (Steyaert *et al.*, 1990, 1997). Mutagenesis studies with residues Arg 121, Leu 145 and Tyr 48 have

confirmed their participation in the protein function (Masip *et al.*, 2001, 2003; Álvarez-García *et al.*, 2006). Arg 121 is involved in the correct orientation of large substrates in the active site and its positive charge is essential for the interaction of the protein with membranes (Masip *et al.*, 2001). Leu 145 interacts with His 137, contributing to the low pK_a of this histidine residue, and keeps relation to the particular orientation of loop 5, which diminishes the accessibility of the active site to the solvent (Masip *et al.*, 2003). Finally, mutation of residue Tyr 48 to Phe gives rise to a variant that is inactive against ribosomes but keeps the ability to degrade ApA, which reveals the essential role of the OH group in the phenolic ring in degradation of polymeric RNA (Álvarez-García *et al.*, 2006). Thus, Tyr 48, Arg 121 and Leu 145 appear to be determinants of the ribotoxin activity of α-sarcin. In the crystal geometric complex of RNase T1 with the minimal substrate 3'-GMP (Loverix and Steyaert, 2001), their counterpart residues Tyr 38, Arg77 and Phe 100 also appear to be part of the catalytic site of the enzyme. There has been speculation that these three residues, together with His 40, would form a prearranged structural and dielectric microenvironment that is complementary in shape, charge and hydrogen-bonding formation to the equatorial oxygens of the transition state, contributing to its optimal solvation/desolvation during the catalysis (Loverix and Steyaert, 2001). Additionally, studies on the crystal structures of complexes of restrictocin with inhibitors led to the proposal that ribotoxins may use base flipping to enable cleavage at the correct site of the SRL (Yang *et al.*, 2001). All studies so far suggest that residues Tyr 48, Arg 121 and Leu 145 would enable the base flipping performed by the catalytic triad that permits RNase cleavage at a unique phosphodiester bond (Yang *et al.*, 2001).

Despite of all these conclusions being obtained throughout dinucleoside phosphates assays, the catalytic efficiency of ribotoxins against these substrates is several orders of

magnitude lower than that of T1-like RNases (Lacadena *et al.*, 1998). One of the main reasons for this behavior is the solvent restriction imposed on the active site by the surrounding loops, especially loop 5. Length and interactions of this loop with the amino terminal hairpin affect the environment of the principal catalytic histidine (His 137 in α-sarcin). This observation would explain the differences in catalytic efficiency, but not in specificity, leading to the suggestion that there might be interactions of the substrate with other loops.

In summary, the main advantage of ribotoxins over T1-like RNases relies on their specificity, which makes them capable of inhibiting protein biosynthesis by cleaving a single bond out of more than 7000 present in the ribosome.

Interaction with the ribosome

This specificity, which makes these proteins extraordinarily efficient toxins, depends on the recognition of a very specific region within the ribosome. The SRL is located at domain VI of 28S rRNA (23S in prokaryotes) and is composed of 30-35 nucleotides of double stranded RNA with a universally conserved sequence and a compact structure that contains several purine-purine base pairs, a GAGA tetraloop, and a bulged guanosine adjacent to a reverse Hoogsteen AU pair. This structure is stabilised by an unusual set of cross-strand base-stacking interactions and imino proton to phosphate oxygen hydrogen bonds (Figure 4) (Szewczak *et al.*, 1993; Szewczak and Moore, 1995; Seggerson and Moore, 1998). Together with the ribosomal protein L11-binding region, the L7/L12 stalk, and the ribosomal proteins L6 and L14 (Figure 4), the SRL constitutes a binding site for elongation factors that is required for correct ribosome function (Endo and Wool, 1982; Cameron *et al.*, 2002; Van Dyke *et al.*, 2002). The L11-binding domain sequence is also universally conserved, in good agreement with its essential role

(Mears *et al.*, 2002). Interestingly, the spatial orientation in the ribosome of both the SRL and the L11-binding domain varies not only among the different *phyla* (Ramakrishnan and Moore, 2001; Mears *et al.*, 2002; Uchiumi *et al.*, 2002), but also during the different steps of peptide bond formation (Gabashvili *et al.*, 2000). These variations might explain why different toxins display different affinities when assayed against distinct ribosomal substrates (Schindler and Davies, 1977; Endo and Wool, 1982; Wool *et al.*, 1992; Uchiumi *et al.*, 2002). Mutations affecting the sequence contained in the SRL result in deffective binding of elongation factors and aminoacyl-tRNA, as well as a decreased translational fidelity (Liu and Liebman, 1996). Some of those mutations are lethal, reinforcing the importance of this region for the translational machinery (Leonov *et al.*, 2003). Studies on the dynamics and kinetics of the ribosome show considerable mobility of this region, known as the GTPase centre, and its potential involvement in conformational changes essential for the correct performance of translation (Nilsson and Nissen, 2005).

Recognition of the SRL by ribotoxins mainly depends on interactions between the protein and both the GAGA tetraloop and the bulged G (Moazed *et al.*, 1988; Glück and Wool, 1996; Munishkin and Wool, 1997; Pérez-Cañadillas *et al.*, 2000). G2655 is the most critical site for binding of elongation factors and it seems to be the only really essential nucleotide for the specific ribonucleolytic activity of ribotoxins (Macbeth and Wool, 1999). However, the primary determinant of recognition does not seem to be the type of nucleotide, but rather the SRL conformation (Munishkin and Wool, 1997; Correll *et al.*, 1999; Correll and Swinger, 2003). As can be deduced from the crystal structure of the restrictocin-SRL analogue complex, loops 1 and 3 of the ribotoxin interact with the bulged G and the S-turn, which includes this nucleotide (Figure 5) (Macbeth and Wool, 1999; Yang *et al.*, 2001). Some residues of loop 5 and loop 2, the

latter comprising an abundance of positive charge residues, would be involved in interactions with the GAGA tetraloop including the target bond. Once the ribotoxin is anchored, it exerts its action on the GA bond 12 Å distant from the bulged G, though participation of a G in the cleaved bond does not seem to be strictly necessary (Glück and Wool, 1996).

Nevertheless, all these interactions with the SRL do not explain by themselves the exquisite specificity of ribotoxins against their target ribosomes. Accordingly, it has been recently shown that the ribosomal context enhances the reaction rate several orders of magnitude, probably due to favorable electrostatic interactions (Korennykh *et al.*, 2006, 2007). Ribotoxins are basic proteins with a high density of charged and polar side chains exposed to the solvent (Pérez-Cañadillas *et al.*, 2000), which would agree with an electrostatic localisation of ribotoxins to the ribosome and subsequent difussion within the ribosomal electrostatic field to the SRL. Additionally, the high internal mobility exhibited by some regions of the ribotoxins structure would enable accessibility of these proteins to other potential recognition sites, which would increase the probability of successful binding. In this regard, one of the regions with the highest conformational flexibility in α-sarcin is the N terminal β-hairpin (amino acids 1-26) (Pérez-Cañadillas *et al.*, 2000; García-Mayoral *et al.*, 2005a). Obtention of the deletion mutants α-sarcin Δ (7-22) and Asp f 1 Δ(7-22) made it possible to assess that these variants maintained the ribonucleolytic activity against dinucleoside phosphates, as well as the ability to specifically degrade oligonucleotides mimicking the sequence and structure of the SRL, but could not act against intact ribosomes, resulting to be much less cytotoxic proteins (García-Ortega *et al.*, 2002, 2005). The three-dimensional structure of the mentioned α-sarcin deletion mutant showed that the general folding of the wild-type protein was preserved, including the spatial conformation of the loops of

non periodic structure (García-Mayoral *et al.*, 2004). Modeling the ribotoxin recognition of ribosomes by both wild-type α-sarcin and its Δ (7-22) mutant, two additional interacting regions were identified (García-Mayoral *et al.*, 2005b). One of them would involve a sequence stretch of loop 2 and ribosomal protein L6, whereas the other would depend on the contact between the N terminal β-hairpin and ribosomal protein L14 (Figure 6). This latter interaction would not be possible for the Δ (7-22) mutant, thus being crucial for the specific recognition of the ribosomes, as proved by the results obtained with this variant. This conclusion is supported by the observation that a sequence homologous to the 11-16 region of α-sarcin can be found in EF-2 from *Saccharomyces cerevisiae* (Kao and Davies, 1999; García-Mayoral *et al.*, 2005b). Additionally, sequence variability in this region among proteins of the L14 family would explain the different specificity exhibited by ribotoxins depending on the species from which the ribosomes assayed are obtained (Schindler and Davies, 1977; Endo and Wool, 1982; Endo *et al.*, 1983; Uchiumi *et al.*, 2002; García-Mayoral *et al.*, 2005b).

In summary, all these interactions contribute to the extraordinary specificity of ribotoxins against their substrate, inactivating it with a second order rate constant (k_{cat} / K_M = 1.7 x $10^{10} M^{-1}s^{-1}$) that matches the catalytic efficiency of the fastest known enzymes (Korennykh *et al.*, 2006). This specific action is so effective that a single molecule of α-sarcin is enough to kill a cell (Lamy *et al.*, 1992).

Interaction with membranes and citotoxicity

In order to completely explain the cytotoxic character of ribotoxins, mention must be made of their ability to interact with cell membranes. This is the main difference between ribotoxins and RNases from the T1 family, as well as the limiting factor for cytotoxicity. Although knowledge about the mechanism of cell entry followed by

ribotoxins is very scarce, the most relevant data concerning this topic have been obtained for α-sarcin.

Studies with vesicle-model systems proved that α-sarcin interacts specifically with acid phospholipid vesicles, such as phosphatidilserine (PS) or phosphatidilglycerol (PG), at neutral or slightly acid pH (Gasset *et al.*, 1989, 1991a). This fact would be in agreement with the preference exhibited by ribotoxins for tumour or virus-infected target cells, where the loss of simmetry in the plasma membrane induces a higher exposure of PS or other acid phospholipids to the extracellular medium (Bergelson *et al.*, 1970; Turnay *et al.*, 1993; Orntoft and Vestergaard, 1999; Ran *et al.*, 2002; Papo and Shai, 2005). The recent discovery of the involvement in malignant transformation of the enzymes responsible for phosphatidic acid synthesis (diacylglycerol kinases) seems to further support this hypothesis (Filigheddu *et al.*, 2007; Griner and Kazanietz, 2007; Mérida *et al.*, 2008). Binding experiments allowed determination of a K_d = 60.0 nM for lipid-protein complexes that caused vesicle aggregation followed by fusion, but whose formation was abolished at basic pH (Gasset *et al.*, 1990). In the initial step of this interaction, α-sarcin acts as a bridge to dimerise vesicles (Mancheño *et al.*, 1994a) and then fusion is triggered by the destabilising effect of the protein, which simultaneously suffers conformational changes upon binding to the vesicles (Gasset *et al.*, 1991b; Mancheño *et al.*, 1994a). α-Sarcin is capable of translocating across the lipid bilayer of the vesicles thanks to a hydrophobic interaction involving region 116-139, as demonstrated by a synthetic peptide with that sequence (Oñaderra *et al.*, 1993; Gasset *et al.*, 1994, 1995b; Mancheño *et al.*, 1995b). Even a peptide comprising only residues 131-139 mimicks the effects of the whole protein in this respect (Mancheño *et al.*, 1998). Passage across membranes is accompanied by structural changes and a decrease

in protein stability, but the protein is ribonucleolytically active once inside the vesicle (Gasset *et al.*, 1991b, 1995b; Oñaderra *et al.*, 1993; Mancheño *et al.*, 1994b).

α-Sarcin Δ (7-22) deletion mutant and some other variants affecting residues in this region of the protein have shown that the N terminal β-hairpin is also involved in the interaction with cell membranes (García-Ortega *et al.*, 2001, 2002) as they display a different pattern of interaction with lipid vesicles, compatible with the absence of one vesicle-interacting protein region (García-Ortega *et al.*, 2002). Restrictocin also behaves differently from α-sarcin as far as lipid-interacting abilities are concerned (García-Ortega *et al.*, 2001), being noteworthy the fact that six residues out of the only 20 differences between both proteins are located in the N terminal β-hairpin. Loop 2 has been proposed by some authors to be involved as well in the interaction with membranes (Martínez del Pozo *et al.*, 1988; Yang and Moffat, 1996; Kao and Davies, 1999; Pérez-Cañadillas *et al.*, 2000). The two triptophan residues of α-sarcin have not been proven necessary for the interaction (De Antonio *et al.*, 2000), but studies with α-sarcin's R121Q mutant have demonstrated the important role played by Arg121 during this process (Masip *et al.*, 2001). This interesting result led to the proposal that proteins that had evolved to interact with RNA, such as ribotoxins, would have developed structural and chemical determinants to recognise polyphosphate lattices that might as well allow recognition of a phospholipid bilayer (Masip *et al.*, 2001). Interestingly, when the crystalline structure of restrictocin was elucidated, Arg 120, the counterpart to α-sarcin's Arg 121, was found to be hydrogen bonded to a cocrystallised phosphate molecule at the active site (Yang and Moffat, 1996).

In general, the basic character of ribotoxins seems to be one of the key factors for cytotoxicity, as has been shown for other RNases (Di Donato *et al.*, 1994; Vatzaki *et al.*, 1999; Ilinskaya *et al.*, 2002). Passage across the cell membrane is the rate-limiting step

for α-sarcin's cytotoxic activity (Turnay *et al.*, 1993), endocytosis being the internalisation mechanism (Olmo *et al.*, 2001). As no protein receptor has been found so far, the toxic specificity must be related to a differential interaction with the lipid components of the membranes. α-Sarcin has been reported to be a powerful inhibitor of protein synthesis in picornavirus-infected cells and several transformed cell lines (Fernández-Puentes and Carrasco, 1980; Carrasco and Esteban, 1982; Turnay *et al.*, 1993; Olmo *et al.*, 2001; Stuart and Brown, 2006). Besides, ionophores, external ATP or phospholipase C treatment made mammalian cells more sensitive to α-sarcin entry (Alonso and Carrasco, 1981, 1982; Otero and Carrasco, 1986, 1988). All these observations were interpreted in terms of the existence of altered membrane permeability. The toxin reaches the cytosol after clathrin-independent transport by acid endosomes and the Golgi (Olmo *et al.*, 2001). In this regard, it has been recently shown that polycationic proteins tend to associate with phosphatidylserine enriched compartments such as endosomes (Yeung *et al.*, 2008). The abundance of anionic phospholipids in the cytosolic leaflet of these organelles might be directly related to ribotoxins' cytotoxicity.

Studies with rhabdomyosarcoma cells proved that apoptosis is the mechanism of cell death, although it does not seem to be a general direct consequence of protein biosynthesis inhibition, as deduced from a comparative analysis of the effects of α-sarcin and cycloheximide (Olmo *et al.*, 2001). However, variants with mutations affecting the enzymatic specificity of the protein showed diminished cytotoxic effects on these cells, revealing the relationship between ribonucleolytic activity and cytotoxicity (Lacadena *et al.*, 1995; García-Ortega *et al.*, 2002).

Evolution

Ribotoxins are an intriguing group of proteins regarding structure-function relationships. Their high degree of sequence and structural similarity with nontoxic fungal RNases of the T1 family has led to the suggestion that both families could have a common ancestor (Lamy *et al.*, 1992; Kao and Davies, 1995). Comparing ribotoxins and T1-like RNases, 25 per cent sequence homology can be found (Sacco *et al.*, 1983), as well as the conservation of the main structural core including the active site responsible for the phosphodiesterase activity of these enzymes (Pérez-Cañadillas *et al.*, 2000) (Figures 1 and 2). RNase U2 stands out as the unspecific fungal extracellular RNase most closely related to ribotoxins (Sacco *et al.*, 1983; Martínez del Pozo *et al.*, 1988; Martínez-Ruiz *et al.*, 1999a). It is 10 residues longer than the rest of the proteins of the T1 family (114 vs 101-106) and displays 34 per cent sequence identity with ribotoxins. These are the reasons why both families are considered as members of the same group of proteins (Aravind and Koonin, 2001).

However, ribotoxins present a number of characteristics that make them unique within this superfamily. They are around 40 residues longer, basic, and show a high specificity for their natural substrate, resulting besides cytotoxic due to their ability to interact with cell membranes (Lacadena *et al.*, 2007). From a structural point of view, the main differences with T1-type RNases lie in length and arrangement of the loops of non periodic structure and the N terminal β-hairpin, these elements being thus considered as the determinants of the extra activities of ribotoxins. Consequently, the study of the evolution of these proteins is of particular interest, as they appear to be naturally engineered target toxins that could have evolved from a nontoxic microbial RNase (Lamy *et al.*, 1992; Kao and Davies, 1995), maybe from a guanine or purine specific one. If so, it would be reasonable to consider the existence of evolutionary intermediates

that could have acquired only some of the extra regions conferring additional activities to these RNases.

After its discovery, hirsutelin A (HtA), an insecticidal protein from the mite fungal pathogen *Hirsutella thompsonii*, appeared as a feasible candidate to be such intermediate (Martínez-Ruiz *et al.*, 1999b). Some of its biological properties resembled those of the ribotoxin family (Liu *et al.*, 1996) and the alignment of the primary structure deduced from the HtA cDNA sequence (Boucias *et al.*, 1998) with those of ribotoxins revealed a significant similarity (Martínez-Ruiz *et al.*, 1999b). Sequence identity between HtA and ribotoxins was instead of only about 25 per cent, a value much lower than that among known ribotoxins (always above 60 per cent), but conservation of the catalytic residues and the four cysteines presumably involved in disulfide-bridges formation was observed (Figure 1). Interestingly, HtA is 20 residues shorter than α-sarcin-type proteins, the deletion of amino acids being presumably located at the protein loops and the N terminal hairpin, where HtA also differs from RNases of the T1 family. Characterisation of its enzymatic properties has shown that HtA specifically inactivates ribosomes releasing the α-fragment characteristic of ribotoxin activity on rRNA (Herrero-Galán *et al.*, 2008). In addition, HtA specifically cleaves oligonucleotides that mimick the SRL, as well as selected polynucleotides and dinucleosides, behaving as a cyclising ribonuclease too (Herrero-Galán *et al.*, 2008). Finally, it interacts with phospholipid membranes and exhibits cytotoxic activity on human tumour cells as the other ribotoxins (Herrero-Galán *et al.*, 2008). Based on all these results, HtA has been considered as a new ribotoxin, resulting to be the smallest member so far described in this family. The characterisation performed (Herrero-Galán *et al.*, 2008) proves that the abilities of ribotoxins can be accommodated into a shorter amino acid sequence of intermediate size between those of T1-type RNases and

previously known ribotoxins. Therefore, comparative studies with HtA can shed light on the structure-function relationships of this family of proteins, maybe revealing unknown roles of the longer loops of the other ribotoxins. In this regard, the current determination of its three-dimensional structure in solution by NMR (A. Viegas *et al.*, unpublished) will be of great help. Additionally, the insecticidal character of HtA opens a new way for exploration of the biological function of ribotoxins, unknown to date. In this sense, it has been suggested that they could avoid destruction of the ribotoxin-producing fungi deterring insect-feeding on their phialides (Brandhorst *et al.*, 1996).

Current trends

Despite the fact that the potential use of ribotoxins as antitumour agents was abandoned early due to high toxicity (Roga *et al.*, 1971), the present accumulation of data about their mechanism of action allows an optimistic view regarding the therapeutic utilisation of these proteins. In relation to this, studies on the allergenic character of some ribotoxins and the production of hypoallergenic mutants, together with the development of immunotoxins based on these fungal ribonucleases, stand out as the most feasible alternatives in the mid-term future. On the other hand, the design of chimaeric ribotoxins with convenient activities must also be considered.

Allergenicity

Allergens are usually identified as substances recognised by IgE antibodies contained in the sera of allergic patients. In this sense, ribotoxins have been related to allergies caused by *Aspergillus*, the main ribotoxin-producing *genus* and the most important pathogen involved in human allergic syndromes provoked by fungi (Kurup *et al.*, 2002; Kurup, 2003). In fact, ribotoxins were found in the urine of patients with disseminated

aspergillosis (Arruda *et al.*, 1990; Lamy *et al.*, 1991) and antibodies have been used to prove that they accumulate in the vicinity of the nodes of fungal infection (Lamy *et al.*, 1991), Asp f 1 from *Aspergillus fumigatus* being the ribotoxin most deeply studied as an allergen. This protein is involved in the pathogenicity of allergic bronchopulmonary aspergillosis (ABPA), the most severe form of allergic inhalant diseases, as high levels of Asp f 1-specific IgE are found in the sera of patients affected by this syndrome (Kurup *et al.*, 1994; García-Ortega *et al.*, 2005). Asp f 1 was, besides, the first recombinant allergen tested *in vivo* (Moser *et al.*, 1992), showing complete concordance with serologic determinations (Moser *et al.*, 1992; Crameri *et al.*, 1998; Hemmann *et al.*, 1999). Unfortunately, the recombinant protein is not devoid of cytotoxic activity and can trigger anaphylaxis.

Attempts to improve diagnosis of allergic diseases are focusing on the employment of homogeneous preparations of recombinantly produced allergens, much easier to standardise than complex fungal extracts (Piechura *et al.*, 1983; Crameri *et al.*, 1998; Kurup *et al.*, 2006). Recent studies with the Asp f 1 Δ (7-22) deletion mutant have shown that one of the major allergenic determinants of this protein is located at the N terminal β-hairpin (García-Ortega *et al.*, 2005). This region displays the highest sequence variability among ribotoxins (Figure 1) (Martínez-Ruiz *et al.*, 1999a, b, 2001), and is highly flexible and solvent exposed (Pérez-Cañadillas *et al.*, 2000; García-Mayoral *et al.*, 2004). Asp f 1 differs from α-sarcin in only 19 residues, but five of these differences are located at this N terminal β-hairpin. Responses of α-sarcin and its deletion mutant against Asp f 1-containing sera were even lower than that of Asp f 1 Δ (7-22) mutant, indicating that the deleted portion, although important, is not the only allergenic epitope within the molecule and that the essential residues for the other determinants of the immunoreactivity are changed in wild-type α-sarcin (García-Ortega

et al., 2002, 2005). Despite this decrease in IgE reactivity, the prevalence of the three Asp f 1 variants remained essentially unaffected, and they retained most of the IgG epitopes (García-Ortega *et al.*, 2005). This fact, together with the absence of cytotoxic activity in these ribotoxins deletion variants, states them as promising molecules for use in immunomodulating therapy and diagnosis of *Aspergillus* hypersensitivity, though this possibility should still be corroborated by *in vivo* assays. With this purpose, development of an allergic murine system sensitised against Asp f 1 is currently under way (E. Álvarez-García *et al.*, unpublished).

Immunotoxins

Immunotoxins have emerged as a powerful alternative for the treatment of a variety of human pathologies because of their ability to specifically direct their action to certain cell types. Immunotoxin design is based on the 'magic bullet' concept, introduced by Ehrlich in 1906, according to which these molecules would consist of a tissue-specific carrier that would deliver toxic agents to neoplasic tissues (Ehrlich, 1956; Sandvig and van Deurs, 2000). The discovery of monoclonal antibodies in 1975 allowed the preparation of new toxins specifically directed against particular tumour cells, thanks to their conjugation to immunoglobulins specific for cancer cell antigens. The targeting moiety of these first-generation immunotoxins was the whole antibody molecule (Kreitman, 2000). As the recognition sites for antigens are on the variable regions of immunoglobulins, further studies were performed to verify that Fab fragments, obtained after IgG papain digestion, retained the ability to interact with the epitopes (Ward *et al.*, 1989; Worn and Pluckthun, 2001), leading to the so called Fab or Fv immunotoxins, which were more easily internalised because of their smaller size (Brinkmann, 2000). The development of new technologies allowed the production of recombinant

immunotoxins, stabilized by a flexible peptide (scFv) or by a disulfide bridge between the variable domains (dsFv). These domains can be easily modified by genetic engineering, are more stable, and can be expressed in several organisms (Kreitman, 2003; Li *et al.*, 2004).

Regarding the toxin moiety, different toxins from bacteria and several ribosome inhibiting proteins (RIPs) from plants or fungi, mainly ricin, have been employed for immunotoxin design (Ghetie *et al.*, 1993; Engert *et al.*, 1997; Schnell *et al.*, 1998). The cytotoxic character of ribotoxins against carcinomas, together with their high thermostability, low immunogenicity and resistance to proteases, states them as ideal candidates for the construction of immunotoxins. Initial attempts were performed by chemical conjugation for restrictocin (Orlandi *et al.*, 1988; Conde *et al.*, 1989; Rathore and Batra, 1997a, b), mitogillin (Better *et al.*, 1992) and α-sarcin (Wawrzynczak *et al.*, 1991; Rathore *et al.*, 1997). Immunotoxins based on this latter protein offered promising results in preliminary assays (Wawrzynczak *et al.*, 1991) but did not proceed to *in vivo* studies probably because of their large size, that could hinder correct internalisation, or because of low structural stability of the immunoconjugates.

Second generation immunotoxins attempt to solve these problems by fusing the toxin to a single chain containing only the variable domains, needed for antigen recognition. Recombinant immunotoxins of this kind have been already obtained based on restrictocin (Rathore and Batra, 1997a, b). These single-chain immunotoxins (scFv-IMTX) can be easily modified by genetic engineering to improve their cytotoxic activity or to diminish immunogenicity or unspecific toxicity *in vivo*. In relation to this, a single-chain immunotoxin composed of the variable domains of the B5 monoclonal antibody bound to α-sarcin through a peptide containing a furine cleavage site (scFv-IMTXαS) has been recently produced in the methylotrophic yeast *Pichia pastoris* (Lacadena *et al.*,

2005). The monoclonal antibody (mAb) B5 belongs to a family of mAbs directed against a Lewis^Y-related carbohydrate antigen that is overexpressed on the surface of many carcinomas, including breast and colon solid tumours (Pastan and FitzGerald, 1991). Different members of the family have been used as the targeting moiety in many immunotoxins, and three of them have been evaluated in phase I trials in cancer patients, with promising results (Pai *et al.*, 1996; Brinkmann, 2000; Woo *et al.*, 2008). As far as the expression system is concerned, *Pichia pastoris* has emerged as a robust heterologous host in which several immunotoxins have already been successfully produced extracellularly (Woo *et al.*, 2002, 2004, 2006; Liu *et al.*, 2005).

In this sense, scFv-IMTXαS produced in *P. pastoris* displays the characteristic ribonucleolytic activity and specific cytotoxicity of α-sarcin against targeted cells containing the Lewis^Y antigen (Lacadena *et al.*, 2005; N. Carreras-Sangrà, unpublished). Studies on genetically engineered variants of this immunotoxin with increased stability and affinity are currently being performed.

Biotechnology

In recent years, oral vaccination using Gram-positive bacteria as probiotics is being developed as a promising approach for treatment of several human pathologies, allergies included, thanks to the 'Generally Regarded As Safe' (GRAS) status of some of these microorganisms (Pouwels *et al.*, 1996; Robinson *et al.*, 1997; Kirjavainen *et al.*, 1999; Maassen, 1999; Steidler *et al.*, 2000). One of these Gram-positive bacteria is *Lactococcus lactis*, a non pathogenic, non invasive, non colonizing microorganism mainly used to produce fermented foods, but also proven useful, for example, in producing IL-10 for the treatment of inflammatory bowel disease in mice (Steidler *et al.*, 2000). Although *L. lactis* passes rapidly through the gastrointestinal tract without

colonization (Gruzza *et al.*, 1994; Klijn *et al.*, 1995), genetically modified versions of this bacterium are still effective in delivering antigens to the mucosal immune system and capable of inducing a local immune response, which seems to happen because *L. lactis* lacks the ability to multiply *in vivo* but can readily be sampled by dendritic cells (Robinson *et al.*, 1997; Maassen, 1999; Adel-Patient *et al.*, 2005; Perez *et al.*, 2005). This process seems to be involved in the development of efficient immune responses (Xin *et al.*, 2003), including the selective induction of IgA (Macpherson and Uhr, 2004). In addition, antigens within *Lactococci* are protected against direct contact with gastric acid and proteolytic enzymes.

Following this idea, the *Lactococcus lactis* MG1363 strain has been recently engineered to produce and secrete wild-type Asp f 1 and α-sarcin, as well as three different mutants with reduced cytotoxicity and/or IgE-binding affinity, such as the above mentioned Δ (7-22) variants of both proteins and H137Q active site mutant of α-sarcin (Álvarez-García *et al.*, 2008). The proteins were secreted in native and active form when the extracellular medium was buffered at pH values around 8.0. Intragastric administration of either the bacterial strain alone or the transformed producing wild-type α-sarcin did not induced any deleterious effect on mice intestinal tract, indicating that even the highly toxic protein could be safely delivered using this vehicle for oral administration (Álvarez-García *et al.*, 2008). *Lactococcus lactis* utilisation as a potential delivery system for hypoallergenic variants of Asp f 1 and for ribotoxins in general as antitumoural agents against gastrointestinal tumours must thus be considered, though this possibility needs further evaluation.

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Web Resources

http://ccvweb.csres.utexas.edu/ccv/gallery/gallery.php?cat0ID=1&cat1ID=2&cat2ID=0&softwareID=4

Excellent movie on protein synthesis by ribosomes.

http://www.fgsc.net

One of the most popular Fungal Genetics web sites. A resource available for the Fungal Genetics research community with links to different *Aspergillus* genome sites (http://www.fgsc.net/aspergenome.htm) and the *Aspergillus* information web site (http://www.fgsc.net/Aspergillus/asperghome.html).

http://www. aspergillus.org.uk

The *Aspergillus* web site. A worldwide comprehensive resource providing information about these fungi and the diseases they can cause.

http://www.cbs.know.nl

The Centraal Bureau Voor Schimmelcultures, an institute of the Royal Netherlands Academy of Science.

http://rna.ucsc.edu/rnacenter/ribosome_movies.html

Different movies on ribosome structure and protein biosynthesis.

http://biochem4.okstate.edu/~biocukm/N1/N14212.html

Definition and description of different GNRA tetraloops such as that one cleaved by ribotoxins.

http://www.ehime-u.ac.jp/~cellfree/english/english_research.html

Link to a robust wheat germ cell-free protein synthesis system.

http://rmn.iqfr.csic.es/

Connection to the RMN group where many of the ribotoxins' three-dimensional structures have been solved.

http://bmb.bsd.uchicago.edu/Faculty_and_Research/01_Faculty/01_Faculty_Alphabetically.php?faculty_id=52

Connection to Ira Wool Home Page, where many of the initial discoveries regarding ribotoxins were made.

Figure Legends

Figure 1. Sequence alignment of several ribotoxins (α-sarcin, gigantin, clavin, restrictocin and hirsutellin A) and RNases T1 and U2. Elements of secondary structure in α-sarcin are delimited at the top of the alignment, as well as the essential catalytic residues and the cysteines involved in disulfide bridges formation in ribotoxins. Positions conserved in the seven or at least four sequences are highlighted in black or grey, respectively.

Figure 2. Representation of the three-dimensional structures of ribonucleases T1 (Martínez-Oyanedel *et al.*, 1991; PDB entry: 9RNT), U2 (Noguchi *et al.*, 1995; PDB entry: 1RTU) and α-sarcin (Pérez-Cañadillas *et al.*, 2000; PDB entry: 1DE3). Colour code for the secondary structure elements is the same as in Figure 1. Superpositions of the RNase U2 structure with those of RNase T1 and α-sarcin fitted to the active site residues are shown. The diagrams were generated with the MOLMOL program (Koradi *et al.*, 1996).

Figure 3. A: Catalytic mechanism proposed for cyclising ribonucleases. The type of substrate (dinucleotide, homo or heteropolynucleotide) is determined by R and R'. The catalytic residues A, B and C are represented within the active site structures of RNase T1 and α-sarcin in **B**. Essential residues are shown in red. The diagrams were generated with MOLMOL (Koradi *et al.*, 1996).

Figure 4. A: Diagram showing the position of the SRL (red) in the structure of the *Haloarcula marismortui* large ribosomal subunit (PDB entry: 1JJ2). Ribosomal proteins L6 (green) and L14 (yellow) are also shown. **B:** Structure of the SRL. Bases are filled in the 5' half and empty in the 3' half of the sequence. Numbers correspond to rat or *E. coli* (in brackets) nucleotide positions within the 28S (23S) rRNA. Ribotoxins cleave the bond between G4325 (2661) and A4326 (2662) (grey). The bulged G4319 (2655) is also indicated. The diagrams were generated with VMD (Humphrey *et al.*, 1996).

Figure 5. Structure of a restrictocin-SRL analogue complex (Yang *et al.*, 2001; PDB entry: 1JBS). The bulged G, interacting with loops 1 and 3 of the protein, and the nucleotides linked by the bond cleaved by ribotoxins are highlighted in black. The GAGA tetraloop is distorted in this substrate analogue and thus is not degraded. The diagrams were generated with VMD (Humphrey *et al.*, 1996).

Figure 6. Minimised docking model showing the interaction of wild-type α-sarcin (red) and its Δ (7-22) deletion mutant (blue) (García-Mayoral *et al.*, 2004; PDB entry: 430D) with the SRL and the *Haloarcula marismortui* ribosomal proteins L6 and L14. The diagrams were generated with MOLMOL (Koradi *et al.*, 1996).

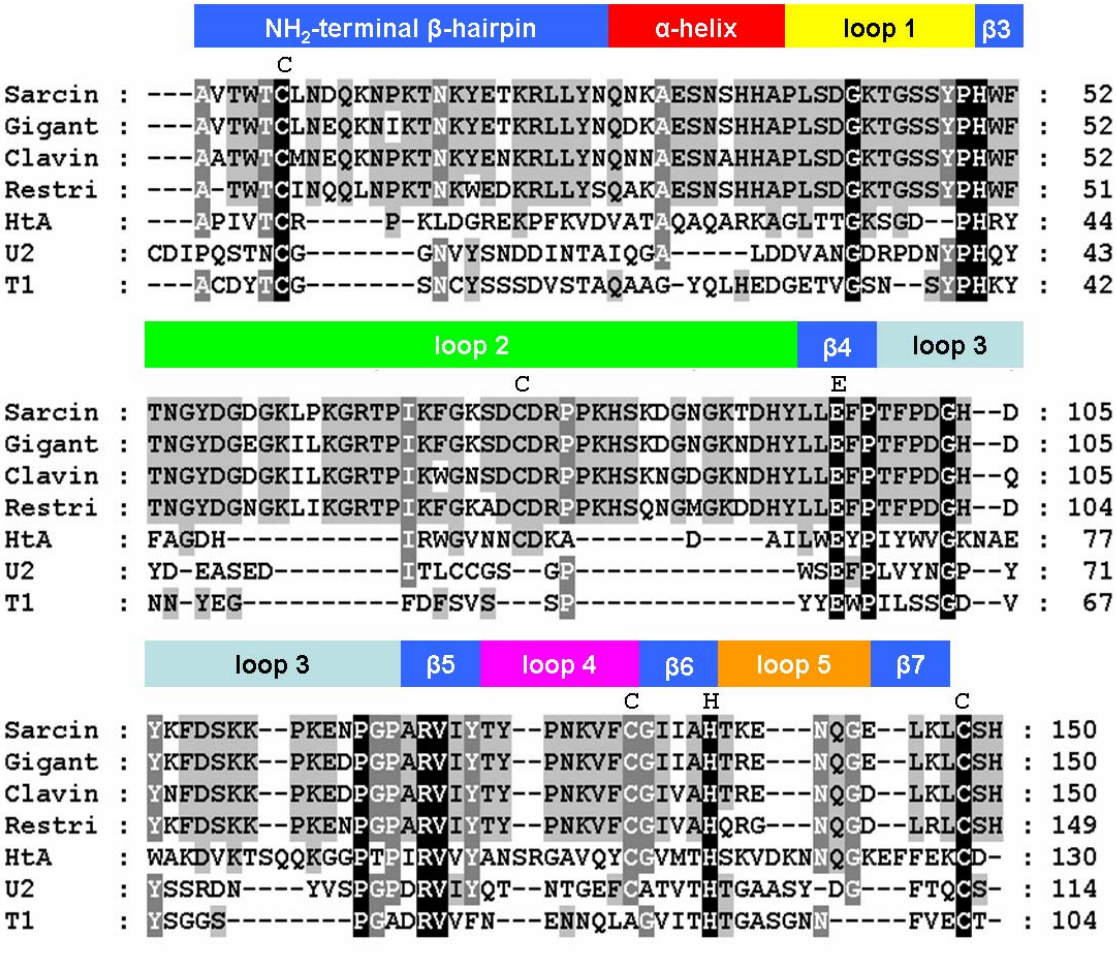
NH2-terminal β-hairpin
α-helix
loop 1
β3
loop 2
β4
loop 3
loop 3
β5
loop 4
β6
loop 5
β7
Sarcin : ---AVTWTCLNDQKNPKTNKYETKRLLYNQNKAESNSHHAPLSDGKTGSSYPHWF : 52
Gigant : ---AVTWTCLNEQKNIKTNKYETKRLLYNQDKAESNSHHAPLSDGKTGSSYPHWF : 52
Clavin : ---AATWTCMNEQKNPKTNKYENKRLLYNQNNAESNAHHAPLSDGKTGSSYPHWF : 52
Restri : ---A-TWTCINQQLNPKTNKWEDKRLLYSQAKAESNSHHAPLSDGKTGSSYPHWF : 51
HtA : ---APIVTCR-----P-KLDGREKPFKVDVATAQAQARKAGLTTGKSGD--PHRY : 44
U2 : CDIPQSTNCG-------GNVYSNDDINTAIQGA-----LDDVANGDRPDNYPHQY : 43
T1 : ---ACDYTCG-------SNCYSSSDVSTAQAAG-YQLHEDGETVGSN--SYPHKY : 42
Sarcin : TNGYDGDGKLPKGRTPIKFGKSDCDRPPKHSKDGNGKTDHYLLEFPTFPDGH--D : 105
Gigant : TNGYDGEGKILKGRTPIKFGKSDCDRPPKHSKDGNGKNDHYLLEFPTFPDGH--D : 105
Clavin : TNGYDGDGKILKGRTPIKWGNSDCDRPPKHSKNGDGKNDHYLLEFPTFPDGH--Q : 105
Restri : TNGYDGNGKLIKGRTPIKFGKADCDRPPKHSQNGMGKDDHYLLEFPTFPDGH--D : 104
HtA : FAGDH----------IRWGVNNCDKA-------D----AILWEYPIYWVGKNAE : 77
U2 : YD-EASED--------ITLCCGS--GP--------------WSEFPLVYNGP--Y : 71
T1 : NN-YEG----------FDFSVS---SP--------------YYEWPILSSGD--V : 67
Sarcin : YKFDSKK--PKENPGPARVIYTY--PNKVFCGIIAHTKE---NQGE--LKLCSH : 150
Gigant : YKFDSKK--PKEDPGPARVIYTY--PNKVFCGIIAHTRE---NQGE--LKLCSH : 150
Clavin : YNFDSKK--PKEDPGPARVIYTY--PNKVFCGIVAHTRE---NQGD--LKLCSH : 150
Restri : YKFDSKK--PKENPGPARVIYTY--PNKVFCGIVAHQRG---NQGD--LRLCSH : 149
HtA : WAKDVKTSQQKGGPTPIRVVYANSRGAVQYCGVMTHSKVDKNNQGKEFFEKCD- : 130
U2 : YSSRDN----YVSPGPDRVIYQT--NTGEFCATVTHTGAASY-DG---FTQCS- : 114
T1 : YSGGS--------PGADRVVFN---ENNQLAGVITHTGASGNN-----FVECT- : 104

Figure 1

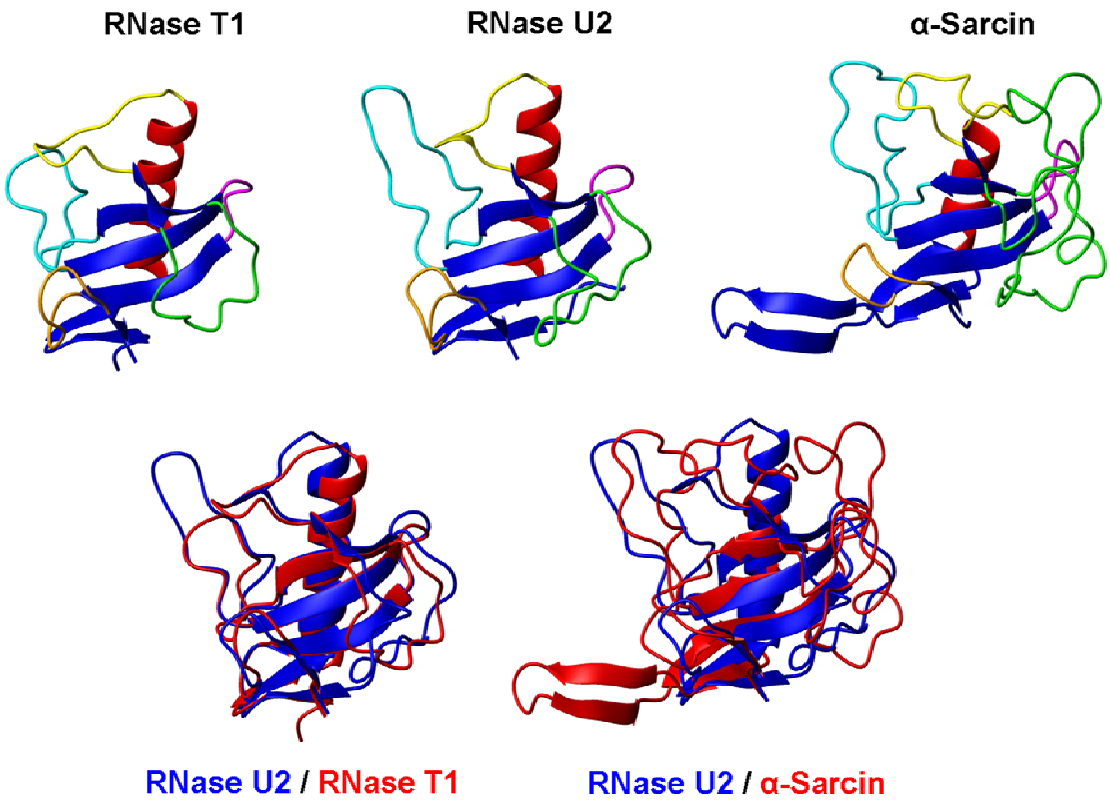

Figure 2

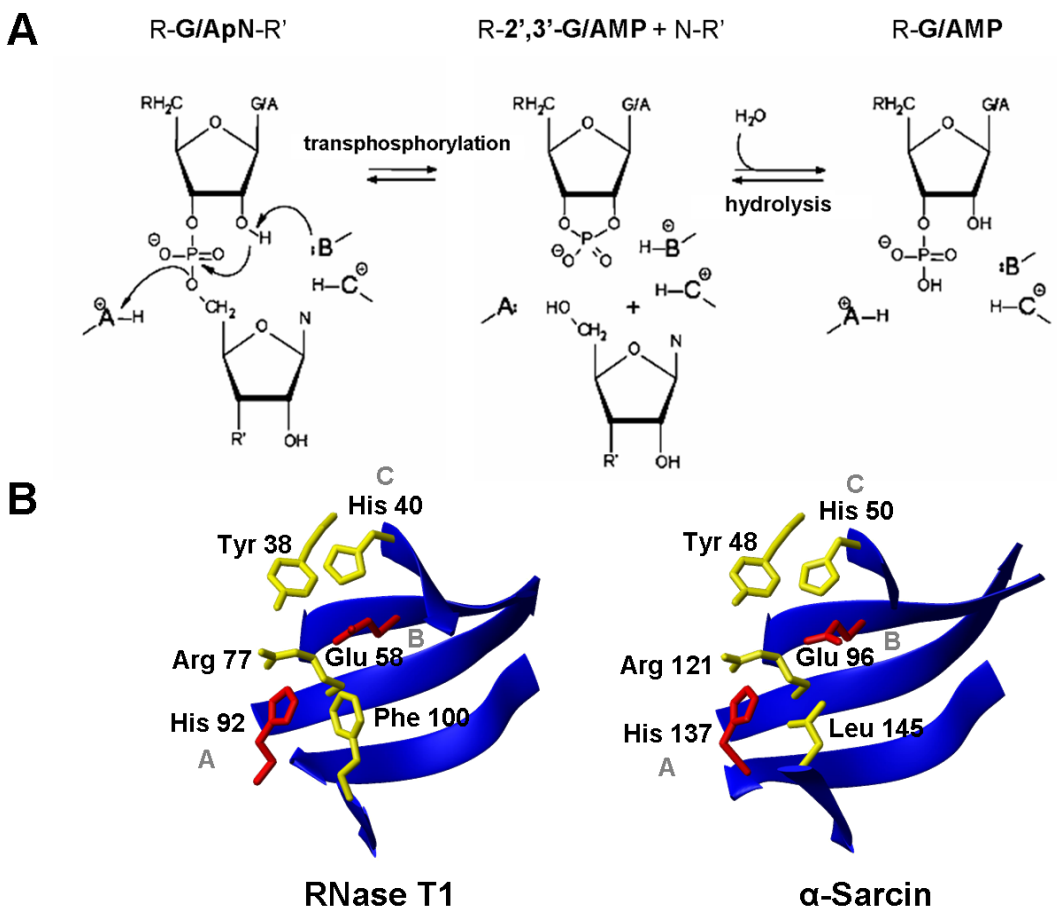
A
R-G/ApN-R'
R-2',3'-G/AMP + N-R'
R-G/AMP
transphosphorylation
hydrolysis
B
His 40
Tyr 38
Arg 77
Glu 58
His 92
Phe 100
RNase T1
His 50
Tyr 48
Arg 121
Glu 96
His 137
Leu 145
α-Sarcin

Figure 3

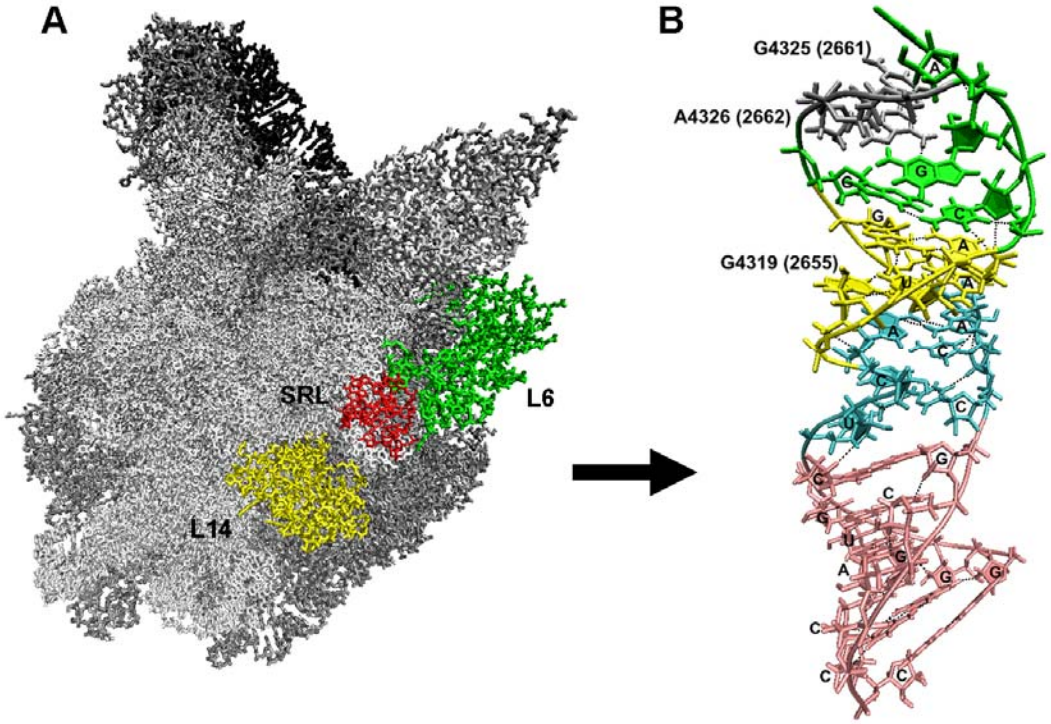

Figure 4

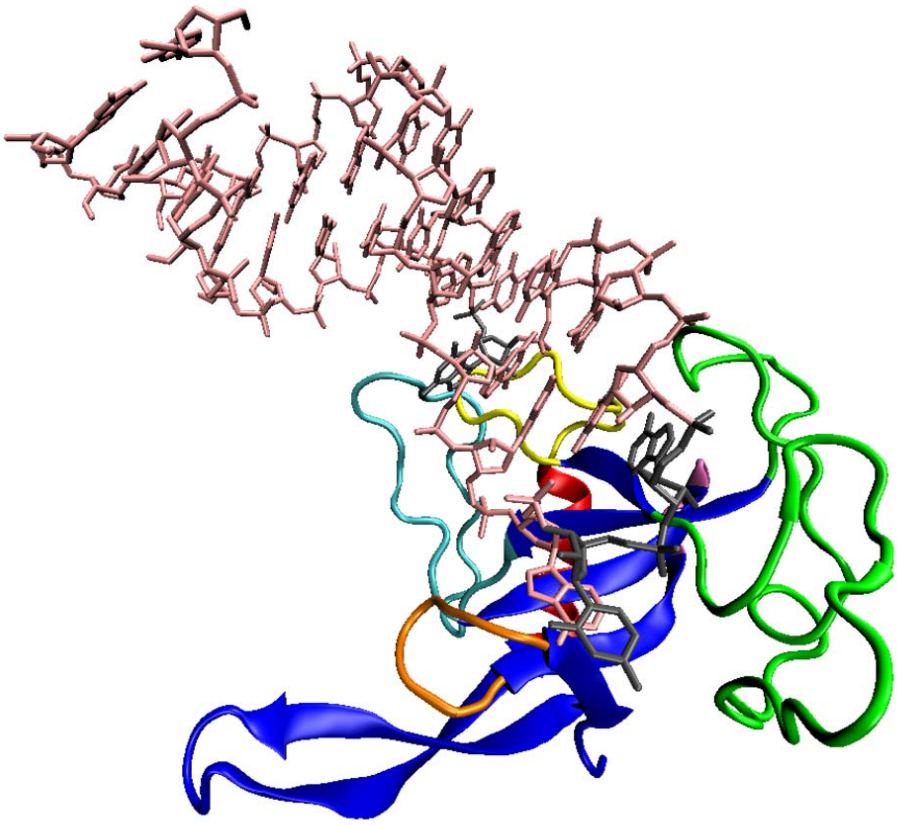

Figure 5

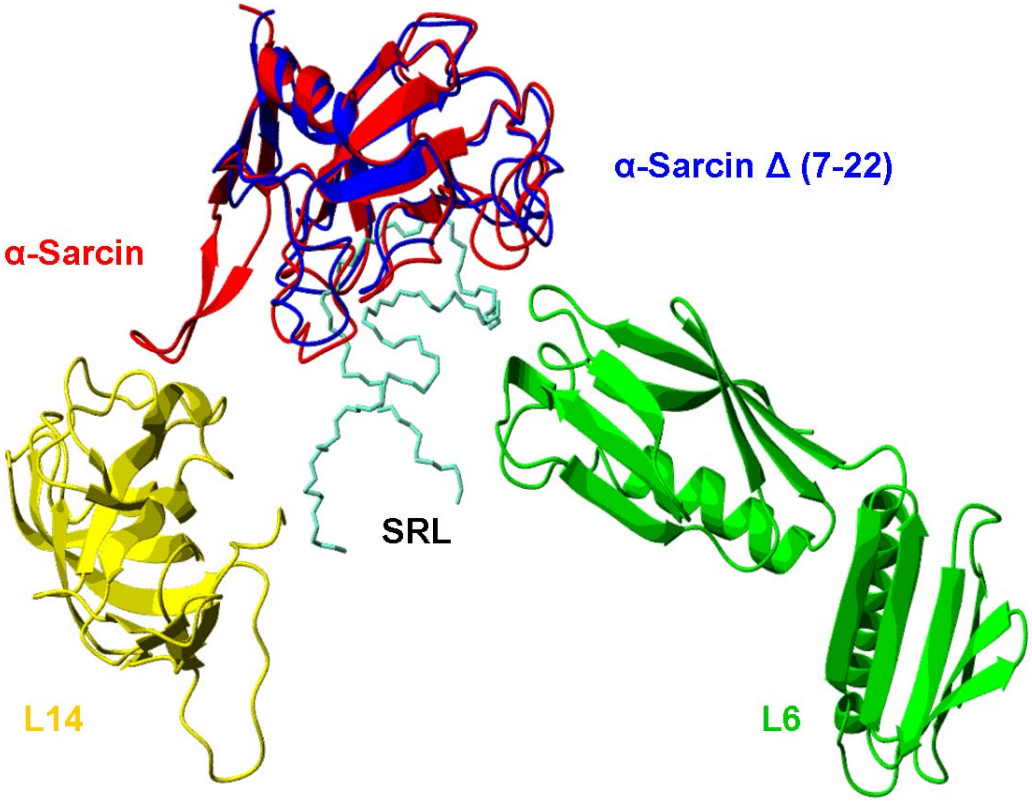

Figure 6