Fungal ribotoxins: molecular dissection of a family of natural killers

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Abstract

RNase T1 is the best known representative of a large family of ribonucleolytic proteins secreted by fungi, mostly *Aspergillus* and *Penicillium* species. Ribotoxins stand out among them by their cytotoxic character. They exert their toxic action by first entering the cells and then cleaving a single phosphodiester bond located within a universally conserved sequence of the large rRNA gene, known as the sarcin–ricin loop. This cleavage leads to inhibition of protein biosynthesis, followed by cellular death by apoptosis. Although no protein receptor has been found for ribotoxins, they preferentially kill cells showing altered membrane permeability, such as those that are infected with virus or transformed. Many steps of the cytotoxic process have been elucidated at the molecular level by means of a variety of methodological approaches and the construction and purification of different mutant versions of these ribotoxins. Ribotoxins have been used for the construction of immunotoxins, because of their cytotoxicity. Besides this activity, Aspf1, a ribotoxin produced by *Aspergillus fumigatus*, has been shown to be one of the major allergens involved in allergic aspergillosis-related pathologies. Protein engineering and peptide synthesis have been used in order to understand the basis of these pathogenic mechanisms as well as to produce hypoallergenic proteins with potential diagnostic and immunotherapeutic applications.

Introduction

Ribotoxins are a family of toxic extracellular fungal RNases that exert ribonucleolytic activity on the larger molecule of RNA in the ribosome, leading to protein synthesis inhibition and cell death by apoptosis (Gasset *et al.*, 1994; Kao *et al.*, 2001; Martínez-Ruiz *et al.*, 2001). Several studies have suggested that their location on the surface of fungal conidiophores correlates with the maturation of the conidia (Brandhorst & Kenealy, 1992; Yang & Kenealy, 1992a, b). Ribotoxins were discovered during a screening program of the Michigan Department of Health, started in 1956, searching for antibiotics and antitumor agents. The culture filtrates of a mold isolated from a sample of Michigan farm soil were found to contain a substance inhibitory to both *sarcoma 180* and *carcinoma 755* induced in mice (Olson *et al.*, 1965b). The mold was identified as *Aspergillus giganteus* MDH18894 (Fig. 1a), and the molecule responsible for these effects proved to be a protein, named α-sarcin (Fig. 1b) (Olson & Goerner, 1965). Two other antitumor proteins, restrictocin and mitogillin, both produced by *A. restrictus*, were later found to have similar activities, and were therefore included within the same group of antitumor molecules. Aspf1, another ribotoxin, produced by *Aspergillus fumigatus*, was much later identified as a major allergen in *Aspergillus*-related diseases (Arruda *et al.*, 1992). Unfortunately, further studies revealed an unspecific cytotoxicity of these proteins, which limited their potential clinical uses (Roga *et al.*, 1971). The study of these toxins was abandoned until the mid-1970s, when it was demonstrated that they inhibited protein biosynthesis in ribosomal preparations at concentrations as low as 0.1 nM by specifically cleaving a single phosphodiester bond of the large rRNA gene fragment (Schindler & Davies, 1977; Endo & Wool, 1982). This bond is of particular interest, because it is located at an evolutionarily conserved site with important roles in ribosome function, elongation factor 1 (EF-1)-dependent binding of aminoacyl-tRNA, and EF-2-catalyzed GTP hydrolysis and translocation (Wool *et al.*, 1992). This specific action of ribotoxins is so effective that a single molecule of α-sarcin is enough to kill a cell (Lamy *et al.*, 1992). It was this unique potency and specificity against ribosomes that prompted us to designate them as 'natural killers'.

Ribosome-inactivating proteins (RIPs) are a group of highly specialized toxic proteins (Stirpe *et al.*, 1988, 1992; Nielsen & Boston, 2001) produced by plants and fungi that inactivate ribosomes by acting on the same unique rRNA gene structure as ribotoxins do (Schindler & Davies, 1977; Endo & Wool, 1982; Endo *et al.*, 1987; Correll *et al.*, 1998, 1999; Mears *et al.*, 2002). Ribotoxins would be also RIPs from this point of view, but some authors claim (Nielsen & Boston, 2001; Peumans *et al.*, 2001) that the name RIP should be restricted to plant *N*-glycosidases, best represented by ricin, that depurinate a single nucleotide contiguous to the phosphodiester bond cleaved by ribotoxins (Endo *et al.*, 1987; Endo & Tsurugi, 1987). These are the reasons why the conserved rRNA gene sequence targeted by RIPs and ribotoxins has come to be universally known as the sarcin–ricin loop, or SRL (Fig. 2).

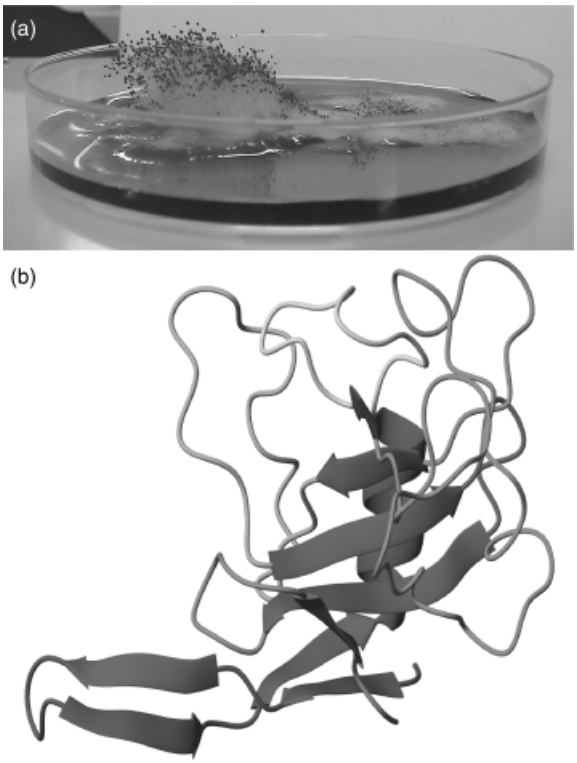

Fig. 1. (a) Photograph of a culture of *Aspergillus giganteus* MDH 18894. (b) Three-dimensional structure of α-sarcin (PDB ID 1DE3): The diagram was generated with the molmol program (Koradi *et al.*, 1996).

Many prokaryotes encode both a labile antitoxin and a stable toxin under the control of a single operon. These toxin–antitoxin (TA) systems constitute another interesting family of toxic endo-RNases (Christensen *et al.*, 2003; Muñoz-Gómez *et al.*, 2005; Condon, 2006; Kamphuis *et al.*, 2006; Luna-Chávez *et al.*, 2006). The antitoxin is synthesized in equimolecular amounts to the toxin, thus inhibiting its ribonucleolytic action. Once the cells are under a variety of stress conditions, the antitoxin is inactivated and the action of the toxin promotes cellular growth arrest in preparation for a more favorable situation. The target of these proteins is not completely established, but mRNA seems to be the best candidate according to the data so far published. It cannot be ruled out, however, that the ribosome, or most probably the translation complex, exerts some type of stimulating or modulating activity (Christensen & Gerdes, 2003). Thus, these endo-RNases can also be considered as modulators of protein biosynthesis and, in this regard, they have a functional connection with fungal ribotoxins. Actually, they share cleavage mechanisms, as they also behave as cyclizing

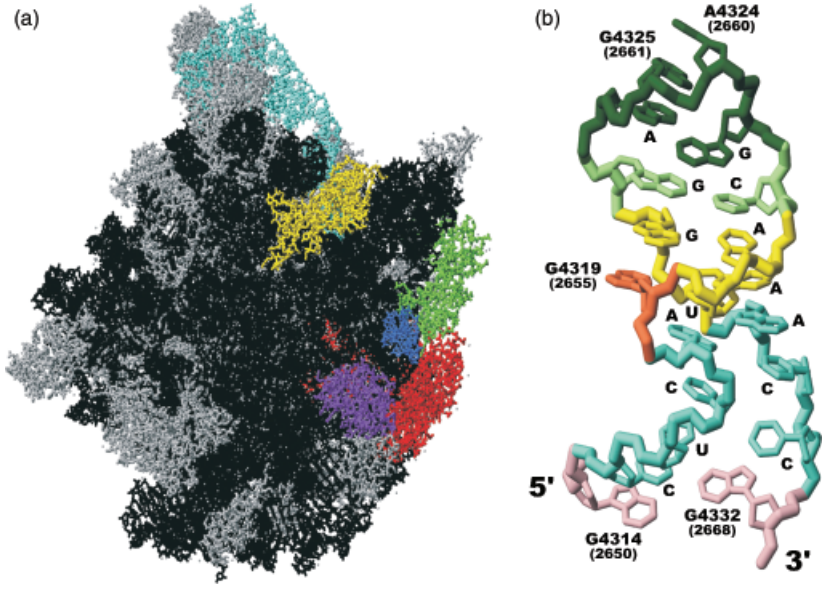

Fig. 2. (a) Diagram showing the structure of the *Halobacterium marismortui* ribosome large subunit (PDB ID 1JJ2): black, 23S RNA gene; cyan, 5S RNA gene; blue, SRL; red, ribosomal protein L3; green, ribosomal protein L6; yellow, ribosomal protein 10e; purple, ribosomal protein L14; gray, other ribosomal proteins. (b) Diagram showing the structure of the SRL (Correll *et al.*, 1998). Numbers correspond to rat or *Escherichia coli* (in brackets) nucleotide positions within the 28S (23S) rRNA gene. The bond cleaved by ribotoxins is that on the 3′-side of G4325 (2661) (dark green). Ricin depurinates A4324 (2660) (dark green). The bulged G is G4319 (2655) (orange). The Watson–Crick region of the hairpin (violet), the flexible region (cyan), the G-bulged cross-strand stack (yellow) and the tetraloop (green) are colored. The diagrams were generated with the molmol program (Koradi *et al.*, 1996).

RNases, as ribotoxins do (see below). However, the similarities end there, as known ribotoxins are not produced by prokaryotes, and use a different pair of side chain residues for the acid–base catalysis, and no specific antitoxin production has been so far reported (Martínez-Ruiz *et al.*, 1998, 2001; Kamphuis *et al.*, 2006).

In addition to their ribonucleolytic activity, ribotoxins cross lipid membranes in the absence of any known protein receptor (Oñaderra *et al.*, 1993; Gasset *et al.*, 1994; Martínez-Ruiz *et al.*, 2001). Thus, although any ribosome could be potentially inactivated by these proteins, owing to the universal conservativeness of the SRL, they are especially active on transformed or virus-infected cells (Olson *et al.*, 1965; Fernández-Puentes & Carrasco, 1980; Olmo *et al.*, 2001). This observation has been explained in terms of altered permeability of these cells combined with the ability of ribotoxins to interact with acid phospholipid-containing membranes (Gasset *et al.*, 1989, 1990; Martínez-Ruiz *et al.*, 2001; Olmo *et al.*, 2001).

α-Sarcin, restrictocin and Aspf1 are the most exhaustively characterized ribotoxins (Arruda *et al.*, 1992; Gasset *et al.*, 1994; Wool, 1997; Kao *et al.*, 2001; Martínez-Ruiz *et al.*, 2001; García-Ortega *et al.*, 2005), but many others have been identified and partially characterized in different fungal species (Lin *et al.*, 1995; Parente *et al.*, 1996; Huang *et al.*, 1997; Wirth *et al.*, 1997; Martínez-Ruiz *et al.*, 1999a, b). These studies have suggested a high degree of conservation among ribotoxins, as those characterized display amino acid sequence similarities above 85% (Fig. 3). However, hirsutellin A (HtA), an extracellular ribonucleolytic protein produced by the invertebrate fungal pathogen *Hirsutella thompsonii*, has been recently demonstrated to be a ribotoxin (Herrero-Galán *et al.*, 2007), and it displays only about 25% sequence identity with previously known family members (Boucias *et al.*, 1998; Martínez-Ruiz *et al.*, 1999a; Herrero-Galán *et al.*, 2007). This suggests that the presence of ribotoxins among fungi is more widespread than initially considered (Martínez-Ruiz *et al.*, 1999b). A specific RNase purified from mature seeds of oriental arbovitae (*Biota orientalis*) has also been reported to cleave a single phosphodiester bond of 28S rRNA gene in rat ribosomes but in a different region from the SRL, although spatially close to it (Xu *et al.*, 2004).

Aspergilli are a ubiquitous and complex group of filamentous fungi containing more than 185 species, including 20 human pathogens as well as others used for the industrial production of foods and enzymes. The publication of the genome sequence of the model organism *Aspergillus nidulans* (Galagan *et al.*, 2005) has created huge expectations regarding advances in our knowledge of the biology of these microorganisms. A comparative genomic study involving two other species, *Aspergillus fumigatus* and *Aspergillus oryzae* (Machida *et al.*, 2005; Nierman *et al.*, 2005), has also been reported. *Aspergillus nidulans* does not produce any ribotoxin, whereas *A. fumigatus*, a serious human pathogen, produces Aspf1, one of the best known ribotoxins (Moser *et al.*, 1992). *Aspergillus oryzae* (Machida *et al.*, 2005) is used in the production of sake, miso and soy sauce, and also of

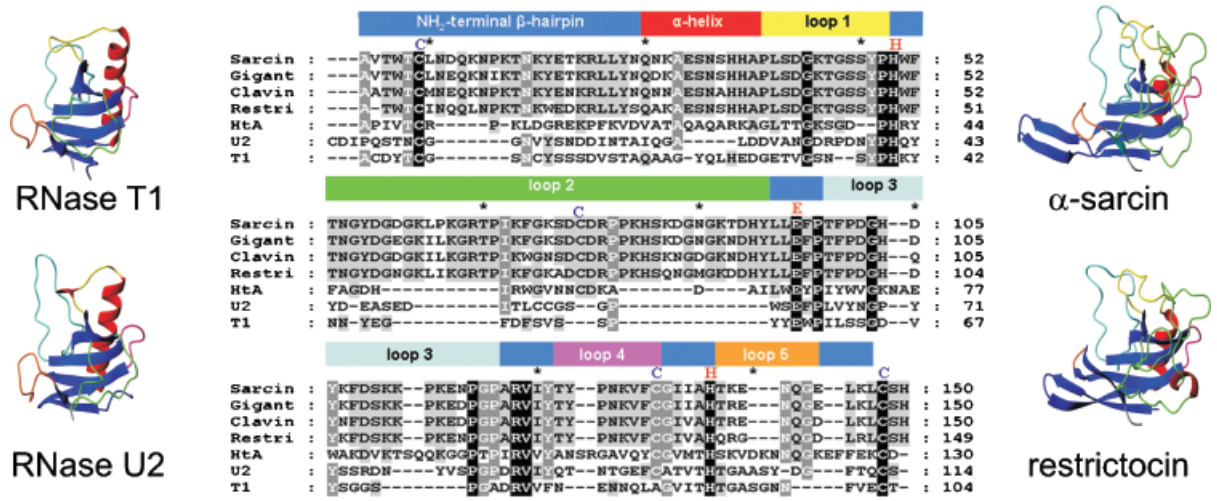

Fig. 3. (a) Sequence alignment of several different ribotoxins (α-sarcin, gigantin, clavin, restrictocin, and HtA) and RNases T1 and U2. Cysteine residues of ribotoxins (blue) and the three proved, or presumed, catalytic residues of all the proteins (in red) are indicated. Diagrams showing the three-dimensional structures of α-sarcin, restrictocin and RNases T1 and U2 are also shown. Color codes are as in Fig. 1. Three-dimensional structures of proteins were fitted to the atomic coordinates of the active site residues (α-sarcin: 48, 50, 96, 121, 137, 145; restrictocin: 47, 49, 95, 120, 136, 144; RNase T1: 38, 40, 66, 77, 92, 100; RNase U2: 39, 41, 62, 85, 101, 110) and common disulfide bridges of the four proteins (α-sarcin, 6–148; restrictocin, 5–147; RNase T1, 6–103; RNase U2, 9–113) (root mean square deviation of the fitting, 1.877). The diagrams and fittings were generated with the molmol program (Koradi *et al.*, 1996).

RNase T1 (Sato & Egami, 1957), one of the most exhaustively characterized proteins. RNase T1 is indeed the best known member of the family of fungal extracellular RNases (Yoshida, 2001; Loverix & Steyaert, 2001), a group that obviously includes ribotoxins. All of them show a high degree of sequence (Sato & Uchida, 1975; Sacco *et al*., 1983; Martínez-Ruiz *et al*., 1999a, b) and structural similarity (Pace *et al*., 1991; Noguchi *et al*., 1995; Yang & Moffat, 1996; Campos-Olivas *et al*., 1996a, b; Pérez-Cañadillas *et al*., 2000) (Fig. 3), but, apart from ribotoxins, none of these RNases has been reported to show cytotoxic activity.

Besides RNase T1, RNase U2 from *Ustilago sphaerogena* (Fig. 3) (Arima *et al*., 1968a, b; Sato & Uchida, 1975) also stands out as the nontoxic microbial extracellular RNase most closely related to ribotoxins from a phylogenetic point of view (Sacco *et al*., 1983; Martínez del Pozo *et al*., 1988; Martínez-Ruiz *et al*., 1999a, b, 2001). RNase U2 is a small and highly acidic protein that shows a strong preference for 3′-linked purine nucleotide phosphodiester linkages (Rushizky *et al*., 1970; Uchida *et al*., 1970), which is rather unusual within the group of microbial RNases. RNase T1, for example, shows strict specificity for the guanyl group. Both enzymes also differ in their optimum pH values, which are acid for RNase U2 and neutral for RNase T1, but both are cyclizing enzymes, cleaving RNA in two separate steps, transphosphorylation and hydrolysis (Yasuda & Inoue, 1982).

Ribotoxins are larger proteins, generally basic, that contain longer and charged loops that are not present in the noncytotoxic fungal RNases (Fig. 3), suggesting that these loops are the structural basis of their toxicity (Martínez del Pozo *et al*., 1988). It would appear that an RNase T1-like RNase had acquired ribosome specificity by the insertion of short recognition domains that would target it to cleave more specific substrates. Thus, the study of the evolution and mechanism of action of ribotoxins is of particular interest, as they appear to be naturally engineered targeted toxins evolved from the other microbial nontoxic RNases to enter cells and specifically inactivate the ribosomes (Lamy *et al*., 1992; Kao & Davies, 1995). Identification of the structural features that have allowed these proteins to become such efficient natural killers would be a major step towards their utilization, native or modified, as weapons against different human pathologies.

Structural features

Ribotoxins are synthesized as precursors that mature into cellular membrane compartments (Endo *et al*., 1993a, b). Consequently, there has been speculation for a long time about the mechanism developed by the producing fungi in order to overcome the toxicity of these proteins, as their own ribosomes are also sensitive to the action of the toxins (Miller & Bodley, 1988). There is no evidence for the simultaneous production of any antitoxin or protein inhibitor that could block their cytotoxic action before they are secreted to the extracellular medium (Martínez-Ruiz *et al*., 1998b), as happens with some bacterial ribonucleolytic toxins (Muñoz-Gómez *et al*., 2005; Condon, 2006; Kamphuis *et al*., 2006; Luna-Chávez *et al*., 2006). In addition, characterization of pro-α-sarcin, produced in *Pichia pastoris*, revealed that it is ribonucleolytically active (Martínez-Ruiz *et al*., 1998). Thus, the data so far accumulated suggest that protection of the producing cells against the toxic effects of ribotoxins must rely on efficient recognition of their signal sequences, followed by adequate compartmentalization before they are secreted to the extracellular medium.

The complete or partial amino acid sequences of several ribotoxins have been determined (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*., 1999a, b). They show a high degree of identity in their *c*. 150 amino acid sequence (Fig. 3), including the conservation of their two disulfide bridges (Martínez del Pozo *et al*., 1988; Martínez-Ruiz *et al*., 2001). This observation includes HtA (Martínez-Ruiz *et al*., 1999a), although it is 20 residues shorter than the other known ribotoxins. Sequence differences are mainly concentrated at the loops of the ribotoxins (Martínez-Ruiz *et al*., 1999a) (Fig. 3).

This similarity is also manifested in the three-dimensional structure of the two ribotoxins studied at this level, restrictocin (Yang & Moffat, 1996; Yang *et al*., 2001) and α-sarcin (Pérez-Cañadillas *et al*., 2000, 2002; García-Mayoral *et al*., 2005a, b). For α-sarcin, nuclear magnetic resonance (NMR) and other techniques have been used to make a very detailed map of its structural and dynamic properties (Campos-Olivas *et al*., 1996a, b; Pérez-Cañadillas *et al*., 2000, 2002; García-Mayoral *et al*., 2005a, b). The elucidation of its three-dimensional structure (Fig. 1) revealed that it folds into an α+β structure with a central five-stranded antiparallel β-sheet and an α-helix of almost three turns. The sheet is composed of the strands β3, β4, β5, β6 and β7 arranged in a $-1, -1, -1, -1$ topology (Figs 1 and 3) (Campos-Olivas *et al*., 1996a, b; Pérez-Cañadillas *et al*., 2000). 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 His50, Glu96, Arg121 and His137, with their side chains projecting outwards from the cleft (Fig. 4). In addition, 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 juts out as a solvent-

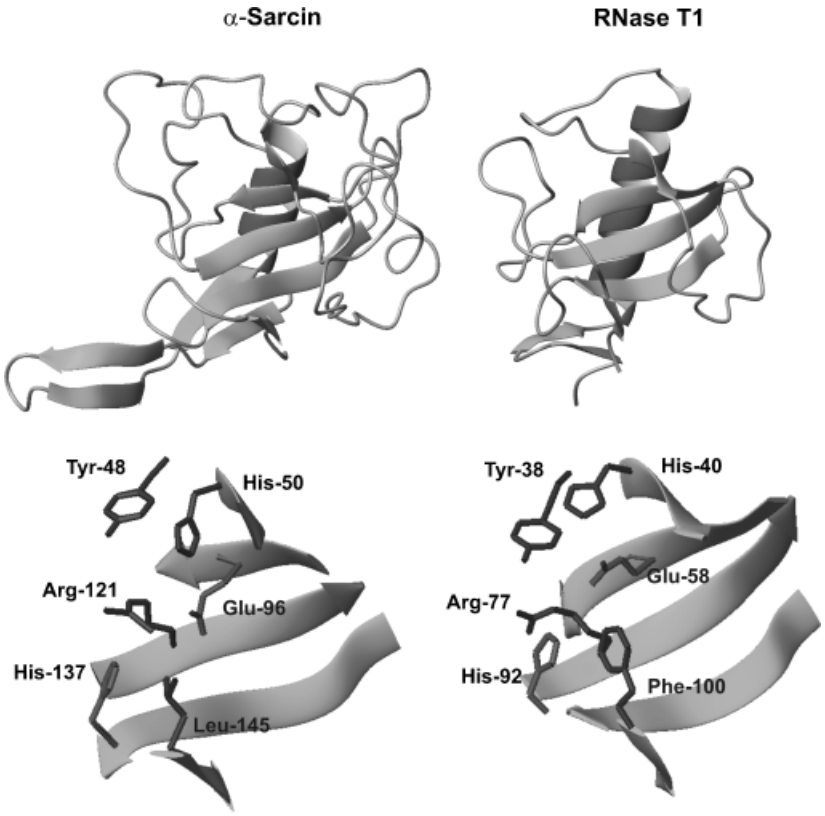

Fig. 4. Representation of the geometric arrangement of the side chain residues found in the active sites of α-sarcin and RNase T1. Only the side chains of the catalytic residues directly involved in the mechanism of general acid–base cleavage are shown. The three-dimensional structures of both proteins are also shown. The diagrams were generated with the molmol program (Koradi *et al.*, 1996).

exposed protuberance, a detail that it is important for its function, as explained below. α-Sarcin and restrictocin show almost identical structures (Fig. 3), but some small differences are observed concerning their long nonstructured loops and especially the above-mentioned N-terminal β-hairpin, a region of high mobility (Pérez-Cañadillas *et al.*, 2002), which is lacking in the restrictocin crystalline structure (Yang & Moffat, 1996). The remaining stretches of its sequence form large loops connecting the secondary structure elements (Fig. 1). Despite the exposed character of these loops and their lack of repetitive secondary structure, their conformations are well defined. They are maintained by networks of intraloop and interloop interactions, including hydrogen bonds, hydrophobic interactions, and salt bridges (Yang & Moffat, 1996; Pérez-Cañadillas *et al.*, 2000). From a dynamic point of view, NMR measurements have shown that α-sarcin behaves as an axial symmetric rotor of the prolate type, and that it is composed of a rigid hydrophobic core and some exposed segments, mostly the loops, which undergo fast (picosecond to nanosecond) internal motions (Pérez-Cañadillas *et al.*, 2002).

Ribotoxins share this structural core with nontoxic RNases of the RNase T1 family, in good agreement with the observed sequence similarity (Figs 3 and 4). For example, comparison of the three-dimensional structures of α-sarcin and restrictocin with those of RNase T1 and RNase U2 reveals that the four proteins share identical regular secondary structure elements despite their different amino acid sequence lengths, including the geometric arrangement of the residues involved in the active site (Figs 3 and 4). Thus, all fungal extracellular RNases whose three-dimensional structure is known exhibit quite different enzymatic specificities, but all of them share this common structural fold concerning the architecture and connectivity of the secondary structure elements (Yang & Moffat, 1996; Campos-Olivas *et al.*, 1996b; Pérez-Cañadillas *et al.*, 2000; Martínez-Ruiz *et al.*, 2001). The most significant structural differences among them are, again, related to both the presence of a longer N-terminal β-hairpin in ribotoxins and the different length and charge of their unstructured loops (Figs 3 and 4).

Loop 2 of α-sarcin (Thr53 to Tyr93) (Fig. 3) deserves especial emphasis, because it exhibits a well-defined conformation with functional implications (Pérez-Cañadillas *et al.*, 2000). It is highly mobile, rich in Gly and positively charged residues, and largely solvent exposed. In this loop, the stretch comprising residues 52–54 is essentially frozen within the molecular framework (Pérez-Cañadillas *et al.*, 2002), Asn54 being 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 Ile69, these protons being very resistant to exchange with the solvent. This interaction is,

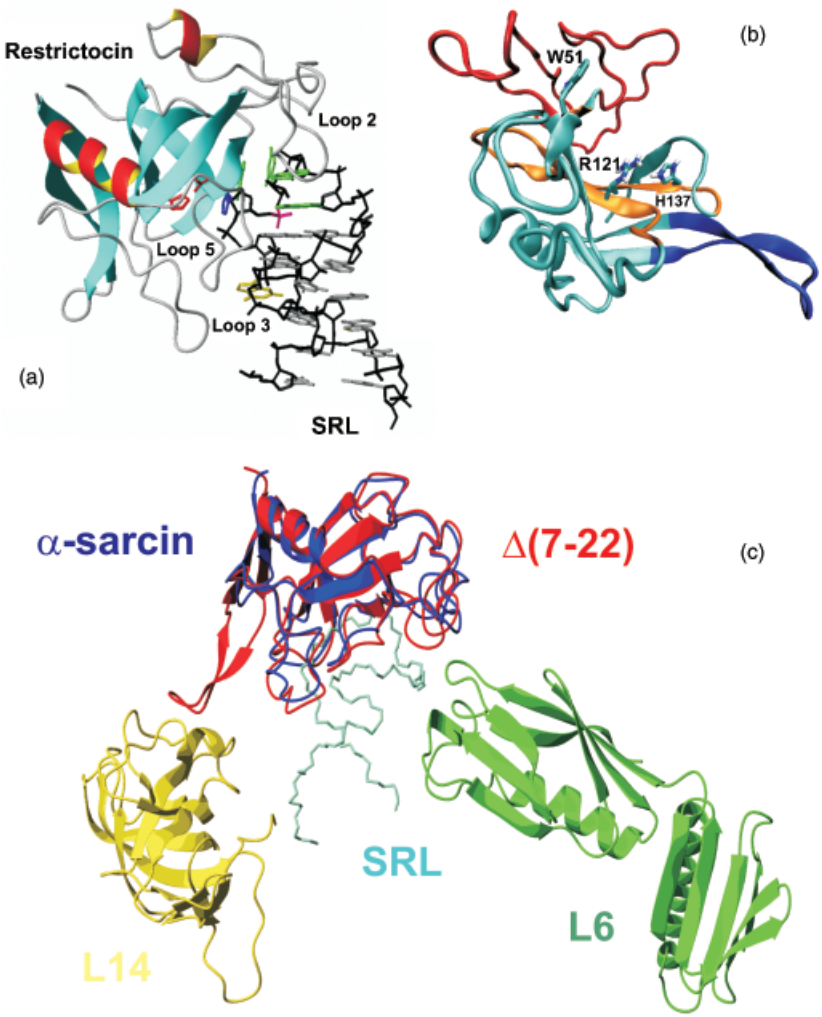

Fig. 5. (a) Diagram showing the crystalline structure of an SRL analog–restrictocin complex (Yang *et al.*, 2001) (PDB accession number 1JBS). The analog structure is distorted in comparison to the wild-type SRL, and this explains the absence of cleavage, which allowed crystallization of the complex. The side chains of His49 (blue), Glu95 (red), and His136 (red) are also shown. The RNA backbone is shown is black, with the bases in gray, except for the bulged G in yellow, the tetraloop in green, and the phosphate group of the bond susceptible to cleavage in magenta. The loops equivalent to α-sarcin's loops 2, 3 and 5 are also indicated. (b) Diagram showing the α-sarcin regions presumably involved in the establishment of interactions with phospholipid bilayers: purple, residues 7–22; orange, residues 116–139 and 51; red, residues 53–93 (loop 2). The side chains of Trp51, Arg121 and His137 are also shown. (c) Minimized docking model showing the interaction of wild-type (PDB ID 1DE3) and Δ(7–22) α-sarcin (PDB ID 1R4Y) with the SRL (PDB ID 430D) and the *Halobacterium marismortui* ribosomal proteins L6 and L14 (García-Mayoral *et al.*, 2005a, b). Diagrams were generated with molmol (a, c) (Koradi *et al.*, 1996) and vmd (b) (Humphrey *et al.*, 1996) programs.

indeed, conserved in the other RNases of the RNase T1 family (Sevcik *et al.*, 1991; Pfeiffer *et al.*, 1997, Hebert *et al.*, 1998). Docking studies have also suggested that the segment formed by residues 51–55 of α-sarcin (Fig. 3) could specifically interact with the SRL in the vicinity of the scissile bond (Pérez-Cañadillas *et al.*, 2000), a prediction that was later confirmed by X-ray crystallography (Fig. 5a) (Yang *et al.*, 2001). In relation to this, three Lys residues of loop 3 (Lys111, Lys112 and Lys114) seem to be of especial importance, as they contact the identity element of the ribosomal SRL region, the bulged-G in the S-turn (see below and Fig. 5a) (Yang & Moffat, 1996; Pérez-Cañadillas *et al.*, 2000; Yang *et al.*, 2001).

It is also remarkable that an N-terminal deletion mutant Δ(7–22) of α-sarcin (García-Ortega *et al.*, 2002) retained the same conformation as the wild-type protein, as ascertained from its spectroscopic characterization (García-Ortega *et al.*, 2002) and three-dimensional structure in solution (García-Mayoral *et al.*, 2004). Docking and enzymatic studies have revealed that this N-terminal β-hairpin (positions 7–22) of α-sarcin could establish interactions with ribosomal proteins in order to direct the ribotoxin to the SRL region of the ribosome (García-Ortega *et al.*, 2002; García-Mayoral *et al.*, 2005b) (Fig. 5).

α-Sarcin is a highly charged protein, with a high isoelectric point. The high content of positively charged residues is probably required for recognizing and binding to not only its highly negatively charged target, the rRNA gene, but also the cellular membranes. It contains eight Tyr and two Trp residues, which have been spectroscopically explored. By using UV absorbance, fluorescence emission and circular dichroism (CD) measurements, five different pH-induced conformational transitions, corresponding to p*K*a values of 2.5, 4.5, 8.0, 10.2, and 11.4, were initially described (Martínez del Pozo *et al.*, 1988). The two latter ones (10.2 and 11.4) corresponded to two different Tyr populations of different solvent accessibility. The transition at p*K*a 8.0 was assigned to the α-amino group of the N-

terminal residue, Asp and Glu residues deprotonated at pH 4.5, and p*K*a values of 2.5 and 10.2 were considered to be denaturing transitions (Martínez del Pozo *et al.*, 1988). This characterization later became much more detailed, when the p*K*a values of all aspartic acid, glutamic acid and histidine residues of α-sarcin were determined by NMR; it was found that many of them, including several at the active site, are highly perturbed (Pérez-Cañadillas *et al.*, 1998). Much more recently, the p*K*a values of all titratable residues have also been systematically measured, or predicted when direct measurement was not possible because of the unfolding of the protein (García-Mayoral *et al.*, 2003). These measurements and predictions were also extended to a series of active site variants (E96Q, H50Q, H137Q, and H50/137Q) (García-Mayoral *et al.*, 2003, 2006). This detailed characterization at the level of individual residues was completed by determining the tautomeric state of all the side chain histidine residues (Pérez-Cañadillas *et al.*, 2003).

α-Sarcin's two Trp residues, at positions 4 and 51, are conserved in all ribotoxins known so far (Fig. 3). Characterization of mutants where one or both of these two residues were substituted by Phe (single mutants W4F and W51F, and the double mutant W4/51F) showed that they are not required for the highly specific ribonucleolytic activity of the protein, although the mutant forms involving Trp51 showed decreased activity (De Antonio *et al.*, 2000). More importantly, it was shown that Trp51 is responsible for most of the near-UV CD of the protein, and also contributes to the overall ellipticity of the protein in the peptide bond region, but does not show fluorescence emission (De Antonio *et al.*, 2000).

Finally, it is also important, from a structural point of view, to mention that the active site of α-sarcin is composed of at least residues Tyr48, His50, Glu96, His137, Arg121, and Leu145, although only three of them (His50, Glu96, and His137) are directly involved in proton transfer steps in the catalytic mechanism (Lacadena *et al.*, 1999; Martínez-Ruiz *et al.*, 2001). As mentioned above, they are located in the central β-sheet, and their side chains point towards the concave face of the protein structure (Fig. 4). The most representative characteristics of this active site would be: (1) high density of charged residues; (2) unusual p*K*a values of His50, Glu96, and His137; (3) unusual Nδ tautomeric forms adopted by His50 and His137, a common feature of microbial RNases; (4) the presence of a structurally important hydrogen bond between the catalytic His137 and a backbone oxygen in loop 5; and (5) low surface accessibility of all titratable atoms (Pérez-Cañadillas *et al.*, 1998, 2000, 2003).

Crossing membranes

The antitumor activity of α-sarcin can be explained by its unique ribonucleolytic activity after its selective passage across some cell membranes. Thus, although the SRL is a universally conserved structure, cells are only killed if ribotoxins cross their membranes to gain access to the ribosomes. As no protein receptors have so far been reported for α-sarcin, the toxic specificity must be related to a differential interaction with the lipid components of the membranes. Long ago, it was shown that α-sarcin was a powerful inhibitor of protein synthesis in picornavirus-infected cells (Fernández-Puentes & Carrasco, 1980), and that ionophores (Alonso & Carrasco, 1981, 1982), external ATP (Otero & Carrasco, 1986) or phospholipase C treatment (Otero & Carrasco, 1988) targeted mammalian cells to α-sarcin entry. All these observations were interpreted in terms of the existence of altered membrane permeability.

The use of lipid model systems proved that α-sarcin interacts specifically with negatively charged phospholipid vesicles at neutral or slightly acidic pH, resulting in protein–lipid complexes that can be isolated by centrifugation in a sucrose gradient (Gasset *et al.*, 1989). Binding experiments revealed a strong ribotoxin–lipid vesicle interaction ($K_d = 60.0$ nM) that caused vesicle aggregation, followed by their fusion into much larger lipidic structures (Gasset *et al.*, 1989). The minimum phosphatidylcholine/phosphatidylglycerol molar ratio required for this behavior was 1 : 10, and it was dependent on neither the length nor the degree of unsaturation of the phospholipid acyl chain, being more effective at temperatures above the melting temperature of the phospholipid used. Saturation was reached at phospholipid/protein molar ratios of 50 : 1, and the effect was maximal at 0.15 M ionic strength. It was, however, abolished at basic pH (Gasset *et al.*, 1990).

Light-scattering stopped-flow kinetic studies of the α-sarcin–vesicle interaction revealed that the initial step was the formation of a vesicle dimer maintained by protein–-protein bridges (Mancheño *et al.*, 1994). Once the aggregation had started, lipid mixing occurred between the bilayers of aggregated vesicles, as would be expected with fusing liposomes. In fact, this fusion was triggered by the destabilizing effect of the protein, which simultaneously suffered conformational changes upon binding to the vesicles (Mancheño *et al.*, 1994), as revealed by CD, fluorescence emission, and attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) (Gasset *et al.*, 1991b). These conformational changes suggested an increase in the α-helix content that, together with the other spectroscopic changes observed, was interpreted in terms of shielding from polar groups caused by the lipids, which would promote intrachain hydrogen bonding and decreased static quenching (Gasset *et al.*, 1991b). Indeed, the peptide bonds of the protein were protected against trypsin hydrolysis upon binding to these vesicles (Gasset *et al.*, 1989; Oñaderra *et al.*, 1989), despite the high number of basic residues present along its sequence (Sacco *et al.*, 1983). Freeze-fracture electron micrographs corroborated this fusogenic

effect, as at the highest phospholipid/protein molar ratio employed (50 : 1), there was a complete absence of small vesicular structures; instead, lipids were exclusively organized in planar sheets, indicating that the fusion processes had gone to completion (Gasset *et al.*, 1990). As a final step, and most probably as a consequence of the formation of these unstable large structures, α-sarcin also modified the permeability of the membranes, causing the leakage of calcein in dye-trapped phosphatidylglycerol vesicles (Gasset *et al.*, 1990). Fluorescence depolarization measurements, differential scanning calorimetry and labeling with photoactive phospholipids revealed that α-sarcin, a water-soluble and hydrophilic protein, interacts with phospholipid bilayers through a combination of electrostatic and hydrophobic forces (Gasset *et al.*, 1991a). According to this hypothesis, the protein would then initially be adsorbed to the charged polar head groups of the phospholipids, and then would partially penetrate the interface of the bilayer to interact with a portion of the lipid hydrocarbon chains (Gasset *et al.*, 1991a). All these observations were consistent with an intercalation of the protein into the lipid matrix, promoting fusion and permeability changes in the bilayers, processes that would presumably be involved in the passage of the protein across the membranes of its target cells. A higher content of negatively charged phospholipids, such as phosphatidylserine, has been reported in transformed cell membranes (Connor *et al.*, 1989; Gasset *et al.*, 1989, 1990; Zachowski, 1993). Unfortunately, there is no direct evidence yet that this abundance of acidic phospholipids is the main explanation for the antitumor activity of α-sarcin.

In good agreement with this hypothesis, the innate ability of α-sarcin to translocate across a phospholipid membrane, if it is acidic enough, in the absence of any other protein was also demonstrated, using two different types of assay (Oñaderra *et al.*, 1993). First, the protein was completely degraded when added externally to asolectin vesicles containing encapsulated trypsin, an experiment that was performed in the presence of such an external excess of trypsin inhibitor that degradation by traces of leaked protease was not possible. Second, externally added α-sarcin was also capable of cleaving encapsulated baker's yeast tRNA molecules in a protein concentration-dependent manner (Oñaderra *et al.*, 1993).

With regard to the protein regions involved in the interaction, the first hints were obtained using water-soluble synthetic peptides corresponding to sequences within the main β-sheet of α-sarcin. Some of these peptides, one of them only nine residues long, were shown to be able to mimic, at least qualitatively, the effects produced by the complete protein on acid phospholipid vesicles, indicating that this region of the protein (residues 116–139) is probably involved in its interaction with the cell membranes (Fig. 5b) (Mancheño *et al.*, 1995b, 1998a). These conclusions were indeed compatible with the observation that a denatured form of α-sarcin, containing β-strands as the only regular secondary structure elements, promoted destabilization of the hydrophobic core of bilayers (Gasset *et al.*, 1995). Using the Trp mutants mentioned above, it was also shown that neither Trp4 nor Trp51 were required for the interaction of α-sarcin with lipid membranes (aggregation and fusion of vesicles) (De Antonio *et al.*, 2000). However, this interaction promoted a large increase in the quantum yield of Trp51, the residue located in the β-sheet of the protein (Fig. 5b), and its fluorescence emission was simultaneously quenched by anthracene incorporated into the hydrophobic region of such bilayers. Furthermore, a study of mutants affecting α-sarcin active site residue Arg121 (R121 K and R121Q), also located at the major β-sheet (Figs 4 and 5b), showed that the loss of the positive charge at that position produced a dramatic impairment of the protein's ability to interact with phospholipid membranes (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 recognize polyphosphate lattices that might, indeed, allow the recognition of a phospholipid bilayer (Masip *et al.*, 2001). Interestingly, when the crystalline structure of restrictocin was solved, the equivalent residue (Arg120) was found to be hydrogen bonded to a cocrystallized phosphate molecule at its active site (Yang & Moffat, 1996). In summary, these results indicated that this β-sheet, predicted to be one of the scarcest apolar regions of the protein (Martínez del Pozo *et al.*, 1988; Mancheño *et al.*, 1995b), was in fact located within the hydrophobic core of the bilayer following protein–vesicle interaction (Fig. 5b) (De Antonio *et al.*, 2000).

Other than this hydrophobic core, mutations affecting single residues located at the N-terminal β-hairpin of α-sarcin (Lys11 and Thr20) and the deleted Δ(7–22) variant suggested that this protein portion would be another region involved in the interaction with cell membranes (García-Ortega *et al.*, 2001, 2002), as they displayed a different pattern of interaction with the lipid vesicles (Fig. 5b). When restrictocin was the protein assayed, it also behaved differently from wild-type α-sarcin (García-Ortega *et al.*, 2001). It is noteworthy that α-sarcin and restrictocin sequences differ in only 20 residues, and six of these changes are concentrated at the N-terminal β-hairpin (Fig. 3). In agreement with this idea, the Δ(7–22) α-sarcin showed behavior compatible with the absence of one vesicle-interacting protein region (García-Ortega *et al.*, 2002).

Finally, loop 2 has been proposed by several authors (Yang & Moffat, 1996; Martínez del Pozo *et al.*, 1988; Kao & Davies, 1999; Pérez-Cañadillas *et al.*, 2000) to also be one of the protein regions involved in the interaction with lipids

(Fig. 5b). The differences between the NMR-refined structure of this loop in α-sarcin and restrictocin (García-Mayoral *et al.*, 2005a, b) could help to explain their distinct behavior when translocating across cell membranes, although this possibility has not been directly studied yet.

Enzymatic properties

The enzymatic activity of ribotoxins remained obscure for a long time after their discovery (Lamy *et al.*, 1992). Then, in 1977, Schindler and Davies published the observation that α-sarcin was able to inactivate both *Saccharomyces cerevisiae* and *Escherichia coli* ribosomes, although with different efficiencies (Schindler & Davies, 1977). Surprisingly, neither intact yeast or bacteria nor HeLa cells were susceptible to the toxicity exerted by α-sarcin, suggesting that they were refractory to the entrance of the protein. A more detailed study concluded that α-sarcin's inactivation of purified ribosomes affected EF-2-catalyzed GTP hydrolysis and translocation. Finally, separation of the rRNA gene species by gel electrophoresis after incubation of yeast 80S ribosomes with the toxin resulted in the appearance of an extra fragment about 300 nucleotides long (the so-called α-fragment), corresponding to the 3′-end of the 28S rRNA gene (Fig. 6a) (Schindler & Davies, 1977). Further experiments showed that α-sarcin cleaved the phosphodiester backbone at the 3′-side of G2661 (*E. coli* numbering) (Endo & Wool, 1982), whereas ricin depurinated the N-glycosidic linkage between the ribose sugar and the base moieties corresponding to the 5′-adjacent A2660 (both positions corresponding to G4325 and A4324 in 28S rRNA gene) (Fig. 2) (Endo & Tsurugi, 1987; Endo *et al.*, 1987).

Therefore, ribotoxins are highly specific RNases against cell-free intact ribosomes, and they retain this specificity when assayed against naked rRNA gene containing the SRL. However, they can also cause extensive progressive digestion of total or 28S rRNA with no formation of the α-fragment, when used at higher concentrations (Endo *et al.*, 1993a, b; Wool, 1996, 1997). Even DNA has been shown to be digested by α-sarcin when large enzyme/substrate ratios are assayed (Wool, 1984; Endo *et al.*, 1993a, b). This nonspecific activity has been taken advantage of in the employment of some other, much less specific, ribonucleolytic assays, apart from those based on following the release of the α-fragment. The lack of biological significance, due to the loss of specificity, and the much higher than catalytic concentrations needed, are compensated for by much easier quantitation of the results, as well as the possibility of analyzing the products, or even intermediates, of the reaction. Thus, although they are less specific, these assays have contributed significantly to the detailed study of the cleavage mechanism of ribotoxins (Lacadena *et al.*, 1994, 1998; Kao *et al.*, 2001; Martínez-Ruiz *et al.*, 2001).

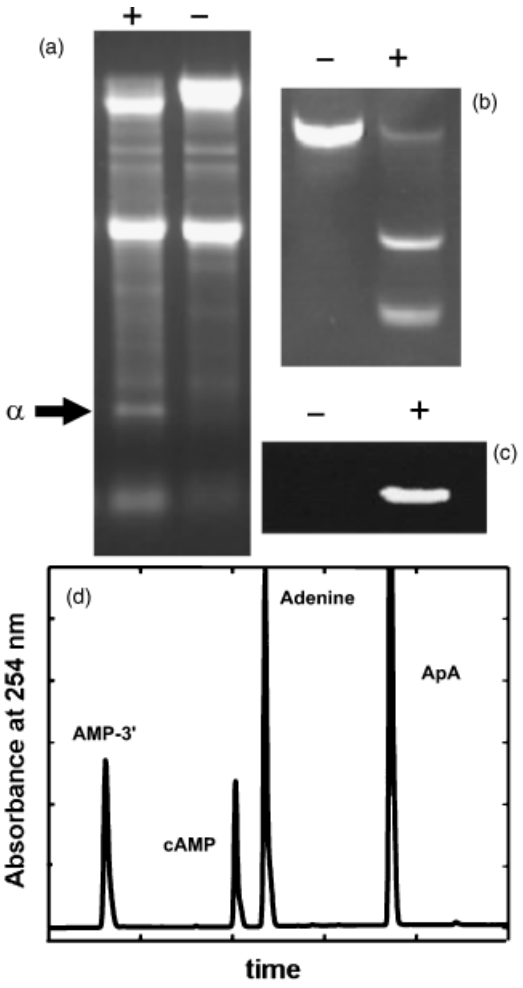

Fig. 6. Examples of the different enzymatic assays used to study ribotoxin ribonucleolytic activity. The presence (+) or absence (−) of α-sarcin in the assay is indicated. (a) Specific cleavage of rabbit ribosomes. The α-fragment is indicated by an arrow. (b) Specific cleavage of a 35-mer SRL-like oligonucleotide. (c) Zymogram against poly(A). (d) HPLC resolution of the products produced after incubation of α-sarcin with ApA.

Four different types of enzymatic assay are usually performed (Kao *et al.*, 2001; Martínez-Ruiz *et al.*, 2001). The first, and most specific, is one that uses natural substrates, purified ribosomes or, at least, a cell-free reticulocyte lysate (Kao *et al.*, 2001). The highly specific action can be then visualized by detecting the release of a 300–400-nucleotide (depending on the ribosome source) α-fragment on a denaturing agarose gel stained with ethidium bromide (Fig. 6a). The sensitivity of this assay has recently been improved by the detection of this α-fragment by hybridization with a specific ^{32}P-radiolabeled DNA probe (Korennykh *et al.*, 2006).

In decreasing order of complexity, and therefore of specificity, the second assay frequently used is based on the

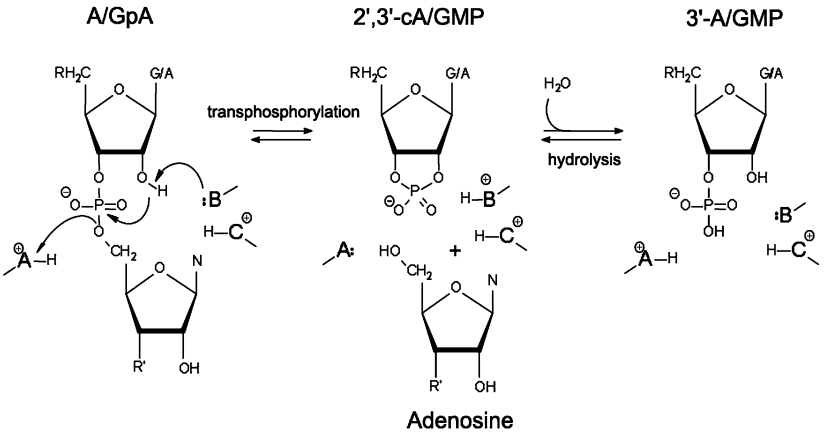

Fig. 7. Proposed mechanism for the catalytic mechanism of cyclizing RNases against a dinucleotide substrate (ApA or GpA). A transphosphorylation process (in which the corresponding 2′,3′ cyclic mononucleotide and adenosine are produced) is followed by hydrolysis of the cyclic nucleotide to produce the corresponding 3′-mononucleotide. (A), (B) and (C) are His92, Glu58 and His40 in RNase T1 (Steyaert, 1997), and His137, Glu96 and His50 in α-sarcin (Lacadena *et al.*, 1999), respectively.

employment of short oligoribonucleotides mimicking the SRL sequence and structure (SRL-like oligos). Ribotoxins cleave these SRL-like oligos specifically, producing only two smaller fragments, which can be fractionated on a polyacrylamide gel (Fig. 6b) (Endo *et al.*, 1988; Wool *et al.*, 1992), although this cleavage is several orders of magnitude less efficient than that produced on intact ribosomes (Glück & Wool, 1996; Wool, 1997).

The third, and much less specific, assay is a zymogram (Fig. 6c), in which the ribonucleolytic activity is shown against a homopolymer, such as poly(A) or poly(I), embedded in a polyacrylamide gel after electrophoretic separation of the proteins by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and convenient refolding by elimination of the detergent. In some instances, this type of assay can be also performed in solution, using ultrafiltration devices to fractionate the small oligonucleotides produced by the ribonucleolytic reaction (Kao *et al.*, 2001). The advantage of the zymogram is its additional use as a homogeneity control of the protein sample.

The fourth assay is based on the fact that ribotoxins are also capable of hydrolyzing different dinucleoside (or dinucleotide) phosphates, such as ApA (or ApAp), although with very low efficiency (Lacadena *et al.*, 1998). This type of substrate should be considered as just containing the minimal and essential elements needed to be cleavable by a RNase. The advantage in this case is that the products, substrates and intermediates of the reaction can be separated and quantitated by HPLC (Fig. 6d), providing information about the different steps (Lacadena *et al.*, 1998).

A combination of all these different activity assays, and the production and characterization of many site-directed and randomly produced mutants (Yang & Kenealy, 1992a, b; Lacadena *et al.*, 1995, 1999; Kao *et al.*, 1998), 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 phosphodiester bond. The non-cytotoxic microbial RNases T1 and U2 have been of great help as reference models. The enzymatic mechanism of RNase T1, for example, has been clearly established (Fig. 7), as have the roles of most of the residues forming its active site (Steyaert, 1997; Loverix & Steyaert, 2001; Yoshida, 2001). Accordingly, this enzyme performs the general acid–-base type endonucleolytic cleavage of RNA in two steps. First, there is a transphosphorylation reaction to form a 2′,3′-cyclic phosphate intermediate. Second, this intermediate is hydrolyzed to the corresponding 3′-phosphate (Fig. 7). The appearance of this cyclic intermediate, common to all RNases of the RNase T1 family so far studied, including RNase U2, is implicit in the denomination of all these enzymes as cyclizing RNases. Analysis of the cleavage reactions performed by α-sarcin against different dinucleoside monophosphates proved that this protein is also a cyclizing RNase (Lacadena *et al.*, 1998, 1999), with an optimum pH of 5.0 (Pérez-Cañadillas *et al.*, 1998; Lacadena *et al.*, 1999). Therefore, ribotoxins follow the same general reaction scheme as the other members of the RNase T1 family. However, the catalytic efficiency of RNases T1 and U2 against naked RNA, homopolynucleotides or dinucleotides is several orders of magnitude higher. On the other hand, when assayed against natural substrates, ribotoxins cleave and consequently inactivate the ribosome with a second-order rate constant (k_{cat}/K_m of $1.7 \times 10^{10}\,M^{-1}\,s^{-1}$) that matches the catalytic efficiency of the fastest known enzymes (Korennykh *et al.*, 2006).

In the case of RNase T1, during the first step of the reaction Glu58 acts as a general base and His92 as a general acid (Figs 4 and 7). The hydrolysis of the cyclic derivative is catalyzed by the same groups, but their roles are reversed (Steyaert, 1997). In fact, the most common pair of catalytic residues found in microbial RNases is this Glu/His combination (Yoshida, 2001). Another His residue, His40, is required in its protonated form to assist the electrostatic stabilization of the transition state and, eventually, seems to be able to adopt the function of the general base, as shown

with Glu58-mutated variants of RNase T1 (Steyaert *et al.*, 1990; Steyaert, 1997). Superposition of the three-dimensional structures of RNases T1 and U2 with those of α-sarcin and restrictocin showed that the counterpart residues were α-sarcin's His137, Glu96, and His50, and restrictocin's His136, Glu95, and His49 (Fig. 4) (Sacco *et al.*, 1983; Martínez del Pozo *et al.*, 1988; Campos-Olivas *et al.*, 1996b). Production of wild-type and several mutant forms of restrictocin in *S. cerevisiae* showed that only the strain producing an H136L mutant was able to grow (Yang & Kenealy, 1992a, b). The same authors produced and partially characterized this same mutant in *A. niger* and *A. nidulans* (Brandhorst *et al.*, 1994), with very similar results. Not much later, another equivalent α-sarcin (H137Q) and restrictocin (H136Y) variants were isolated and characterized in detail from the structural and functional points of view; this confirmed that their lack of toxicity was due to the absence of ribonucleolytic activity, and not to major conformational changes (Kao & Davies, 1995; Lacadena *et al.*, 1995). Thanks to the production and further characterization of more mutants, affecting these three residues in α-sarcin and restrictocin, it is now well known that α-sarcin His137 and Glu96 are the only residues that are essential for performing the acid–base type reaction (Brandhorst *et al.*, 1994; Kao & Davies, 1995, 1999; Lacadena *et al.*, 1995, 1999; Sylvester *et al.*, 1997; Kao *et al.*, 1998); His50 would also contribute to the stabilization of the transition state but, in this case, would not be able to substitute for Glu96 as the general base in E96Q mutants (Fig. 7) (Lacadena *et al.*, 1999). This was inferred because substitution of His50 (or His49 in restrictocin) with different residues did not completely inactivate the enzyme, but rather decreased its k_{cat} values, so that it showed only residual enzymatic or cytotoxic activity, depending on the nature of the assay used (Nayak & Batra, 1997; Sylvester *et al.*, 1997; Lacadena *et al.*, 1999). In addition, it was proved that the three mentioned residues are required for the specific inactivation of the ribosomes, as each individual variant assayed, as well as the double and triple mutant versions, lacked this particular activity (Lacadena *et al.*, 1999).

The profile obtained for the pH dependence of the α-sarcin activity is typical for an acid–base catalyst but significantly different from that described for RNases T1 or U2 (Arima *et al.*, 1968a, b; Sylvester *et al.*, 1997; Pérez-Cañadillas *et al.*, 1998; Lacadena *et al.*, 1999). The α-sarcin H50Q mutant also shows quite different behavior, probably due to the absence of a positive charge in the Glu96 environment. Finally, whereas α-sarcin displays a low efficiency in hydrolyzing the cyclic intermediate, as most cyclizing RNases do, its H50Q variant is much more efficient in producing the 3′-AMP product at pH 7.0 (Lacadena *et al.*, 1999). The NMR measurements mentioned above were also used to calculate how these active site residues display p*K*a values far from their intrinsic values, which would explain these different behaviors in terms not only of specificity but also of pH dependence (Pérez-Cañadillas *et al.*, 1998; Lacadena *et al.*, 1999).

In the crystal complex of RNase T1 with the minimal substrate 3′-GMP (Loverix & Steyaert, 2001), Tyr38, Arg77 and Phe100 also appear to form the catalytic site of the enzyme. There has been speculation about these three residues, together with His40, forming a prearranged structural and dielectric microenvironment that is complementary in shape, charge and hydrogen-bonding capacity to the equatorial oxygens of the transition state, contributing to its optimal solvation/desolvation (Loverix & Steyaert, 2001). Tyr48, Arg121 and Leu145 are their three corresponding structural counterparts in α-sarcin (Figs 3 and 4), and therefore have been also studied.

RNase T1 Arg77 is located in the vicinity of the substrate phosphate moiety, but its potential functional role is not known, as all attempts to isolate any RNase T1 with a mutation affecting that residue have been unsuccessful (Steyaert, 1997). Thus, it has long been proposed that Arg77 of RNase T1 might facilitate the nucleophilic attack, but this has not been directly proven by site-directed mutagenesis (Steyaert, 1997). On the other hand, Arg121 of α-sarcin has been replaced by Gln or Lys, mutations that did not modify the conformation of the protein, but abolished its ribosome-inactivating activity. Unexpectedly, these mutants were still active against a small and nonspecific substrate such as ApA (similar K_m and lower catalytic efficiency than the wild-type protein) (Masip *et al.*, 2001). In addition, as mentioned above, the loss of the positive charge at that position produced dramatic changes in α-sarcin's ability to interact with phospholipid membranes (Masip *et al.*, 2001).

Regarding RNase T1 Phe100, a Leu residue (Leu145) occupies the equivalent position in α-sarcin (Figs 3 and 4). The side chain of Phe100 is an apolar catalytic element, stabilizing charge separations that occur in the transition state by controlling the dielectric environment (Doumen *et al.*, 1996). Characterization of an L145F variant of α-sarcin revealed that it was still an active RNase (the mutant exhibited a similar K_m and slightly lower catalytic efficiency against the ApA substrate), but displayed lower specificity than the wild-type protein against rRNA gene and SRL-like substrates (Masip *et al.*, 2003). Leu145 was also shown to be essential to preserve the electrostatic environment of the active site required to maintain the anomalously low p*K*a value reported for the catalytic His137 (Masip *et al.*, 2003).

One of the residues showing the largest NMR chemical shift variation in the L145F mutant of α-sarcin was Asn54, a conserved residue located in loop 2 (Fig. 3). It not only contributes to the high stability of ribotoxins, but is also required for their highly specific action on ribosomes, according to the results obtained after the characterization

of five different α-sarcins with mutations at this position (Siemer *et al.*, 2004). These results suggest that Asn54 is involved in local conformational arrangements at the substrate-binding pocket. The mutations at this position resulted in less efficient RNases, especially against nonspecific substrates such as poly(A). The RNase residues involved in the interaction with the base 5′ to the cleaved phosphodiester bond are usually referred as the base recognition residues. The results obtained with the α-sarcin Asn54 mutants are in agreement with the idea that residues 53–56 (52–55 in restrictocin) would form that recognition pocket in ribotoxins (Yang & Moffat, 1996). However, recognition of ribosomes involves a much more complex network of interactions, most of which would not be disturbed by the mutation of Asn54, which would explain why most of the mutants still retained the ability to specifically release the α-fragment (Siemer *et al.*, 2004). Overall, these results are in perfect agreement with the idea of local conformational rearrangements of the Asn54 and Leu145 mutants' active site, leading to less specific and less cytotoxic enzymes (Masip *et al.*, 2003; Siemer *et al.*, 2004). Another important observation was that ribotoxins lack a residue equivalent to RNase T1 Glu46, involved in discriminating guanine from adenine (Gohda *et al.*, 1994). The lack of this residue could explain why this ribotoxin is only purine-specific when assayed against naked RNA, and not guanine-specific, as is the case for RNase T1 (Endo *et al.*, 1983, 1988).

Residue Tyr48 in α-sarcin is conserved not only within the ribotoxin family, but also within the larger group of fungal extracellular RNases (Fig. 3) (Martínez-Ruiz *et al.*, 1999a, b). Tyr38 of RNase T1 forms a short hydrogen bond with one of the phosphate oxygens in the RNase T1/3′–GMP complex, an interaction that may be more favourable in the transition state (Loverix & Steyaert, 2001). A Y48F mutant of α-sarcin was shown to be inactive against polymeric RNA substrates, revealing the essential role of the OH group in the Tyr48 phenolic ring (Álvarez-García *et al.*, 2006). This mutant was, again, active against ApA, revealing that it retained ribonucleolytic activity at this level. In summary, the removed OH group only contributes slightly to the catalytic efficiency against ApA, but is essential for the characteristic ribotoxin activity (specific degradation of rRNA gene and SRL-like substrates).

Thus, Tyr48, Arg121 and Leu145 appear to be determinants of the ribotoxin activity of α-sarcin. Studies of the crystal structures of complexes of the α-sarcin-like ribotoxin restrictocin with inhibitors led to the proposal that these ribotoxins may use base flipping to enable cleavage at the correct site of the SRL substrates (Yang *et al.*, 2001). All studies so far suggest that these three residues would enable the base flipping performed by His50/Glu96/His137 that permits RNase cleavage at a unique phosphodiester bond (Yang *et al.*, 2001).

In addition, the N-terminal hairpin has been shown to modulate the catalytic activity of ribotoxins, in studies with different mutants of mitogillin, another ribotoxin with only a single substitution relative to restrictocin (Kao & Davies, 1999; 2000), and α-sarcin (García-Ortega *et al.*, 2001). These studies included deletion variants in which this hairpin had been eliminated without affecting the overall three-dimensional structure of the protein (García-Ortega *et al.*, 2002, 2005; García-Mayoral *et al.*, 2004). These mutants [α-sarcin Δ (7–22) and Aspf1 Δ (7–22)] retained their nonspecific ribonucleolytic activity as well as their ability to specifically cleave SRL-like oligonucleotides, but were not able to specifically inactivate rabbit ribosomes, and therefore were much less cytotoxic (García-Ortega *et al.*, 2002, 2005).

In conclusion, it is important to note the differences exhibited by HtA. As expected, because it is a ribotoxin, this protein caused the specific cleavage not only of rabbit 28S rRNA gene, but also of the SRL-like oligonucleotides used as substrates (Herrero-Galán *et al.*, 2007). However, when less specific substrates were employed, HtA showed quite distinct behavior, as reflected by the fact that it is not active against poly(A) but is active against poly(C) (Herrero-Galán *et al.*, 2007). This behavior must be linked to the structural differences displayed by HtA, but the interpretation is not obvious, as the behavior has also been observed with other wild-type and mutant ribotoxins (Nayak *et al.*, 2001). Most probably, this behavior reflects as yet unknown elements of the catalytic mechanism.

Interaction with the SRL and the ribosome

Ribosomes are different in terms of their components among the three phylogenetic domains, *Archea*, *Bacteria*, and *Eukarya*, but several functional regions are always conserved, probably because they are essential to preserve the protein biosynthesis machinery (Mears *et al.*, 2002; Uchiumi *et al.*, 2002). One of them is the SRL (Szewczak & Moore, 1995; Glück & Wool, 1996). This region is of particular interest, owing to its crucial role in elongation-related events in both prokaryotic and eukaryotic ribosomes. It contains the longest known universally conserved ribosomal sequence (A2654–A2665 in the *E. coli* 23S rRNA gene, and A4318–A4329 in the rat 28S rRNA gene), and shows a unique RNA shape, which is structurally preserved. It is so conserved that when the crystalline structure of the *Halobacterium marismortui* large ribosomal subunit was elucidated, the sequence of the 23S rRNA gene was fitted into the electron density map, nucleotide by nucleotide, starting from its SRL sequence (Ban *et al.*, 2000). This SRL is a distorted hairpin, with an unusually stiff central part, and a GAGA tetraloop, a G-bulged cross-strand A-stack, a flexible region, and a terminal A-form duplex (Fig. 2). It is

not associated with any deep electrostatic potential pockets of the ribosomes, and is not a major binding motif. However, together with the L11-binding region, the L7/L12 stalk, and the ribosomal proteins L6 and L14 (Figs 2 and 5c), it constitutes an elongation factor-binding site that is required for correct functioning of the ribosome (Endo & 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 & 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 differences might explain why different RIPs display different affinities when assayed against different ribosomal substrates (Schindler & Davies, 1977; Endo & Wool, 1982; Wool *et al.*, 1992; Uchiumi *et al.*, 2002). Mutations affecting the sequence contained in the SRL result in defective binding of elongation factors and aminoacyl-tRNA, as well as a decrease in translational fidelity (Liu & Liebman, 1996). Some of these 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 center, and its possible involvement in conformational changes essential for the correct performance of translation (Nilsson & Nissen, 2005).

Extensive studies using small synthetic oligoribonucleotides mimicking the SRL sequence (Correll *et al.*, 1998, 1999, 2003; Correll & Swinger, 2003) have shed light on the rRNA identity elements needed for ribotoxin recognition of the phosphodiester bond to be cleaved. These SRL analogs are indeed specifically recognized and cleaved by the ribotoxins (Endo *et al.*, 1988; Kao *et al.*, 2001), although larger amounts of enzyme need to be employed, as mentioned in the previous section, indicating that the recognition is not as specific as with the whole ribosome. Unquestionably, they have been of great help, because they do maintain the structural features of the SRL within the complete ribosome and have been used to establish the structural determinants needed for the recognition between the SRL and ribotoxins. Thus, docking models and kinetic experiments were used to predict rRNA and protein regions capable of establishing interactions with the ribotoxins (Yang & Moffat 1996; Pérez-Cañadillas *et al.*, 2000; Correll *et al.*, 2004; García-Mayoral *et al.*, 2005b). Some of these predictions were confirmed by the determination of the crystal structures of restrictocin–inhibitor complexes made with several SRL-like RNA oligo variants (Fig. 5a) (Yang *et al.*, 2001). These studies included the resolution of the structures of two mutant versions of the oligonucleotides that mimic the 28S rRNA gene SRL motif (Correll *et al.*, 2003), as well as of three other different SRL analogs in complex with restrictocin (Yang *et al.*, 2001). According to these results, there are two SRL areas that are recognized by both toxins and elongation factors, the GAGA tetraloop and the bulged G2655 (Fig. 2) (Moazed *et al.*, 1988; Glück & Wool, 1996; Munishkin & Wool, 1997; Pérez-Cañadillas *et al.*, 2000). Thus, G2655 represents the most critical site for binding of elongation factors (Munishkin & Wool, 1997). However, the primary determinant of recognition does not seem to be the nucleotide type, but rather the SRL conformation (Munishkin & Wool, 1997; Correll *et al.*, 1999, 2003). Molecular dynamics simulation of two SRL structures based on crystal structures of *E. coli* and rat SRL motifs revealed that the GAGA tetraloop is the most dynamic part of this motif (Špačková & Šponer, 2006). In fact, GNRA tetraloops adopt an unfolded geometry upon binding of elongation factors and/or toxins, as was observed in the above-mentioned SRL–restrictocin complexes (Yang *et al.*, 2001). It has already been mentioned that these studies led to the proposal that ribotoxins may use base flipping to enable cleavage at the correct site of the SRL substrates (Yang *et al.*, 2001), and that this base flipping may be a common cleavage mechanism for endonucleases acting on folded substrates, as is the case for ribotoxins (Yang *et al.*, 2001). In summary, according to the above-mentioned results, two distant regions of the ribotoxin molecules participate in their specific interaction with the SRL. The Lys-rich region corresponding to loop 3 of α-sarcin interacts with the negatively charged phosphodiester bond around the bulged G, and the sequence stretch of loop 2, comprising residues 51–55 (a sequence stretch that includes the mentioned Asn54) and some residues from loop 5, contacts the conserved GAGA tetraloop that contains the sequence cleaved by the toxin (Fig. 5s).

It is, however, quite obvious that these interactions with the SRL do not by themselves explain the exquisite specific activity displayed by ribotoxins against intact ribosomes. Consequently, some other interactions with additional ribosomal elements are required. In this regard, it has recently been shown that the ribosomal context enhances the reaction rate by several orders of magnitude. This catalytic advantage seems to arise from favorable electrostatic interactions with the ribosome (Korennykh *et al.*, 2006). The positively charged ribotoxins bind with high affinity and speed, thereby enhancing the rate of SRL cleavage by several orders of magnitude, matching the catalytic efficiency of the fastest known enzymes (Korennykh *et al.*, 2006). α-Sarcin's surface, for example, is highly charged: 39% of the surface is composed of charged side chains and 26% of polar side chains (Pérez-Cañadillas *et al.*, 2000). Therefore, these results suggest a mechanism of target location whereby α-sarcin encounters ribosomes randomly and diffuses within the ribosomal electrostatic field to the SRL. Long ago, it was

reported not only that the ribonucleolytic activity of α-sarcin was completely inhibited by NH_4^+, K^+, or Na^+ at concentrations higher than 0.2 M, as well as by millimolar levels of some divalent cations such as Ca^{2+}, Mn^{2+}, or Mg^{2+} (Endo *et al*., 1983; Martínez del Pozo *et al*., 1989), but also that it binds Zn^{2+}, Cd^{2+}, and Co^{2+}, with an affinity corresponding to dissociation constants in the millimolar range (Martínez del Pozo *et al*., 1989). This binding was proposed to be mediated by interactions with the active site His side chains, and affected fluorescence emission, most probably modifying the microenvironment of Trp51. Whereas in these studies it was shown that Zn^{2+} cations were effective inhibitors of its ribonucleolytic activity, it was also shown that the inhibition promoted by most of the other cations studied was due to the establishment of interactions with the substrates used, rather than to the existence of a specific interaction with the protein (Martínez del Pozo *et al*., 1989).

Within this context, it must be considered that internal motions allow recognition elements to screen a significant part of the conformational space, increasing the chances of successful binding. As explained earlier, residues 1–26 in α-sarcin form a long β-hairpin that can be considered as two consecutive minor β-sheets connected by a hinge region. The second β-sheet, coincident with residues 7–22, is one of the regions with the highest conformational flexibility, appearing to be folded independently from the protein core (Pérez-Cañadillas *et al*., 2000, 2002; García-Mayoral *et al*., 2004). The results obtained with the previously mentioned Δ (7–22) α-sarcin mutant suggested that this protein would interact with the ribosome in at least two regions, i.e. the well-known SRL of the rRNA gene, and a different region recognized by the β-hairpin of the protein (García-Ortega *et al*., 2002). Its three-dimensional structure in solution (García-Mayoral *et al*., 2004) showed that the folding of wild-type α-sarcin was preserved, including the spatial conformation of the loops. The most significant differences were concentrated in loop 2, the new orientation of loop 3, and the dynamics of loop 5, where conformational heterogeneity was observed as a consequence of the removal of important interactions with residues in the native motif (García-Mayoral *et al*., 2004). Thus, its structural integrity and ability to specifically cleave SRL-like oligos was preserved, but the α-sarcin-specific recognition of the ribosome disappeared. Modeling the highly specific ribotoxin recognition of ribosomes using three-dimensional structures of wild-type and Δ(7–22) α-sarcins suggested two more hitherto unidentified interactions (García-Mayoral *et al*., 2005b). One of them would occur between a short sequence stretch of the α-sarcin loop 2 and the ribosomal protein L6 (Fig. 5c). The second would occur between the residues corresponding to the deleted distal part of the β-hairpin and protein L14 (Fig. 5c). These two prokaryotic ribosomal proteins are immediate neighbors of the SRL (Fig. 2a), are present in the three living phyla (Ban *et al*., 2000), and seem to undergo the most substantial changes, according to the X-ray and cryo-electron microscopy maps of the ribosome (Ban *et al*., 2000; Gabashvili *et al*., 2000). Obviously, the interaction involving the β-hairpin would not be possible for the deletion mutant, and appears to be crucial for the specific ribosome recognition (García-Mayoral *et al*., 2005b). This hypothesis was reinforced by the observation that a region homologous to the 11–16 sequence of α-sarcin can be found in (EF-2) from *S. cerevisiae* (Kao & Davies, 1999; García-Mayoral *et al*., 2005b). Both groups of polypeptides, ribotoxins and ribosomal proteins L14 (and their L23 counterparts in eukaryotic organisms), represent families of highly homologous proteins. The interacting regions of α-sarcin and L14 are conserved but show some degree of variability (García-Mayoral *et al*., 2005b), especially regarding the L14 residues involved (L23 proteins have sequences that are only distantly related). This fact helps to explain not only the extraordinary catalytic efficiency of ribotoxins against ribosomes, but also their different potencies, depending on the origin of the ribosome assayed (Schindler & Davies, 1977; Endo & Wool, 1982; Endo *et al*., 1983; García-Mayoral *et al*., 2005b).

Ribotoxins as natural killers of intact cells

It has already been explained how α-sarcin is able to inactivate ribosomes in cell-free systems of a great variety of organisms (Endo *et al*., 1993a, b; Kao & Davies, 1995), but it displays marked selectivity when intact cells are used as targets. This specificity seems to be determined by its ability to penetrate cells. Thus, α-sarcin is active against transformed or virus-infected mammalian cells, in the absence of any other permeabilizing agent (Fernández-Puentes & Carrasco, 1980; Carrasco & Esteban 1982; Olmo *et al*., 1993, 2001; Turnay *et al*., 1993; Stuart & Brown, 2006). The protein was also cytotoxic, inhibiting protein biosynthesis, when assayed against eight different human and rat tumor cell lines of mesenchymal, glial or epithelial origin (Turnay *et al*., 1993): This effect was saturable and consistent with passage across the cell membrane being the rate-limiting step, but no membrane damage or mitochondrial activity alterations were detected (Turnay *et al*., 1993). Again, these experiments confirmed that α-sarcin exhibits an intrinsic and rather specific cytotoxic character, in the absence of any external permeabilizing agent, virus included, when assayed against some transformed cell lines. The particular reasons for this selectivity at the molecular level have not been completely established yet; however, as mentioned above, the presence of acidic phospholipids on the outer leaflet of the membrane seems to be one of the determining factors (Connor *et al*., 1989; Gasset *et al*., 1989, 1990; Zachowski, 1993).

The mechanism of internalization of α-sarcin into intact human rhabdomyosarcoma cells and the cellular events resulting in the induction of cell death have been studied (Olmo *et al.*, 2001). According to these results, the toxin is internalized via endocytosis involving acidic endosomes and the Golgi, as deduced from the ATP requirement and the effects of NH_4Cl, monensin and nigericin on its cytotoxicity. In addition to the specific cleavage of 28S RNA associated with protein biosynthesis inhibition, α-sarcin killed rhabdomyosarcoma cells via apoptosis. This apoptosis was not just 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). Furthermore, experiments with a catalytically inactive α-sarcin mutant (H137Q), which is neither toxic nor apoptotic, revealed that it was directly related to the catalytic effects of the toxin on the ribosomes, as this mutant displays identical lipid-interacting abilities to those of the wild-type protein (Lacadena *et al.*, 1995).

The loss of the positive charge at the position corresponding to α-sarcin Arg121 produced a dramatic impairment of its ability to interact with phospholipid membranes (Masip *et al.*, 2001), supporting the conclusion that Arg121 is a crucial residue for the characteristic cytotoxicity of α-sarcin and presumably of the other fungal ribotoxins. In agreement with their altered ribonucleolytic and lipid-interaction activities, all of the mutants studied with mutations affecting the enzymatic specificity of the protein, especially the deletion Δ(7–22) mutant, showed diminished cytotoxic effects on human rhabdomyosarcoma cells (García-Ortega *et al.*, 2002). Even restrictocin shows a lower phospholipid-interacting ability, which is correlated with decreased cytotoxicity (García-Ortega *et al.*, 2001), as mentioned earlier in this review. On the other hand, HtA shows a very similar cytotoxic potency, in terms of IC_{50} (concentration required to produce 50% of protein biosynthesis inhibition) (Herrero-Galán *et al.*, 2007).

Ribotoxins as allergens

Fungi represent one of the principal sources of allergens. *Aspergillus fumigatus*, a human pathogenic species, has been well studied as an opportunistic pathogen (Walsh & Pizzo, 1988; Bodey & Vartivarian, 1989). Invasive infection is usually fatal unless treated early, and even then, antifungal therapy is often unsuccessful. The incidence of fungal infections has risen lately, owing to the increase in the number of immunocompromised patients, and *A. fumigatus* infection is common postoperatively (Pasqualotto, 2006). A link between fungal allergy and severe asthma is generally accepted, but is still poorly understood (Ronning *et al.*, 2005; Denning *et al.*, 2006). Among the reasons why *A. fumigatus* can behave as a human pathogen is its ability to grow quickly at temperatures as high as 50 °C, it being the most thermophilic of the *Aspergillus* spp. (Ronning *et al.*, 2005); all the other known ribotoxin-producing fungi can scarcely grow when cultured above 30 °C. It is also remarkable that *A. fumigatus* is a more common source of allergy and asthma than either *A. nidulans* or *A. oryzae*, the other two *Aspergillus* spp. whose genome sequences have been determined (Galagan *et al.*, 2005; Machida *et al.*, 2005; Nierman *et al.*, 2005). Interestingly, all *A. fumigatus* allergens have close homologs in the other two species, with the exception of the ribotoxin Aspf1 and the metalloprotease Aspf5 (Ronning *et al.*, 2005), but it is not yet clear whether Aspf1 is a critical factor in triggering an allergic response. In this regard, it should be remembered that allergens are usually identified as those proteins recognized by the IgE antibodies contained in the sera of allergic patients. In relation to this, restrictocin was also found in the urine of patients with disseminated aspergillosis (Arruda *et al.*, 1990, 1992; Lamy *et al.*, 1991), and antibodies have been also used to prove that it accumulates in the vicinity of nodes of fungal infection (Lamy *et al.*, 1991). Although it has been proven that Aspf1 is not a major virulence factor in *A. fumigatus* infections (Paris *et al.*, 1993; Smith *et al.*, 1993, 1994), this protein is clearly involved in the pathogenicity of allergic bronchopulmonary aspergillosis (ABPA), the most severe form of allergic inhalant diseases, as these patients show high levels of Aspf1-specific IgE (Kurup *et al.*, 1994; García-Ortega *et al.*, 2005). ABPA has a prevalence of 1–2% in patients with persistent asthma, but this value increases to 15% in cystic fibrosis patients (Greenberger, 2002; Kurup *et al.*, 2006). The explanation for *A. fumigatus* being the mold usually involved in these diseases seems to be, again, its ubiquity and its small spores, which grow optimally at 37 °C. Thus, it can colonize the respiratory tract of the host, leading to the onset of the pathologic events (Banerjee & Kurup, 2003).

Extracts of *A. fumigatus* are frequently used to diagnose allergic reactions, but they are highly complex mixtures, containing up to 200 different proteins and glycoproteins and low molecular weight compounds (Piechura *et al.*, 1983), and are very difficult to standardize. Attempts to improve diagnosis are focusing on the employment of homogeneous standard preparations of recombinantly produced allergens (Crameri *et al.*, 1998; Kurup *et al.*, 2006). In this regard, it is therefore important to point out that Aspf1 was the first recombinant allergen tested *in vivo* (Moser *et al.*, 1992), and showed complete concordance with serologic determinations (Moser *et al.*, 1992; Crameri *et al.*, 1998; Hemmann *et al.*, 1999). Unfortunately, the recombinant native allergen is not devoid of cytotoxic activity, and can trigger anaphylaxis.

As mentioned before, ribotoxins have much longer loops than the other nontoxic fungal RNases, which are supposedly involved in their specificity, toxicity and antigenicity.

Once again, it must be noted that the Δ(7–22) region, which contains the ribotoxin-characteristic N-terminal β-hairpin, shows the highest amino acid sequence variability among ribotoxins (Fig. 2) (Martínez-Ruiz *et al*., 1991a, b, 2001), and is highly flexible and exposed (Pérez-Cañadillas *et al*., 2000; García-Mayoral *et al*., 2004). Indeed, Aspf1 differs from α-sarcin in only 19 (87% sequence identity) residues, but five of these 19 amino acid differences are located at this N-terminal β-hairpin. As it is generally assumed that the exposed and highly flexible regions are usually good candidates to be B-cell epitopes in proteins, this β-hairpin could certainly be a major determinant of the immunoreactivity of these proteins. This was confirmed through the production and characterization of Aspf1, α-sarcin, and their corresponding Δ(7–22) variants (García-Ortega *et al*., 2002, 2005). First, these data confirmed the significant prevalence of Aspf1-specific IgE antibodies in sera from patients sensitized to *Aspergillus* (García-Ortega *et al*., 2005), as reported before by other authors (Kao *et al*., 2001; Greenberger, 2002; Kurup *et al*., 2006). This result was particularly important in the ABPA patients studied, as anti-Aspf1 IgE antibodies were detected in 100% of them. Second, and although several earlier studies with synthetic peptides overlapping the above-mentioned region produced controversial results regarding its antigenic behavior (Kurup *et al*., 1998, Madan *et al*., 2004), the three proteins studied, Aspf1Δ (7–22), α-sarcin, and α-sarcin Δ (7–22), showed marked decreases in their reactivity to Aspf1 IgE antibodies (García-Ortega *et al*., 2005), indicating that the deleted portion was involved in at least one allergenic epitope. However, although important, this cannot be the only allergenic epitope within this molecule, as deduced from enzyme-linked immunosorbent assay (ELISA) inhibition experiments. The essential residues for epitopes in Aspf1 are changed in wild-type α-sarcin, as inferred from the fact that the response against the sera of the patients was even lower for the latter than for the Aspf1Δ (7–22) mutant. Despite this decreased IgE reactivity, the prevalence of the three Aspf1 variants remained essentially unaffected, and they retained most of the IgG epitopes (García-Ortega *et al*., 2005). Thus, these noncytotoxic deletion variants of ribotoxins are promising molecules for use in immunomodulating therapies for *Aspergillus* hypersensitivity and diagnosis. However, *in vivo* assays are still required to assess this possibility.

Immunotoxins

One of the goals of antitumor therapy is the preparation of immunotoxins according to the idea described by Ehrlich in 1906, who introduced the concept of targeting cancer cells with a 'magic bullet' consisting of a tissue-specific carrier that would deliver toxic agents to neoplastic tissue (Ehrlich, 1956). In the past decade, an increased amount of clinically oriented research involving immunotoxins has been published (Reiter & Pastan, 1998; Kreitman *et al*., 1999; Brinkmann, 2000; Li *et al*., 2004). Immunotoxins are therapeutic agents with a high degree of specificity, composed of targeting moieties, such as antibodies or physiologically important ligands (such as growth factors or cytokines), linked to toxins, chiefly toxic proteins from plants or bacteria (Brinkmann & Pastan, 1994; Reiter & Pastan, 1998; Kreitman, 2001).

Initially, immunotoxins were prepared by conjugating toxins to monoclonal antibodies. The targeting moiety of these first-generation immunotoxins was the whole antibody molecule (Kreitman, 2000). As the binding sites for antigen are on the variable regions of antibodies, further studies were performed to verify that Fab fragments, obtained after IgG papain digestion, retained the ability to interact with antigens (Ward *et al*., 1989; Wörn & Plückthun, 2001), leading to the so-called Fab or Fv immunotoxins, which were easily internalized because of their smaller size (Brinkmann, 2000). The development of advanced technologies allowed the production of recombinant immunotoxins, stabilized by a flexible peptide (scFv) or by a disulfide bridge between the variable domains (dsFv), that can be expressed in several model organisms, are easily modified by genetic engineering, and are more stable (Kreitman, 2003; Li *et al*., 2004).

Regarding the toxin moiety, the most representative toxins employed have been ricin from plants and *Pseudomonas* exotoxin A (PE) or diphtheria toxin (DT) from bacteria. Ricin is composed of two subunits linked together by a disulfide bond, chain A being responsible for the glycosidase activity, leading to the inactivation of ribosomes (Olsnes & Pihl, 1973a, b; Endo *et al*., 1987), and is the one usually used to make immunotoxins (Ghetie *et al*., 1993; Engert *et al*., 1997; Schnell *et al*., 1998). Ricin depurinates a single nucleotide contiguous to the phosphodiester bond cleaved by ribotoxins (Endo & Tsurugi, 1987; Endo *et al*., 1987), a catalytic action that renders the ribosome inactive too. Different immunotoxins have been obtained that contain the whole blocked ricin or deglycosylated chain A (Pastan *et al*., 1992; O'Toole *et al*., 1998). Regarding bacterial toxins, PE and DT are single-chain proteins that inhibit protein synthesis by ADP-ribosylating EF-2 (Carroll & Collier, 1987). Among PE- and DT-based immunotoxins, the most commonly used involve truncated versions of the toxins, produced by genetic excision of their binding domain, resulting in PE38 or PE40 variants (Kondo *et al*., 1988; Kreitman *et al*., 1990, 1993; Pastan, 2003), and DT388 or DT389 variants (Foss *et al*., 1998; LeMaistre *et al*., 1998), respectively.

Ribotoxins have several advantages for use in the design of immunotoxins, namely, their small size, high thermostability, resistance to proteases, and highly efficient

ribonucleolytic activity (Gasset *et al.*, 1994; Kao *et al.*, 2001; Martínez-Ruiz *et al.*, 2001). Poor immunogenicity and low toxicity in mice have also been described in relation to restrictocin (Rathore & Batra, 1996). Thus, different ribotoxins have been used as components of immunotoxins (Orlandi *et al.*, 1988; Conde *et al.*, 1989; Hertler & Frankel, 1989; Wawrzynczak *et al.*, 1991; Better *et al.*, 1992; Rathore & Batra, 1996; 1997a, b; Rathore *et al.*, 1997). Initially, ribotoxin-based immunotoxins were constructed by chemical conjugation, as described for, among others, mitogillin (Better *et al.*, 1992), restrictocin (Orlandi *et al.*, 1988; Conde *et al.*, 1989; Rathore & Batra, 1996) and α-sarcin (Wawrzynczak *et al.*, 1991). Second-generation immunotoxins were later designed, mostly related to restrictocin single-chain immunotoxins produced by fusing restrictocin cDNA with that encoding the scFv region of the monoclonal antibody directed to the human transferrin receptor (anti-TFR) (Rathore & Batra, 1997a, b), joined by a linear flexible peptide to promote the independent folding of the two immunotoxin moieties. These constructions were further engineered to enhance the intracellular processing and delivery of restrictocin (Goyal & Batra, 2000).

A few immunotoxins containing α-sarcin have been described (Wawrzynczak *et al.*, 1991; Rathore *et al.*, 1997), with α-sarcin chemically coupled to anti-TFR or anti-Fib75. The α-sarcin used in these constructions was obtained either from *A. giganteus* cultures (Wawrzynczak *et al.*, 1991) or from heterologous expression in *E. coli* cultures (Goyal & Batra, 2000). Promising results were obtained when cytotoxicity was measured, with IC_{50} values similar to those obtained with plant or bacterial toxin-based immunotoxins (Goyal & Batra, 2000). The α-sarcin immunotoxin showed equal stability and specific activity on the target cells and similar pharmacokinetics to those of analogous immunotoxins (Wawrzynczak *et al.*, 1991). However, further studies with α-sarcin-based immunotoxins, including clinical trials, were not performed, probably because of the large size of the immunotoxin, which could hinder correct internalization in solid tumors, or because of the low structural stability of the immunoconjugates. It must be noted that these α-sarcin immunotoxins were not made as recombinant second-generation immunotoxins, such as the single-chain immunotoxins (scFv-IMTX) described later for restrictocin (Rathore & Batra, 1997a, b), which gave better results in terms of stability and cytotoxicity assays *in vivo*. Moreover scFv-IMTX can be easily modified by genetic engineering to improve the cytotoxic activity or to diminish unspecific toxicity *in vivo* or immunogenicity.

In relation to this, a single-chain immunotoxin has been recently produced in the methylotrophic yeast *P. pastoris*, composed of the variable domains of the B5 monoclonal antibody, specific against $Lewis^Y$ carbohydrates, which are very abundant in carcinomas, bound to α-sarcin through a peptide containing a furin cleavage site (scFv-IMTXαS) (Lacadena *et al.*, 2005). *Pichia pastoris* has emerged as a robust heterologous expression host, owing to the efficient secretory expression of complex recombinant proteins with correct intramolecular and intermolecular disulfide bonds that do not require additional *in vitro* unfolding and refolding strategies, unlike most immunotoxins that are heterologously expressed in bacteria (Cregg *et al.*, 1993; Gurkan & Ellar, 2003, 2005). Indeed, *P. pastoris* possesses tightly regulated promoters, such as that of the *alcohol oxidase 1* gene (AOX1), which is uniquely suited for the controlled expression of foreign genes (Cregg *et al.*, 1989). Thus, several immunotoxins have been successfully produced extracellularly in *P. pastoris* (Woo *et al.*, 2002, 2004, 2006; Lacadena *et al.*, 2005; Liu *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 tumors (Pastan & Fitzgerald, 1991). Different members of the family have been used as the targeting moiety in many immunotoxins, such as mAb B3 (Brinkmann *et al.*, 1991, 1993; Pai *et al.*, 1991, 1996; Benhar & Pastan, 1995a; Bera & Pastan, 1998), mAb B1 (Pastan & Fitzgerald, 1991; Benhar & Pastan, 1995b; Kuan & Pastan, 1996), and mAb B5 (Benhar & Pastan, 1995a, b). Indeed, mAb BR96 and mAb 3S193 have also been evaluated for targeted immunotherapy (Trail *et al.*, 1993; Rosok *et al.*, 1998; Scott *et al.*, 2000). At least three of these immunotoxins or immunoconjugates have recently been evaluated in phase I trials in patients with cancer, with promising results (Pai *et al.*, 1996; Brinkmann, 2000).

scFv-IMTXαS produced in *P. pastoris* displays the characteristic ribonucleolytic activity of α-sarcin and specific cytotoxicity against targeted cell lines containing the $Lewis^Y$ antigen (Lacadena *et al.*, 2005). Furthermore, studies on the characterization of genetically engineered immunotoxins based on that mentioned above, with increased stability and affinity, are being performed (Lacadena *et al.*, 2005).

Conclusions and future prospects

Ribotoxins are unique RNases in terms of specificity and cytotoxicity. Their remarkable and exquisitely specific ribonucleolytic action, as well as their innate ability to cross membranes, have been subjects of study for many years, and are now quite well understood in molecular terms, through the combination of a wide variety of structural, spectroscopic, biochemical, and cellular techniques, together with the production and characterization of a large number of mutants (Lamy *et al.*, 1992; Gasset *et al.*, 1994; Wool, 1997; Kao *et al.*, 2001; Martínez-Ruiz *et al.*, 2001). The determination of several high-resolution ribosomal structures and the

use of different lipid model vesicles and transformed cell lines, susceptible or not to the action of these toxins, have been of great help in deciphering the details of the cytotoxic mechanism of ribotoxins at the molecular level. The existence of very similar and well-known noncytotoxic fungal extracellular RNases, such as RNase T1, has been also very useful. Presumably, these RNases are noncytotoxic because they lack the above-mentioned ability to cross a phospholipid bilayer. Thus, they have been, and still are, excellent reference models with which to approach the study of the behavior of this family of proteins. Unfortunately, the natural function of ribotoxins still remains unknown, and it is definitively one of the most interesting questions that needs to be answered. In this regard, studies on the regulation of ribotoxin production within the context of their natural environment are still required. In fact, much indirect evidence suggests that these proteins are synthesized under a variety of stress conditions (Olson *et al.*, 1965b; Meyer & Stahl, 2002, 2003; Meyer *et al.*, 2002), but further direct characterization of these mechanisms and their regulation is still needed. In relation to this, the eventual functional connection with the prokaryotic TA systems (Condon, 2006) must not be dismissed. Indeed, the discovery and characterization of HtA, a singular ribotoxin from the structural and functional points of view, has opened a new door to the acquisition of additional clues about the origin and functionality of fungal ribotoxins. Further characterization of this protein and similar ones, which will eventually be discovered, given the increasingly rapid discovery of new ribotoxins, will greatly improve our understanding of ribotoxin action in the natural context, mostly involving the filamentous fungi.

This lack of knowledge about their natural function does not, however, preclude their employment as therapeutic agents. Despite the fact that their use as antitumor agents was abandoned early, due to high toxicity (Roga *et al.*, 1971), it is also true that the actual accumulation of data about their mechanism of action allows the optimistic view that these ribotoxins, or probably some modified variants of them, might be used with therapeutic aims. In relation to this, the production of hypoallergenic mutants and immunotoxins stand out as the most feasible alternatives in the mid-term future.

Regarding the first approach, it must be remarked how *Lactococcus lactis*, a primary constituent of many industrial and artisanal starter cultures used for the manufacture of a wide range of fermented dairy products, has been exploited through applications as a cell factory for metabolite and membrane protein production and as a delivery system for therapeutic molecules in the gastrointestinal tract (Steidler *et al.*, 2000, Kunji *et al.*, 2005). The status of *L. lactis* as a 'generally regarded as safe' (GRAS) organism confers this system with the features required to try immunotherapeutic protocols for Aspf1-related allergic diseases. In relation to this, *L. lactis* strains capable of secreting the above-mentioned hypoallergenic variants of Aspf1 have been obtained (Alegre-Cebollada *et al.*, 2005; García-Ortega *et al.*, 2005), and although their use as potential delivery systems has yet to be tested, they constitute one of the research directions that should be immediately explored.

Immunotoxins are another promising alternative for the employment of ribotoxins as therapeutic agents against tumorigenic processes. The production in large amounts of optimized immunotoxin versions of α-sarcin (Lacadena *et al.*, 2005) and other microbial RNases is well under way, and it is definitively one of the research paths to be followed in the near future.

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