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The Acidic Ribosomal Stalk Proteins Are Not Required for the Highly Specific Inactivation Exerted by α-Sarcin of the Eukaryotic Ribosome

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Supporting Information

ABSTRACT: The ribosomal sarcin/ricin loop (SRL) is the target of ribosome-inactivating proteins like the *N*-glycosidase ricin and the fungal ribotoxin α-sarcin. The eukaryotic ribosomal stalk directly interacts with several members of the *N*-glycosidase family, favoring their disruption of the SRL. Here we tested this hypothesis for the ribotoxin α-sarcin. Experiments with isolated ribosomes, cell-free translation systems, and viability assays with *Saccharomyces cerevisiae* strains defective in acidic stalk proteins showed that the inactivation exerted by α-sarcin is independent of the composition of the ribosomal stalk. Therefore, α-sarcin, with the same ribosomal target as ricin, seems to access the SRL by a different pathway.

Ribosome-inactivating proteins have been extensively studied because of their extraordinary efficiency against their target cells. They are classified into two groups: those with *N*-glycosidase activity (RIPs), like ricin,[1] and ribotoxins, a family of fungal ribonucleases best represented by α-sarcin.[2] Considering their unrelated sequence, structure, and catalytic activity, it is striking how they recognize the same element in the ribosome, the sarcin/ricin loop (SRL), essential for translation factor binding and their GTPase activation.[3,4] Despite the universality of the SRL, ribotoxins and RIPs show important differences in their efficiencies against ribosomes of different origins.[1] Most of them prefer eukaryotic ribosomes, suggesting a specific recognition of additional elements in their structure.[5,6] Moreover, it has been shown how the ribosomal stalk can be used as an important interacting element for RIPs like ricin, trichosanthin, and Shiga-like toxins.[7,8] This protruding structure in the ribosome has been described as being involved in the recruitment and turnover of elongation factors during translation.[9,10]

The composition of the stalk is fairly similar among organisms, although important differences in the sequences of the corresponding proteins have been identified[11−13] (Figure S1 of the Supporting Information). It is based on a central protein, P0 in eukaryotes (L10 in prokaryotes), and dimers of acidic proteins bound to it (P1 and P2 in eukaryotes and L7/12 in prokaryotes). In particular, *Saccharomyces cerevisiae* displays two heterodimers formed by P1 and P2 isoforms: P1αP2β and P1βP2α. These acidic proteins are extremely dynamic, including their exchange with the cytoplasmic pool, their C-terminal regions being responsible for the interaction, recruitment, and regulation of supernatant translation factors and ribosome-inactivating proteins like ricin and trichosanthin.[9,14] However, nothing is known about ribotoxins in this regard.

Ribotoxins make up an interesting family of basic ribonucleases secreted by filamentous fungi.[2] They harbor in their small structure the ability to penetrate into target cells by interacting with membrane lipids and their extraordinary efficiency against the SRL in the ribosome. α-Sarcin is the most representative member of the family and has been exhaustively characterized. However, the mechanism of ribosome recognition is still far from being well understood. On the basis of detailed kinetic analyses, it was shown how the electrostatic character of the ribosomal surface promotes a rapid guiding of ribotoxins toward their target, a concept that was later extended to several other RIPs.[15]

Considering the role of the P1 and P2 proteins of the eukaryotic ribosomal stalk in the positioning of several RIPs to the SRL,[7,8,14,16−18] we have studied the potential interaction of ribotoxins with them. Unexpectedly, the results obtained suggest a different mode of action for fungal ribotoxins.

To first study the role of the ribosomal stalk in the specific recognition and cleavage of the SRL by α-sarcin, purified ribosomes with (wild type) and without P1 and P2 proteins (ΔP1P2) were chosen (Figure S1 of the Supporting Information). Quantification of this cleavage by primer extension analysis gave very similar results in both cases (Figure 1A). Considering that salt concentration is critical for ribosome stability and functionality, as well as the effect of electrostatics in its interaction with ribotoxins and RIPs,[15,18] the effect of changes in Mg^{2+} concentration was tested. The increment from 1 to 5 mM Mg^{2+} made both types of ribosomes less accessible to α-sarcin, as expected, but no differences were observed between them (Figure 1A). Interestingly, the characteristic ribonucleolytic activity of α-sarcin on ribosomes was higher when α-sarcin was assayed in the presence of additional translating factors (by including a ΔP1P2 S100 extract), but substantial differences were not found between wild-type (WT) and mutant ribosomes (Figure 1B). Finally, it was also demonstrated that free cytosolic

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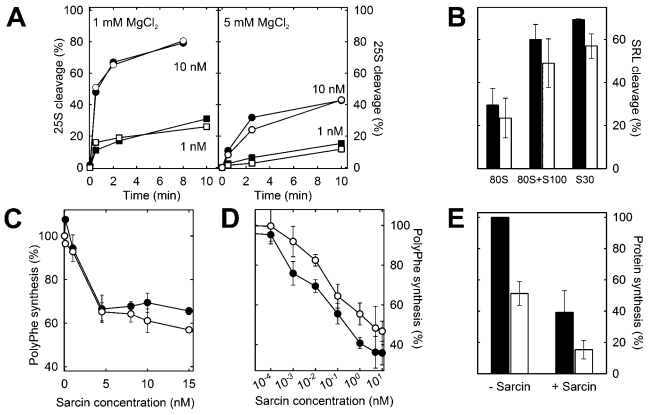

Figure 1. (A) *In vitro* SRL cleavage of ribosomes from WT and ΔP1P2 yeast strains treated with α-sarcin. (B) α-Sarcin (10 nM) against isolated ribosomes in the presence of polyU (80S), supplemented with ΔP1P2 S100 (80S+S100), or against S30 lysates. (C) Effect of α-sarcin in polyPhe synthesis by WT or ΔP1P2 ribosomes supplemented with ΔP1P2 S100. PolyPhe was quantified by precipitating [^{3}H]Phe peptides. (D) Identical experiment as in panel C but with WT or ΔP1P2 S30 extracts. (E) Endogenous mRNA translation by WT or ΔP1P2 S30 extracts in the presence (+) or absence (−) of α-sarcin. Protein synthesis was quantified by the amount of [^{35}S]Met incorporated. Black symbols and bars are used for WT and white symbols and bars for ΔP1P2.

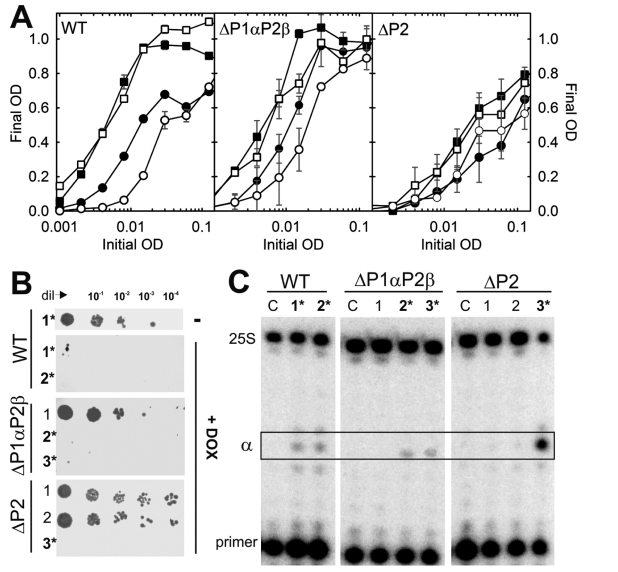

Figure 2. (A) Effect of WT (circles) and H50/137Q/E96Q (squares) α-sarcin expression in WT, ΔP1αP2β, and ΔP2 *S. cerevisiae* strains. Growth curves in the presence (white) or absence (black) of doxycycline. (B) Viability assay spotting serial dilutions of pCM175αS transformed strains in SC-W without (−) and with (+DOX) doxycycline. Several clones are shown, and those marked with asterisks are sensitive to α-sarcin toxicity. (C) Primer extension analysis of RNA extracted from liquid cultures of clones shown in panel B. Clones transformed with an empty control vector (C) were included. Bands corresponding to intact 25S, α-sarcin specific cleavage (α), and an excess of primer are indicated.

P proteins do not have any impact in the SRL cleavage by α-sarcin by using S30 extracts in the assay (Figure 1B).

Measurement of the effect of ribotoxins on ribosome function is more sensitive than direct quantification of SRL cleavage. Moreover, as ribosomes defective in stalk proteins are less efficient in translation, a similar extent of SRL cleavage by ribotoxins may be more efficient in terms of the inhibition of protein synthesis. Therefore, different cell-free translation systems were designed and assayed for α-sarcin inhibition. System A included isolated WT and ΔP1P2 ribosomes and ΔP1P2 S100 extract to translate polyU into polyPhe (Figure 1C). This assay measures the effect of the absence of the stalk proteins. System B includes a more efficient system of polyPhe synthesis using WT and ΔP1P2 S30 lysates, where the cytosolic P protein pool is also a variable (an important fact considering that it has been postulated for ricin that cytosolic P proteins help in the recruitment of these RIPs to the SRL binding site[7]) (Figure 1D). In system C, the translation of the endogenous mRNAs included in the S30 lysates was also assayed for the action of α-sarcin, where steps like initiation are taken into account (Figure 1E). Results from these experiments showed that the absence of P proteins did not affect the inhibitory action of α-sarcin against translating ribosomes when tested in cell-free systems.

To perform an *in vivo* analysis, doxycycline inducible intracellular expression of WT α-sarcin was accomplished in *S. cerevisiae* W303 cells using a catalytically inactive mutant as a negative control (α-sarcin H50/137Q/E96Q, sar3M). Toxicity was analyzed by means of cell growth impairment that, for the WT protein, was observed even in the absence of induction, in agreement with the existence of a minimal, but not negligible, basal expression in this system[19] (Figure 2A). This result is especially remarkable considering that how the intracellular presence of just one single molecule of α-sarcin can be lethal has been described and agrees with previously published data.[20] The specific SRL cleavage on host ribosomes by α-sarcin was confirmed to discard other side effects as a cause of the observed toxicity (Figure S2 of the Supporting Information). The production of α-sarcin was also studied in two *S. cerevisiae* strains defective in the acidic P proteins: ΔP1αP2β (lacking one heterodimer) and ΔP2 (lacking both heterodimers) (Figure S1 of the Supporting Information). Growth curves showed smaller differences when WT ribotoxin was expressed in the ΔP1αP2β strain, and not a considerable defect in growth was detected in the strain ΔP2 (Figure 2A). Because the *in vitro* results suggested that the ribosomal stalk did not directly participate in the ribotoxin inactivation of the ribosome, it seemed that the slower-growing defective strains would favor the appearance of ribotoxin resistance. To further analyze this idea, six individual clones of each strain were tested in solid medium viability assays (Figure 2B). In accordance, this assay showed that α-sarcin was lethal in all WT clones, while 30 and 80% of resistant clones were obtained for the ΔP1αP2β and ΔP2 strains, respectively.

Interpretation of these results in terms of the involvement of the stalk proteins in α-sarcin toxicity required the analysis of the ribotoxin resistance in ΔP1αP2β and ΔP2 strains. Ribosomal RNA was analyzed for all clones, and the α-sarcin cleavage band was present only in those cases where there was a toxic effect inhibiting yeast growth (Figure 2C). Therefore, α-sarcin specific ribonucleolytic activity was always linked to the lethal phenotype of the hosting clones. Finally, all clones were subjected to α-sarcin cDNA amplification analyses. Surprisingly, only those clones for which α-sarcin showed a lethal phenotype in agar plates maintained its cDNA (Figure S3 of the Supporting Information). The difference between WT and stalk defective strains in maintaining the ribotoxin gene may be due to additional factors not involving a direct interaction between the ribosomal stalk and α-sarcin. For example, P protein knockout strains show defective growth, and their pattern of protein

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expression changes.[21] Consequently, resistance to α-sarcin expression did not arise from an incomplete ribosomal stalk but rather from a selection pressure to delete the α-sarcin gene.

This study focuses on the ribosomal stalk, involved in recruiting and activating different translation factors during protein synthesis.[9,10,12] As mentioned above, it also serves as an anchoring platform for several RIPs like ricin and trichosantin to further recognize their target, the SRL.[7,8,14,16−18] This interaction explains the specificity of these toxins for eukaryotic ribosomes, based on their different sequences with respect to their bacterial counterparts. However, not all RIPs behave identically. For example, for the pokeweed antiviral protein, no interaction with the ribosomal stalk has been found.[22] Along those same lines, and in agreement with the results shown here, a recent study has shown that the P1/P2 C-terminal peptide SDDDMGFGLFD does not interact with α-sarcin (a ribotoxin) or saporin (a RIP) but does interact with Shiga-like toxin A1.[23] These differences have been explained in terms of their different surface charge distributions. In the case of ricin, positively charged arginine residues on the opposite side of the active center seem to be essential for its interaction with the stalk.[18] Overall, this heterogeneity has led to the suggestion that evolution of RIPs has been recent and convergent, with the aim of interacting with the ribosomal stalk.[24]

Here we focus on fungal ribotoxins.[2] The idea of the stalk participating in helping α-sarcin reach the SRL was plausible considering previous observations for ricin. First, α-sarcin shares an identical target with ricin despite being a completely different enzyme. Second, the strong basic character of α-sarcin suggests it as a good candidate to interact with the acidic proteins of the stalk. Moreover, as for ricin, it is generally accepted that α-sarcin is more active against eukaryotic ribosomes, although an extensive comparison has not been performed.[5] Finally, interactions with stalk proteins do not seem to be highly selective in terms of docking specific structures, because models with trichosanthin and eEF2 show how these two very different proteins are able to interact with overlapping regions of P proteins.[16] However, our results show that the acidic ribosomal stalk P proteins do not participate in α-sarcin inactivation of the ribosome. The exhaustive *in vitro* characterization performed has not been able to reveal any influence of these proteins on the action of α-sarcin (Figure 1). Furthermore, *in vivo* experiments have shown the extraordinary toxicity of α-sarcin when it is expressed in yeast, which is independent of the composition of the stalk (Figure 2).

In conclusion, this study reveals that a fully assembled ribosomal stalk does not favor the ribotoxin specific cleavage of the yeast ribosome as opposed to the observations reported for other *N*-glycosidases like ricin, trichosantin, or Shiga-like toxins.[7,8,14] In addition, *in vitro* translation inhibition experiments suggest a preferred action of α-sarcin against translating ribosomes (Figure 1B). Therefore, the molecular basis for ribotoxin specific recognition of the SRL within the ribosome is still to be discovered.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures and Figures S1−S3. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interests.

■ DEDICATION

‖This article is dedicated to the memory of María Rodríguez-Mateos, who passed away while this study was being performed.

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