The Cytotoxins \( \alpha \)-Sarcin and Ricin Retain Their Specificity when Tested on a Synthetic Oligoribonucleotide (35-Mer) That Mimics a Region of 28 S Ribosomal Ribonucleic Acid*

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An oligoribonucleotide (35-mer) that mimics the \( \alpha \)-sarcin and the ricin region of eukaryotic 28 S rRNA was transcribed in vitro from a synthetic template with T7 RNA polymerase and was used to test whether the specificity of the hydrolysis by the toxins was retained. \( \alpha \)-Sarcin, at a low concentration, cleaved a single phosphodiester bond on the 3' side of a guanosine residue in the synthetic oligomer that corresponds to G-4325 in 28 S rRNA, the site of action of the toxin in intact ribosomes. At a high concentration of \( \alpha \)-sarcin, the substrate (35-mer) was hydrolyzed after each of its purines. \( \alpha \)-Sarcin was without an effect on a synthetic RNA (20-mer) that reproduces the near universal sequence of nucleotides in the loop, but lacks the stem, of the toxin's domain. Thus, the specificity of the attack of \( \alpha \)-sarcin on a precise region of 28 S rRNA appears to be contingent on the sequence of the nucleotides and the structure of the domain. Ricin depurinated a nucleotide in the synthetic oligomer (35-mer), and in the presence of aniline the phosphoribose backbone was cleaved at a position that conforms to A-4324 in 28 S rRNA, the site of action of the toxin in vitro.

The cytotoxic proteins that inactivate eukaryotic ribosomes include \( \alpha \)-sarcin which is produced by a fungus (1, 2); ricin, abrin, and mudeccin which are derived from plants (3); and Shiga toxin which is formed by a bacterium (4, 5). These toxins catalytically inactivate 60 S ribosomal subunits and in this way inhibit peptide chain elongation. The cytotoxicity of \( \alpha \)-sarcin is a reflection of its enzymatic activity. The toxin is a novel ribonuclease that hydrolyzes the phosphodiester bond on the 3' side of the guanosine at position 4325 in 28 S rRNA. This is the sole cleavage catalyzed by \( \alpha \)-sarcin when the substrate is intact 80 S ribosomes or 60 S subunits, and presumably this single break accounts entirely for its cytotoxicity (6-9). If the concentration of \( \alpha \)-sarcin is high compared to what is required to inactivate ribosomes, and if the substrate is naked RNA, then \( \alpha \)-sarcin cleaves on the 3' side of nearly all of the purines in the nucleic acid (8). Ricin A-chain and related proteins, abrin, mudeccin, Shiga toxin, and VT2 toxin, are also unusual enzymes; they are RNA N-glycosidases (10-12). This group of toxins inactivates 60 S ribosomal subunits by hydrolyzing the N-glycosidic bond of the guanosine residue (position 4324 in 28 S RNA) adjacent to the deoxyribose that \( \alpha \)-sarcin attacks (7-11). It is extraordinary that all of these toxins inactivate eukaryotic ribosomes by affecting a pair of adjacent nucleotides in a highly conserved, indeed, near universal, sequence (9, 13). This region of the large rRNA in the large ribosomal subunit is single-stranded (14). Ricin A-chain retains its activity and its specificity when the substrate is a fragment of 553 nucleotides derived from the 3' end of 28 S rRNA that has the nucleotide A-4324 (15).

We have sought to determine the minimal requirement for the specificity of the enzymatic activity of the two types of toxins, \( \alpha \)-sarcin and ricin. For this purpose, we prepared an oligoribonucleotide (a 35-mer) that mimics the \( \alpha \)-sarcin-ricin region of 28 S rRNA and assessed the effect of the toxins on this substrate.

**EXPERIMENTAL PROCEDURES**

**Synthesis of an Oligoribonucleotide Corresponding to the \( \alpha \)-Sarcin-Ricin Domain in 28 S rRNA—**The RNA oligoribonucleotides were synthesized using phage T7 RNA polymerase (Promega Biotech) and synthetic DNA oligomers as templates (16). The latter were synthesized with an Applied Biosystems model 380B synthesizer and purified by electrophoresis in 20% polyacrylamide gels containing 7 M urea. The two synthetic DNA oligomers used to form the template were annealed at 96 °C for 3 min followed by cooling on ice. The template DNA has a double-stranded T7 promoter and the sequence to be transcribed in a single-stranded region (Fig. 1). The transcription reaction was at 37 °C for 1 h in a solution containing 40 mM Tris-HCl, pH 8.0, 9 mM MgCl₂, 1 mM spermidine, 5 mM dithiothreitol, 1.5 mM each of the four nucleoside triphosphates (Pharmacia LKB Biotechnology Inc.), and including 0.5 μCi/mmol [α-\( ^{32} \)P]ATP, 50 μM DNA template, and 3 units/μL T7 RNA polymerase.

The transcription reaction mixture was extracted with phenol and chloroform and the nucleic acids precipitated with ethanol. The precipitate was dissolved in distilled water and heated at 90 °C for 1 min prior to electrophoresis in 20% polyacrylamide gels containing 7 M urea. The RNA transcript was separated from the template DNA during electrophoresis. The band containing the \( ^{32} \)P-labeled RNA was excised and eluted by soaking the gel slice for a few hours in a solution containing 500 mM ammonium acetate, 0.1% sodium dodecyl sulfate, and 0.1 mM EDTA. The RNA transcript was recovered from the supernatant by ethanol precipitation.

**Analysis of the Effect of \( \alpha \)-Sarcin and Ricin on Synthetic Oligoribonucleotides—**The RNA oligonucleotides (35- or 20-mer) were heated at 90 °C for 2 min in buffer (10 mM MgCl₂, 10 mM Tris-HCl, pH 7.6) and renatured by cooling at 4 °C overnight. Samples of the synthetic RNA (0.1 μg) were incubated in 25 μl of buffer (50 mM KCl, 5 mM MgCl₂, 10 mM Tris-HCl, pH 7.6). The concentrations of the substrate RNAs and of the cytotoxins (details are given in the legends to the figures) were chosen to approximate those employed with the adenosine residue (position 4324 in 28 S rRNA) adjacent to the deoxyribose that \( \alpha \)-sarcin attacks (7-11). It is extraordinary that all of these toxins inactivate eukaryotic ribosomes by affecting a pair of adjacent nucleotides in a highly conserved, indeed, near universal, sequence (9, 13). This region of the large rRNA in the large ribosomal subunit is single-stranded (14). Ricin A-chain retains its activity and its specificity when the substrate is a fragment of 553 nucleotides derived from the 3' end of 28 S rRNA that has the nucleotide A-4324 (15).

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with ethanol, washed with ethanol, and analyzed by electrophoresis as above.

**Determination of the Sequence of Nucleotides in RNA**—The synthetic RNA (35-mer) was prepared with unlabeled nucleotide triphosphates and was incubated (as described above) with 5.9 \times 10^{-7} M a-sarcin. The RNA fragments generated by the toxin were extracted with phenol and made radioactive at the 5' end in a reaction with [\gamma-32P]ATP and T4 kinase but without prior treatment with phosphatase. In these circumstances, only the 14-mer formed as a result of a-sarcin action is radioactive. The latter was purified by electrophoresis in a polyacrylamide gel and the sequence of nucleotides determined by an enzymatic method (17).

**RESULTS AND DISCUSSION**

**Substrate Specificity of a-Sarcin**—The purpose of these experiments was to determine whether the exquisite specificity of a-sarcin (it cleaves only one of almost 7000 phosphodiester bonds in ribosomes (7)) is dependent on the complex ordered structure of 28 S rRNA and the presence of the nucleic acid in a particle containing proteins. A contingent purpose was to establish the minimal substrate on which a-sarcin retains its specificity. To accomplish this, we synthesized a small RNA oligonucleotide (35-mer) using a synthetic DNA template and the phage T7 RNA polymerase. This oligomer has the nucleotide sequence and should have the secondary structure of the domain in eukaryotic 28 S rRNA that is attacked by a-sarcin (G-4325) and by ricin (A-4324) (Fig. 1).

Treatment of the synthetic oligonucleotide with lower concentrations of a-sarcin, i.e. 5.9 \times 10^{-8} or 5.9 \times 10^{-7} M, led to the formation of two fragments suggesting that a single phosphodiester bond had been hydrolyzed (Fig. 2A, lanes 1–3). In contrast, a higher concentration of a-sarcin (5.9 \times 10^{-6} M) led to cleavage of the substrate at all, or nearly all, of the purines (Fig. 2A, lane 4), as is evident from the marked reduction in the amount of substrate present at the origin and the appearance of numerous bands. It is assumed that some of the fragments were so small that they were lost from the gel. This conforms with the observation that higher concentrations of a-sarcin, i.e. higher than is necessary to inactivate ribosomes, led to the hydrolysis of almost every purine in naked 5 S rRNA (8).

The orientation of the two fragments and the site of cleavage of the synthetic RNA by lower concentrations of a-sarcin were reconstructed, in the first instance, from the radioactivity in bands B and C (Fig. 2A). We assume for the moment that a-sarcin has the same specificity with the synthetic substrate as it has on ribosomes. In this instance, the 5' fragment would have 21 nucleotides and would have 6 radioactive phosphates derived from the [\alpha-32P]ATP used in the synthesis of the substrate, whereas the putative 3' 14-mer would have 3 (cf. Fig. 1) and the ratio of radioactivity in the two fragments, B:C, would be 2. The ratio was 2.11 for the radioactivity in the two fragments in lane 2 and 2.01 for those in lane 3.

**Fig. 1. Synthesis of a-sarcin-ricin domain RNA oligonucleotides and a schematic of the products of the hydrolysis of the substrates by the toxins.** The oligoribonucleotides, either a 35-mer or a 20-mer, corresponding to the a-sarcin-ricin domain in 28 S rRNA were made in a transcription reaction using phage T7 RNA polymerase and a synthetic DNA template containing a T7 promoter. The predicted secondary structure of the oligomer (35-mer), based on a proposal for the structure of 28 S rRNA (14), and the fragments expected from treatment of this substrate with a-sarcin (21- and 14-mer) or with ricin followed by addition of aniline at acid pH (20- and 15-mer) are shown. The fragments (13- and 7-mer) anticipated from the treatment of a second synthetic oligonucleotide (20-mer) with a-sarcin are also shown. The arrows designate the sites expected to be affected by a-sarcin (S) and ricin (R). The asterisks indicate radioactive residues.

**Fig. 2. Effect of a-sarcin on synthetic RNA oligonucleotides (35- and 20-mer).** The a-sarcin-ricin domain RNAs were synthesized in a reaction mixture containing [\alpha-32P]ATP and nonradioactive nucleoside triphosphates. In A, the oligomer (0.36 \muM) having 35 nucleotides was incubated for 10 min at 37 °C without a-sarcin (lane 1) or with different concentrations of the toxin: 5.9 \times 10^{-8} M (lane 2); 5.9 \times 10^{-7} M (lane 3); 5.9 \times 10^{-6} M (lane 4). The products of the digestion were separated by electrophoresis. The arrows specify the intact oligoribonucleotide (A), the 5' fragment (B), and the 3' fragment (C) (cf. Fig. 1). An alkaline digest of the substrate was analyzed in lane 5. In B, a radioautograph of the sequencing gel that was used to determine the order of nucleotides in the 14-mer produced by a-sarcin action on the 35-mer. The details of the method used for the analysis are given under "Experimental Procedures" and in the text. Lane 1, the intact oligoribonucleotide (14-mer); lane 2, an alkaline digest of the 14-mer; lane 3, a ribonuclease U2 digest of the 14-mer; lane 4, a ribonuclease T1 digest of the 14-mer. The sequence of nucleotides in the 14-mer is at the right. In C, the effect of a-sarcin on an oligomer of 20 nucleotides that mimics the sequence at the site of attack of 28 S rRNA by the toxin is shown (cf. Fig. 1 for the structure). The concentration of a-sarcin was: 0, lane 1; 2.9 \times 10^{-7} M, lane 2; 5.9 \times 10^{-7} M, lane 3; 2.9 \times 10^{-6} M, lane 4; 5.9 \times 10^{-6} M, lane 5; 1.2 \times 10^{-5} M, lane 6. The arrow designates the 20-mer.
in lane 3 (Fig. 2A). Thus, the B fragment is likely to have 21 nucleotides and to be at the 5' end of the substrate and the C fragment to be the 3' 14-mer. To confirm these deductions a separate experiment was done with a nonradioactive sample of the oligoribonucleotide substrate. After treatment of the 35-mer with a-sarcin the products were made radioactive with viral T4 kinase and [γ-32P]ATP. Only the 5' terminus of the oligoribonucleotide substrate. After treatment of the 3' (15-mer) fragments produced by ricin cleavage of the N-glycosidic bond and subsequent chain scission with aniline.

A smaller substrate was prepared. This one had 20 residues and corresponded to the sequence of nucleotides in the loop in the toxin domain (Fig. 1); this 20-mer lacked the stem but retained the 5'-hydroxyl, whereas fragment B does not (cf. Fig. 1). The radioactive fragment was separated by gel electrophoresis, excised, and the sequence of the 5' terminus was determined to be AGGAACCGCAGG (Fig. 2B). This established that it has in vivo. This is surprising and a contradiction of our previous assumption (8, 13) that the toxin's specificity was contingent on the presence of ribosomal proteins.

This should allow a more precise definition of the sequence of nucleotides and the higher order structure that prescribes the binding of the toxins to the substrate (i.e. the interaction of the proteins with the nucleic acid) and the catalysis of hydrolysis by α-sarcin and ricin.

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