The Mechanism of Action of Ricin and Related Toxins on Eukaryotic Ribosomes

THE SITE AND THE CHARACTERISTICS OF THE MODIFICATION IN 28 S RIBOSOMAL RNA CAUSED BY THE TOXINS*

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Ricin is a potent cytotoxic protein derived from the higher plant *Ricinus communis* that inactivates eukaryotic ribosomes. In this paper we have studied the mechanism of action of ricin A-chain on rat liver ribosomes in vitro. Our findings indicate that the toxin inactivates the ribosomes by modifying both or either of two nucleoside residues, G43,59 and A43,249 in 28 S rRNA. These nucleotides are located close to the 28 S rRNA cleavage site and become resistant to all ribonucleases tested. The examination of the lability of phosphodiester bonds of these nucleotides to both mild alkaline digestion and aniline treatment at acidic pH suggests that the base of A43,249 is removed by the toxin.

This unique activity of ricin A-chain was also observed when naked 28 S rRNA is used as a substrate, indicating that the toxin directly acts on the RNA. Similar activity on 28 S rRNA is also exhibited by abrin and modeccin, ricin-related toxins, suggesting a general mechanistic pathway for ribosome inactivation by lectin toxins.

There is a group of cytotoxic proteins acting on eukaryotic ribosomes including those from fungi (α-sarcin) and higher plants (ricin, abrin, and modeccin). These toxins have been known to catalytically and irreversibly inactivate 60 S ribosomal subunits affecting the activities in peptide elongation reaction (see Ref. 1 for a review). However, the molecular mechanisms of their action have not been elucidated except for the case of α-sarcin, which has been known to hydrolyze a single phosphodiester bond between G43,59 and A43,249 in an evolutionarily conserved region of 28 S rRNA (2–5). Ricin and other plant lectins, e.g. abrin and modeccin, consist of two peptide chains, A and B, linked together by a disulfide bond while α-sarcin is a single peptide (6). The B-chain binds the toxins to receptors on the cell surface, and the A-chain enters the cytoplasm and inactivates the 60 S ribosomes including those from fungi (α-sarcin) and higher plant (7). The B-chain binds the toxins to receptors on the cell surface, and the A-chain enters the cytoplasm and inactivates the 60 S ribosomes including those from fungi (α-sarcin) and higher plant (7). The B-chain binds the toxins to receptors on the cell surface, and the A-chain enters the cytoplasm and inactivates the 60 S ribosomes including those from fungi (α-sarcin) and higher plant (7).

Irrespective of the structural differences, the mode of action of ricin and the related lectins is known to be identical with that of α-sarcin in the following aspects: (i) they affect EF-1’, and EF-2-associated functions of 60 S subunits and (ii) they do not require energy and any cofactors. These lines of evidence suggested to us that they also act on rRNA rather than on ribosomal proteins in 60 S ribosomal subunits like α-sarcin. The possibility that the toxins are endonucleases was once ruled out in 1976 by Mitchell et al. (7) who reported that ricin does not change the sizes of any rRNA species of L cell polysomes in vitro, but more recently it was suggested by Obrig et al. (8) who demonstrated that the lectins, ricin and phytolaccin, and Shiga toxin are able to hydrolyze naked 5 S and 5.8 S rRNAs.

Recently, to test whether ribonuclease activity of ricin, if any, is involved in the inactivation of ribosomes, we determined the sequences of 5’ and 3’ termini of each rRNA species after ricin treatment and found that even 100 times molar excess of the toxin over ribosomes did not hydrolyze any rRNA species both exo- and endonucleolytically, consistent with the results of Mitchell et al. (7) instead, we noticed that 28 S rRNA from ricin-treated ribosomes always migrates more slowly in gel electrophoresis than control and demonstrated that catalytic amounts of ricin and other related toxins modify probably one nucleotide of 28 S RNA located adjacent to the α-sarcin site. The possible reactions catalyzed by the toxin are discussed.

**EXPERIMENTAL PROCEDURES**

Materials—The following enzymes were purchased from Pharmacia P-L Biochemicals: polynucleotide kinase and RNA ligase from the virus T4, ribonuclease T1, ribonuclease U2, ribonuclease from Phasystopharum polycephalum, and ribonuclease from Bacillus cereus. Ribonuclease T1 was obtained from Behring Diagnostics, and [γ-32P] ATP (3000 Ci/mmol) and cytidine-3’-5’-(2’p) diphosphate (3000 Ci/ mmol) were from Amersham Corp. Ricin and abrin were purchased from E. Y. Laboratories, Inc. (CA) and modeccin was from Pierce Chemical Co. Ricin A-chain, the catalytic subunit of ricin, was purified by the method of Olsones (9). Abrin and modeccin were activated with 2-mercaptoethanol before use (10).

Preparation of Ribosomes and Treatment with Toxins—Ribosomes were isolated from rat liver (11) and from *Escherichia coli* strain K-A19 (12); the preparation from rat liver contained mainly polysomes (11, 13). The ribosome preparations were suspended in buffer (25 mM Tris/HCl, pH 7.6, 25 mM KCl, and 5 mM MgCl2) to give a concentration of 150 A260 units/ml. Treatment of rat liver ribosomes (1.5 A260 units) with ricin A-chain (1 ng), abrin (2 ng), or modeccin (20 ng) was carried out in 100 μl of buffer (25 mM Tris/HCl, pH 7.6, 25 mM KCl, 5 mM MgCl2) incubated for 10 min at 37 °C. The activity of ribosomes was measured in poly(U)-directed polyphenylalanine synthesis by the same method as described by Olsones et al. (14) except for a pretreatment of the ribosomes with puromycin KCl to make them run off (15). For the treatment of bacterial ribosomes with ricin A-chain, the toxin amount was raised up 10,000 times higher (10 ng) than that for rat liver ribosomes. The reaction was stopped by the addition of 0.5 ml of 0.5% sodium dodecyl sulfate in 50 mM Tris/HCl, pH 7.6.

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2 The abbreviations used are: EF-1 and EF-2, eukaryotic elongation factors 1 and 2, respectively; α-fragment, the oligonucleotide of 488 bases that is cleaved from the 3’ end of 28 S rRNA by the action of α-sarcin; β-fragment, the polynucleotide of the remaining 5’ end of 28 S RNA after the removal of the α-fragment.

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For the treatment of naked RNA with ricin A-chain, isolated rRNA was incubated with a high concentration of the toxin for a prolonged time period. Total RNA (1.5 A260 units) was dissolved in 100 µl of the same buffer as for ribosomes and incubated with 10 ng of ricin A-chain for 60 min at 37 °C. Denaturation of rRNA was done in 25 mM Tris/ HCl, pH 7.6, by heating at 95 °C for 2 min followed by quick chilling on ice.

Preparation and Analysis of rRNA—RNA was extracted from reaction mixtures with 0.5% sodium dodecyl sulfate and phenol (3) and analyzed by electrophoresis, either on 2.5% acrylamide-0.5% agarose composite gel (16) or on polyacrylamide gels (3).

Preparation of the 3' Terminal Fragment of 28 S rRNA Modified by Ricin A-chain—RNA from ricin A-chain-treated ribosomes was dissolved in buffer (25 mM Tris/ HCl, pH 7.6, 0.1 M NaCl, 0.2 mM EDTA) and layered on a 10-30% linear sucrose gradient in the same buffer. The several species of RNA were separated by centrifugation, either on 2.5% acrylamide-0.5% agarose composite gel (16) or on 20% polyacrylamide gels (3). This modified fragment was localized by brief exposure of the gel to x-ray film, excised from the gel, and eluted from the polyacrylamide (19).

Determination of the Nucleotide Sequences of the Modified Fragment—The sequence at both the 5' and 3' termini of the fragment was determined with an enzymatic method (20, 21). The alkaline digestion was carried out in 40 mM sodium carbonate buffer, pH 9.0, at 90 °C for 15 min or, in some experiments, for 1 min. In some experiments, 5'-labeled RNA was treated with the solution of 1 M aniline/acetate, pH 4.5, at 60 °C for 20 min in the dark according to Peattie (22).

RESULTS AND DISCUSSION

The Effect of Ricin and Related Toxins on 28 S rRNA in Rat Liver Ribosomes—When rat liver ribosomes were treated with ricin A-chain at a molar ratio of 1:960 (ricin:ribosomes), the activity of the ribosomes was decreased by greater than 85% in poly(U)-directed polyphenylalanine synthesis (data not shown), confirming that the toxin catalytically inactivates ribosomes. To gather evidence that RNA is the toxin target, total rRNAs were extracted from the ricin-treated ribosomes and analyzed by composite gel electrophoresis. As shown in Fig. 1A, comparison of electrophoretic mobility of treated (lane 2) versus untreated (lane 1) rRNAs showed identical mobility of 18 S rRNA while a mobility difference between the two 28 S rRNAs was clearly visible, as indicated by the arrow. This small but definitive difference between the two 28 S rRNAs was reproducible from experiment to experiment. The same mobility shift of 28 S rRNA was observed when run-off ribosomes were used as a substrate (data not shown). This finding suggested that ricin A-chain modifies 28 S rRNA which results in slow migration on the gel, without cleaving the RNA. The resistance of RNA cleavage by ricin observed here is in disagreement with the previously reported data (8).

To identify the site of modification on the 28 S rRNA, we searched for a modified rRNA fragment among the many RNA fragments that are normally generated by the contaminating ribonuclease(s) which associate ribosomes during their preparation. A fragment of 550 nucleotides clearly had slower mobility compared to ricin-untreated ribosomes, as shown by an arrow in lane 2 of Fig. 1B. This difference in rate of migration disappeared when both treated and untreated samples were analyzed by gel electrophoresis in the presence of 7 M urea (data not shown). This observation suggested that the mobility shift of the ricin-treated rRNA was due to the possible change in the conformational or chemical modification rather than change in length. Other ricin-related toxins, abrin and modeccin, also resulted in the similarly modified 550-nucleotide fragment as shown in lanes 3 and 4 of Fig. 1B. Thus, it appears that related toxic lectins (ricin, abrin, and modeccin), isolated from different sources, exhibit the same RNA-modifying activity. These results exclude the possibility that the modifying activity is due to a contaminant in the toxin preparation because it is highly unlikely that the same contaminant was associated with these different toxin samples that were isolated from different sources. Furthermore, the 28 S rRNA-modifying activity was exhibited by the purified ricin A-chain but not by the purified B-chain (cell-binding subunit).

Characterization of the Altered Fragment—In order to determine the origin of the modified fragment, we isolated the fragment and determined the nucleotide sequences of both 5' and 3' terminal regions (Fig. 2). Both modified and unmodified 550-nucleotide fragments revealed identical 5' and 3' end sequences by the enzymatic digestion method and we localized this fragment to the 3' terminal 553 nucleotides of the 28 S rRNA (Fig. 2). The occurrence of this fragment in a ribosomal preparation has been reported previously (24). This result indicated that a modified region may be located in the middle portion of the modified fragment. Further nucleotide sequence analysis revealed absence of bands corresponding to G4323 and A4324 in the modified fragment, whereas these bands were present in the unmodified fragments as shown by arrows in Fig. 3A. This striking observation immediately suggested that these two nucleotides have been modified by an unknown enzymatic activity of the ricin A-chain. A possibility of known type of modification in the residue A4324 can be excluded because of its resistance to hydrolysis by ribonuclease T2 which is known to recognize most kinds of modified bases (25) (Fig. 3A, lanes 1 and 2). However, it is unclear about the G4323 residue which remained resistant to hydrolysis by ribonuclease T1 before and after ricin treatment. The higher

![Fig. 1. Analysis by gel electrophoresis of RNAs from toxin-treated rat liver ribosomes.](image-url)
susceptibility to hydrolysis of C4322 by B. cereus ribonuclease of the modified over unmodified fragment (Fig. 3A, lane 10) suggests that, as a result of the modification of either or both G4323 and A4324, the neighboring C residue has been widely exposed to digestion by the ribonuclease.

Since it is possible that modification of either the G4323 or A4324 results in the ribonuclease resistance of both of the nucleotides, we examined the cleavage behavior of the modified fragment by the chemical methods. It is known that various amines and hydroxide ion cleave RNA strand by β-elimination reaction if the base of the nucleoside residue is removed, leaving the aldehyde radical at C1 of ribose (26). As shown in Fig. 3A, lanes 13 and 14, partial alkaline hydrolysis shows stronger radioactive bands corresponding to the G4323 and A4324 residues compared to other nucleotides (as shown by arrowheads). This result suggests that the modification imparts increased lability of the phosphodiester bonds surrounding A4324, since the 5' end of the fragment was radiolabeled. Treatment of the modified and unmodified fragments with aniline at acidic pH according to Peattie (22) also resulted in chain scission at positions apparently corresponding to the G4323 and A4324 residues. This result also indicated that the fragment was sensitive at both phosphodiester bonds surrounding the A4324 residue. If the G4323 residue is also modified, the band of G4323 will appear on the gel by these treatment. These results suggested that the base of A4324 is cleaved leaving the aldehyde radical at C1 of ribose rather than that the bases of both residues are missing or severely damaged.

Examination of the Site of Modification in Total rRNAs of the Toxin-treated Ribosomes—Examination of the total rRNAs for the ricin A-chain-induced modification by aniline treatment showed that only 28 S rRNA was modified (Fig. 4A). Results similar to ricin A-chain modification were observed when α-sarcin, abrin, and modeccin were used as the toxins as shown in Fig. 4A, lanes 1–5. These results demonstrated that 28 S rRNA is the only target RNA for all toxin treated and that in each case the site of modification is located close to the α-sarcin cleavage site. The data presented in Fig. 4B indicate that inactivation of ribosomes by ricin A-chain is specific for eukaryotic ribosomes because rRNA of E. coli ribosomes remained resistant to aniline treatment.
Effect of Ricin A-chain on Naked 28 S rRNA—To understand the mechanism of these toxins and the role of ribosomal proteins in allowing toxins to modify the rRNA, we examined the effect of ricin A-chain on naked 28 S rRNA. The native total rRNA was isolated (27) and incubated with ricin A-chain followed by treatment with aniline. Analysis of the reaction mixture by gel electrophoresis, showed the presence of the same size fragment (553 nucleotides, Fig. 5, lane 2) as generated from the ribosomes on treatment with ricin A-chain. This result clearly shows that the site of modification of the naked rRNA by ricin A-chain is similar to when rRNA is part of the ribosomes. However, the rate and the amount of modification of the naked RNA was slow and far less than the RNA present in the ribosomal particle. This may be partly caused by the fact that the isolated rRNA may have lost some of the native secondary structure resulting in less than quantitative modification of the 28 S rRNA. This study strongly suggested an important role of ribosomal proteins in inducing and maintaining the secondary structure recognized by the ricin A-chain. This suggestion was further supported by the fact that denatured 28 S rRNA was not modified by the ricin A-chain (Fig. 5, lane 3). Furthermore, the 553-nucleotide fragment alone did not serve as a substrate for modification by the ricin A-chain (compare lanes 1, 2, and 4).

The question of the nature of the specific modification by the ricin A-chain was examined next. A previous study dealing with the ricin A-chain-induced inactivation of ribosomes proposed that ricin A-chain acts as a ribonuclease (8). Our data presented above, however, show that the RNA chain remains intact after treatment with ricin A-chain. In other words, ricin A-chain does not catalyze hydrolysis of the RNA but instead modifies a specific nucleotide which renders the surrounding phosphodiester bonds highly susceptible to hydrolysis. The nature of modification could involve removal of adenine from the A324 in a manner, similar to N-glycosidases act, which causes its phosphodiester bonds to be sensitive to chemical hydrolysis. However, rRNA N-glycosidases have not yet been described but ricin A-chain acting as a specific N-glycosidase appears to be a very attractive possibility. These studies strongly suggest that inactivation of eukaryotic ribosomes by toxins such as ricin A-chain occurs by specific modification of the A324 residue of 28 S rRNA. Although the nature of the modification has not yet been established, it is very likely that ricin A-chain acts as a specific N-glycosidase. The results also show that this specific N-glycosidase activity is not nucleotide sequence-specific—because the ricin A-chain target sequence AGUACGAGGAAAC, which is conserved between eukaryotes (rat, yeast, etc.) and prokaryotes (E. coli), is only modified when this sequence is part of the eukaryotic ribosomes. This observation emphasizes the role of the ribosome particles in the toxin-induced inactivation of the ribosomes.

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REFERENCES
Mechanism of Action of Ricin and Related Toxic Lectins