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The mechanism of action of trichosanthin on eukaryotic ribosomes—RNA N-glycosidase activity of the cytotoxin

Jin-song Zhang and Wang-yi Liu*
Shanghai Institute of Biochemistry, Academia Sinica, 320 Yue-yang Road, Shanghai 200031, China

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ABSTRACT

Trichosanthin is a ribosome-inactivating protein from root tubers of *Trichosanthes kirilowii* Maxim. In this paper, the mechanism of action of trichosanthin on eukaryotic ribosomes was studied. A fragment of about 450 nucleotides was released from 28S ribosomal RNA after treatment of rat liver ribosome with trichosanthin and its isolated ribosomal RNAs were treated with aniline. Analysis of nucleotide sequence of 5′ terminus of this fragment revealed that the aniline-sensitive site of the phosphodiester bond was between positions A_{4324} and G_{4325} in the 28S rRNA. Adenine was recovered by ion-exchange column chromatography from the 50% ethanol soluble fraction of the reaction mixture in which rat liver ribosomes were treated with trichosanthin. Thin-layer chromatographic analysis indicated that 1 mol of adenine was released from 1 mol of ribosomes. When the ribosomes were incubated with trichosanthin in the presence of inorganic [^{32}P]phosphate, little incorporation of radioactivity into 28S rRNA was observed, indicating that the release of adenine was not mediated by phosphorolysis. These results demonstrate that trichosanthin inactivates the ribosomes by cleaving the N-C glycosidic bond of adenylic acid at 4324 of 28S rRNA in a hydrolytic fashion.

INTRODUCTION

Ribosome-inactivating protein (RIP) is a group of cytotoxic proteins acting on eukaryotic ribosomes (1–3). Apart from α-sarcin from fungi (4, 5), Shiga toxin and Shiga-like toxin from bacteria (6, 7), most RIPs are from higher plants. RIPs have been known to catalytically and irreversibly inactivate 60S ribosomal subunits by affecting the activities in peptide elongation reaction. Further studies have demonstrated that α-sarcin is a novel ribonuclease which inactivates 60S ribosomal subunits by only hydrolyzing a single phosphodiester bond between the guanosine residue at position 4325 (G_{4325}) and the adenosine residue at position 4326 (A_{4326}) in 28S rRNA (8–10). A group of other cytotoxic proteins, such as ricin, abrin, modeccin, Shiga toxin and Shiga-like toxin, etc. are unusual enzymes, i.e. RNA N-glycosidases (11–14). This group of cytotoxins inactivates 60S ribosomal subunits by hydrolyzing a single N-C glycosidic bond of the adenosine residue at position 4324 (A_{4324}) in 28S rRNA adjacent to the guanosine that α-sarcin attacks. Up to now, about 25 RNA N-glycosidases have been determined (15).

Trichosanthin is an abortifacient plant protein purified from the Chinese medicinal herb Tian-hua-fen, obtained from root tubers of the Chinese medicinal plant *Trichosanthes kirilowii* Maxim (16–18). For a long time, Tian-hua-fen has been used to induce midterm abortion. Recently, the active ingredient has been purified, shown to be a basic protein of MW 27,000 and named trichosanthin. Based on structural and functional properties, the protein belongs to the family of single-chain ribosome-inactivating proteins, which inhibit *in vitro* translation in cell-free systems (17, 19, 20). Recently, it was found that trichosanthin can selectively kill choriocarcinoma cells *in vitro* (21) and has potent inhibitory activity against human immunodeficiency virus (HIV) *in vitro* (22). These effects of trichosanthin can be ascribed to its inhibitory action on protein synthesis (20–22). However, the molecular mechanism of action of trichosanthin on ribosomes has not yet been determined. Casellas *et al.* purified another single-chain ribosome-inactivating protein from the seeds of *Trichosanthes kirilowii*, which they named trichokirin (23). Trichokirin has been proved to be a RNA N-glycosidase (14). In the present paper, we report that trichosanthin is a RNA N-glycosidase, inactivating eukaryotic ribosomes by hydrolyzing the N-C glycosidic bond of adenylic acid at 4324 site in 28S rRNA of rat liver.

MATERIALS AND METHODS

Materials

T4 polynucleotide kinase, bacterial alkaline phosphatase, ribonuclease U_2, ribonuclease T_1 were purchased from Bethesda Research Laboratories Inc., USA. [γ-^{32}P]ATP (~3000 Ci/mmol), [8-^{3}H] adenine (25 Ci/mmol) and KH_2[^{32}P]PO_4, are products of Amersham Corp., USA. Ricin is a product of Sigma, St Louis, USA. Trichosanthin was prepared by the method of Jin *et al.* (16). Rat liver ribosomes were prepared according to the method of Wettstein *et al.* (24).

Preparation of stock solution of ricin A-chain. The ricin A-chain was prepared from ricin by reduction (25). For this purpose, 100 μl of ricin (1.8 mg/ml) was activated by the addition of 100 μl of 10% 2-mercaptoethanol. The mixture was left at room

* To whom correspondence should be addressed

temperature overnight. The next day the solution was stored at 4°C. In the following experiments, ricin A-chain was used without separation from B-chain in this stock solution. The concentration of ricin A-chain in the solution was roughly calculated as 450 ng/μl. The RNA N-glycosidase activity of ricin A-chain in the solution is stable for many months.

Analysis of the ribosomal RNA fragment produced by trichosanthin/aniline treatment

Treatment of ribosomes with ricin A-chain or trichosanthin, extraction of rRNA with phenol/sodium dodecyl sulfate, aniline-induced chain scission and 2.5% acrylamide-0.5% agarose composite gel electrophoresis were performed according to the methods of Endo *et al.* (11, 12).

Sequencing the 5'-terminal nucleotides of the fragment

RNA extracted from cytotoxin-treated ribosomes was treated with aniline and separated by electrophoresis on 5% acrylamide gels (4.75% acrylamide, 0.25% bis-acrylamide, 8 M urea, 89 mM Tris−Borate, pH 8.3, 2 mM EDTA). The RNA bands were visualized by ultraviolet shadowing (26) and the fragments (Figure 1, lanes 3 and 5; arrow) were excised from the gel and eluted by diffusion. The 5'-terminal sequence of each fragment was determined enzymatically (27). The procedure is briefly described as below. The fragment was treated with bacterial alkaline phosphatase and the 5' end was labelled with [γ-^{32}P]ATP and T4 polynucleotide kinase. The radioactive oligonucleotide was separated from contaminants by 5% polyacrylamide gel electrophoresis. The fragment was localized by brief exposure of the gel to X-ray film, excised from the gel and then eluted. The sequence at the 5' terminus of the radioactive fragment was determined.

Determination of adenine released from trichosanthin-treated ribosomes

Rat liver ribosomes were washed with 0.5 M KCl (8). 100 A_{260} units of the washed ribosomes were treated with ricin A-chain (450 ng) or trichosanthin (4500 ng) in 4 ml of 25 mM KCl, 5 mM $MgCl_2$ at 37°C for 30 min. To calculate the recovery of adenine, 0.1 μCi of [8-^{3}H]adenine was added to the reaction mixture as an internal standard prior to the start of incubation. Recovery of the bases in the reaction mixture, identification and quantitative determination of adenine released from ribosomes were performed according to the method of Endo and Tsurugi (12).

Measurement of incorporation of inorganic phosphate into 28S rRNA

Ribosomes were incubated with cytotoxin in the presence of 10^{-4} M KH_2 [^{32}P]PO_4 (1.1×10^8 cpm/μmol). After incubation,

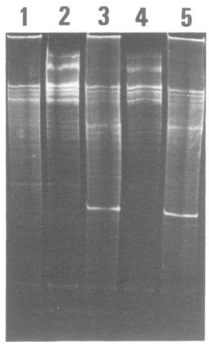

Figure 1. Analysis of rRNA from trichosanthin-treated rat liver ribosomes by gel electrophoresis. Rat liver ribosomes (3.49×10^{-7} M) were incubated with trichosanthin (6.6×10^{-9} M) or ricin A-chain (7.35×10^{-9} M) for 15 min at 37°C. The ribosomal RNA was extracted and separated by 2.5% acrylamide-0.5% agarose composite gel electrophoresis before or after treatment with aniline. Lane 1, aniline without cytotoxin treatment; lane 2, without cytotoxin or aniline treatment; lane 3, ricin A-chain/aniline treatment; lane 4, trichosanthin without aniline treatment; lane 5, trichosanthin/aniline treatment. The arrow denotes the new fragment of about 450 nucleotides produced by aniline cleavage after trichosanthin or ricin A-chain treatment. RNA bands were visualized with ethidium bromide.

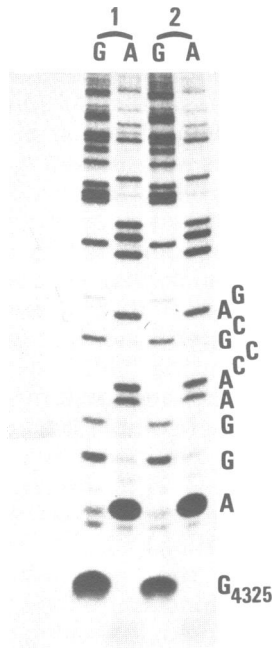

Figure 2. Radioautograph of 20% polyacrylamide sequencing gel of the 5' terminal region of the fragment produced by aniline cleavage after trichosanthin or ricin A-chain treatment on 28S rRNA of rat liver ribosomes. Each fragment (shown with arrow in Figure 1) was isolated by electrophoresis. The RNA was treated with bacterial alkaline phosphatase and labeled at the 5' end with [γ-^{32}P]ATP and T4 polynucleotide kinase. The repurified fragment was partially digested with either ribonuclease T_1 (G) or ribonuclease U_2 (A). Lanes 1 (G and A): 5'-terminal sequence of the fragment produced by trichosanthin/aniline treatment; Lanes 2 (G and A): 5'-terminal sequence of the fragment produced by ricin A-chain/aniline treatment (used as a reference). Nucleotides in 28S rRNA are numbered from its 5' terminal end according to Chan *et al.* (29).

RAT 28S rRNA

Trichosanthin site

5' C U C A G U A C G A G A G G A A C C G C A G 3'

4315 4324 4336

Figure 3. Part of the sequence of rat liver 28S rRNA showing the location of the fragment sequenced in Figure 2, and the position of the adenine catalytically removed by trichosanthin. The nucleotides underlined show the part of G, A sequence obtained in Figure 2. Nucleotides in 28S rRNA are numbered from its 5' terminal end according to Chan *et al.* (29).

ribosomal RNA was extracted with phenol/sodium dodecyl sulfate and a portion of rRNA was applied to agarose gel electrophoresis. The 28S rRNA band visualized with ethidium bromide was excised and the incorporated radioactivity was counted by a liquid scintillation counter. Another part of the extracted RNA was treated with aniline and analyzed by electrophoresis to confirm the complete cleavage of the N-C glycosidic bond of A_{4324}. The amount of inorganic phosphate incorporated into 28S rRNA was calculated from the specific radioactivity of [^{32}P] phosphate.

RESULTS AND DISCUSSION

RNA N-glycosidase activity of trichosanthin

As the mechanism of action of ricin A-chain has been known (11, 12), we can use it as a reference in investigating the mechanism of action of trichosanthin. After incubation of ribosomes with catalytic amounts of ricin A-chain or trichosanthin, the extracted rRNA was analyzed by the composite gel electrophoresis. As seen in Figure 1, when the rRNA from trichosanthin-treated ribosomes was treated with aniline at acidic pH before loading onto the gel, a new fragment of about 450 nucleotides was produced (lane 5, arrow). This fragment did not occur from trichosanthin-treated ribosomes which had not been subjected to aniline-catalyzed hydrolysis (lane 4) or from toxin-untreated ribosomes (lanes 1 and 2). Incubation of ribosomes with or without trichosanthin treatment (lanes 4 and 2) showed identical patterns of band distribution. This indicates that trichosanthin has practically no endonuclease activity. The new fragment was indistinguishable in size from the corresponding fragment produced by ricin A-chain/aniline treatment (lane 3). The susceptibility of RNA to aniline-catalyzed hydrolysis is known to occur at a site in a polynucleotide which is missing a purine or pyrimidine base (28). These results suggest that trichosanthin has RNA N-glycosidase activity as ricin A-chain does. Aniline-catalyzed hydrolysis of treated rRNA should produce two fragments: 5'-terminal fragment and 3'-terminal fragment. Actually, only 3'-terminal fragment was apparently visible in gel (Figure 1, lanes 3 and 5; arrow). The reason may be that contaminating aniline in rRNA samples interfered with the entry of the larger fragment into gel during electrophoresis, resulting in a significant amount remaining at the loading position.

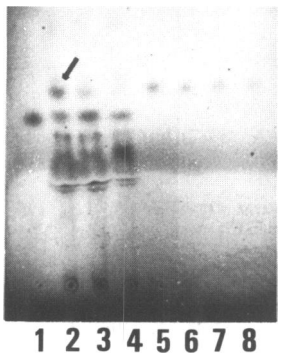

Figure 4. Thin-layer chromatographic identification of the base liberated from the ribosomes by the action of trichosanthin. Bases were isolated from the reaction mixture and separated on a silica gel plate with 1-butanol/methanol/water/ammonia (60/20/20/1) as solvent. Lane 1: standard uracil; Lane 2: bases from trichosanthin-treated ribosomes; Lane 3: bases from ricin A-chain-treated ribosomes; Lane 4: bases from toxin-untreated ribosomes; Lanes 5–8: various amounts of standard adenine. Lanes 5–8 contain 1.85, 1.48, 1.11, 0.74 nmol, respectively. Arrow represents adenine released from the ribosomes by the action of trichosanthin. The ultraviolet absorption band (from lane 1 to 8) below the uracil standard is the impurities from solvent.

Action site of trichosanthin on 28S rRNA

To determine the action site of trichosanthin on 28S rRNA, we have analyzed the 5'-terminal nucleotide sequence of the fragment produced by trichosanthin/aniline treatment. Since the complete sequence of rat 28S rRNA and 5'-terminal nucleotide sequence of the fragment produced by ricin A-chain/aniline treatment are known (29, 11, 12), the 5'-terminal nucleotide sequence of the fragment produced by trichosanthin/aniline treatment can be deduced just from the G, A sequence using the corresponding sequence from ricin A-chain treatment as a reference in sequencing gel, and comparing with the complete sequence of rat 28S rRNA. The fragments (Figure 1, lanes 3 and 5; arrow) were isolated and their 5'-terminal sequences were determined with an enzymatic method. As seen in Figure 2, the 5'-terminal nucleotides of the two fragments have the same G, A sequence. Upon comparing with the complete sequence of rat 28S rRNA, the two fragments occupy the same region of rat 28S rRNA (Figure 3). Thus, the two fragments have identical 5'-terminal nucleotide sequences. The 5' ends of both fragments are G_{4325} of 28S rRNA (Figure 2). These results demonstrate that

Table 1. Search for incorporation of phosphate into 28S rRNA during cleavage of the N-C glycosidic bond of A_{4324}

Reaction conditions	phosphate pmol	phosphate incorporated pmol	modified 28S rRNA pmol	incorporation ratio %
ribosomes, 37°C, 10 min	3.55	–		
ribosomes and 45 ng ricin A-chain, 37°C, 10 min	4.31	0.76	69.8*	1.1
ribosomes and 45 ng trichosanthin, 37°C, 10 min	4.25	0.70	69.8*	1.0

Ribosomes (69.8 pmol) were incubated with trichosanthin or ricin A-chain in the presence of $KH_2[^{32}P]PO_4$ (10^{-4} M, 1.1×10^8 cpm/μmol) in 200 μl of buffer (25 mM Tris–HCl, pH 7.5, 25 mM KCl, and 5 mM $MgCl_2$). The amount of phosphate molecules incorporated into 28S rRNA was calculated from the specific radioactivity of [^{32}P]phosphate. The moles of phosphate incorporated during cleavage of the N-C glycosidic bond were represented after subtraction of those of the toxin-untreated ribosomes (nonspecific adsorption).

*A portion of the extracted rRNA was treated with aniline and analyzed by electrophoresis to confirm the complete cleavage of the N-C glycosidic bond of A_{4324} by the method of Endo *et al.* (11) (data not shown). The result indicated that 69.8 pmol of modified 28S rRNA were produced by either trichosanthin or ricin A-chain treatment.

trichosanthin cleaves the N-C glycosidic bond of A_{4324} in 28S rRNA just as ricin A-chain does, because the β-elimination reaction catalyzed by aniline at acidic pH on the 28S rRNA isolated from trichosanthin-treated ribosomes resulted in chain scission on both 3′ and 5′ sides of A_{4324}, producing pG_{4325} at the 5′ end of the fragment.

Quantitative release of adenine from trichosanthin-treated ribosomes

In order to determine the adenine released from trichosanthin-treated ribosomes, bases were recovered by ion-exchange column chromatography from the 50% ethanol soluble fraction of the reaction mixture in which rat liver ribosomes were treated with trichosanthin. Thin-layer chromatographic analysis showed marked increase of adenine in trichosanthin-treated sample as well as in ricin A-chain-treated sample (Figure 4, lanes 2–4). No other released bases were found in trichosanthin-treated sample. These results provide another evidence that trichosanthin cleaves the N-C glycosidic bond of A_{4324} of 28S rRNA in ribosomes.

To quantify the released adenine from trichosanthin-treated ribosomes, we measured the intensity of the spot of adenine relative to those of standard samples of adenine by densitometry, and calculated the amount of adenine in the reaction mixture making a correction for recovery of adenine. The result showed that trichosanthin released 1.03 mol adenine/mol ribosomes. This value is fairly close to the expected value for cleavage of only one among approximately 7000 N-C glycosidic bonds in eukaryotic rRNAs.

Hydrolytic fashion of trichosanthin on ribosomal 28S RNA

The N-C glycosidic bond of the nucleoside residue in 28S rRNA can be enzymatically cleaved by either phosphorolysis or hydrolysis. In the phosphorolytic mechanism, phosphate should be incorporated into nucleoside residue A_{4324} forming ribose 1-phosphate. In order to determine by which fashion the N-C glycosidic bond of A_{4324} is cleaved by trichosanthin, we measured the amount of inorganic phosphate incorporated into 28S rRNA during cleavage. Ribosomes were treated with trichosanthin or ricin A-chain in the presence of inorganic [^{32}P]phosphate. As shown in Table 1, little incorporation of phosphate into 28S rRNA was observed in either trichosanthin-modified or ricin A-chain-modified 28S rRNA. This demonstrates that trichosanthin cleaves the N-C glycosidic bond by a non-phosphorolytic mechanism as ricin A-chain does.

From the above results, we conclude that trichosanthin inactivates eukaryotic ribosomes by cleaving the N-C glycosidic bond of A_{4324} in 28S rRNA in a hydrolytic fashion.

Existence of aldehyde group in rRNA from trichosanthin-treated ribosomes

RNA N-glycosidase cleaves a special N-C glycosidic bond of 28S rRNA in ribosomes in a hydrolytic fashion and therefore generates a hemiacetal radical in 28S rRNA. Dynamic equilibrium exists between the configuration of hemiacetal and aldehyde group. Aldehyde group can be either reduced to hydroxyl group by $NaBH_4$ or converted to Schiff base by nucleophilic addition reaction with alanine. These reactions break the equilibrium and convert most hemiacetal group into hydroxyl group or Schiff base. If tritium is introduced by [^{3}H]$NaBH_4$ or [^{3}H]alanine, the final products should be radioactive, whereas the unmodified 28S rRNA is not radioactive. The RNA N-glycosidase activity can then be determined quantitatively by counting the radioactivity in the labeled RNA. The modified 28S rRNA could not be labeled if the N-C glycosidic bond were cleaved in a phosphorolytic fashion, producing ribose 1-phosphate which could not react with $NaBH_4$ or alanine. Based on the above principle, Wang *et al.* established a new method to assay the RNA N-glycosidase activity using radioisotope labeling (30). They successfully labeled the rRNA extracted from rat liver ribosomes after trichosanthin or ricin A-chain treatment using either[^{3}H]$NaBH_4$ or [^{3}H]alanine. These results demonstrate the existence of hemiacetal radical in rRNA extracted from trichosanthin-treated ribosomes, indicating that trichosanthin inactivates ribosomes by cleaving a N-C glycosidic bond of rRNA in a hydrolytic fashion, leaving a hemiacetal radical at C_1 of ribose, and thereby provide a circumstantial evidence for results obtained in this paper.

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