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Single-chain ribosome inactivating proteins from plants depurinate *Escherichia coli* 23S ribosomal RNA

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The rRNA *N*-glycosidase activities of the catalytically active A chains of the heterodimeric ribosome inactivating proteins (RIPs) ricin and abrin, the single-chain RIPs dianthin 30, dianthin 32, and the leaf and seed forms of pokeweed antiviral protein (PAP) were assayed on *E. coli* ribosomes. All of the single-chain RIPs were active on *E. coli* ribosomes as judged by the release of a 243 nucleotide fragment from the 3' end of 23S rRNA following aniline treatment of the RNA. In contrast, *E. coli* ribosomes were refractory to the A chains of ricin and abrin. The position of the modification of 23S rRNA by dianthin 32 was determined by primer extension and found to be A_{2660}, which lies in a sequence that is highly conserved in all species.

Ribosome inactivating protein; Depurination; *E. coli*; 23S ribosomal RNA

1. INTRODUCTION

Many plant tissues contain an enzyme (*N*-glycosidase) which catalytically removes a specific adenine residue from a highly-conserved, surface- exposed stem-loop structure present in the large rRNA of the 60S subunit of eukaryotic ribosomes [1,2]. Usually these ribosome inactivating proteins (RIPs) exist as monomers of a molecular weight around 30 kDa (Type 1 or single chain RIPs) [3]. Although single-chain RIPs readily inactivate mammalian ribosomes, they are not cytotoxic because they are unable to bind to and enter cells. In certain instances the RIP (in this case called the A chain) is joined by a single disulphide bond to a second polypeptide (the B chain) which in all examples studied to date is a galactose-binding lectin. These heterodimeric proteins can bind to and enter target cells and are amongst the most potent cytotoxins known (Type II RIPs or cytotoxic lectins) [4].

It has been known for some time that single-chain RIPs or the A chains cytotoxic lectins vary markedly in their ribosomal specificity. Research predominantly utilizing ricin A chain has shown that in general mammalian ribosomes are particularly sensitive, plant ribosomes less so, and, of particular significance to the present report, *E. coli* ribosomes are insensitive [5,6]. This latter finding has led to the widely accepted view that prokaryotic ribosomes are insensitive to plant RIPs. In keeping with this *E. coli* has been successfully used as host for the production of biologically active recombinant ricin A chains [7,8] and abrin A chain [9]. These proteins were produced cytoplasmically where they accounted for up to 10% of the total bacterial protein without affecting bacterial growth. It was therefore surprising when attempts to express the single chain RIP *Mirabilis* antiviral protein (MAP) cytoplasmically in *E. coli* resulted in severely inhibited growth of the host caused by the recombinant product, the yield of which was very low [10]. Subsequently MAP was shown to inhibit protein synthesis of *E. coli* ribosomes, in marked contrast to the effect of ricin A chain [11].

In the present report we show that the effect of MAP on *E. coli* ribosomes is not exceptional and that four other single chain RIPs, the leaf and seed forms of pokeweed antiviral protein (PAP and PAP-S respectively) from *Phytolacca americana* and dianthins 32 and 30 from the leaves of *Dianthus caryophyllus* depurinate 23S rRNA in *E. coli* ribosomes. Furthermore, the depurination site is A_{2660} in helix 90 of domain VI, the homologous base to that removed by RIPs in eukaryotic 26S/28S rRNA. We also confirm the findings of others [4,11] that the catalytic A chains of ricin and abrin are without effect on *E. coli* ribosomes.

2. MATERIALS AND METHODS

2.1. *Materials*

Recombinant ricin A chain [7] was a gift from I.C.I. Pharmaceuticals (Alderley Park, UK). Abrin, from the seeds of *Abrus precatorius*, was purchased from Inland Laboratories (Austin, Texas) and was reduced by incubation in 10 mM β-mercaptoethanol for 1 h before use. PAP was purified from the leaves of *Phytolacca americana* as described by Irvin [12]. Dianthins 30 and 32 from the leaves of *Dianthus caryophyllus* and PAP-S from the seeds of *Phytolacca americana* were gifts from Prof. F. Stirpe. AMV reverse transcriptase was from Life

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Sciences (St. Petersburg, Florida). Oligonucleotides were synthesized on an Applied Biosystems 380 B DNA synthesizer and purified by HPLC.

2.2. *Preparation of ribosomes*

E. coli ribosomes were prepared from mid-exponential cultures of *E. coli* PR-7 (RNase⁻, polynucleotide phosphorylase⁻) [13] as described by Traub et al. [14]. They were resuspended in 25 mM KCl, 5 mM $MgCl_2$, 25 mM Tris-HCl, pH 7.6, at 18 mg/ml and stored at −80°C.

Yeast ribosomes were prepared from the vacuolar protease deficient *S. cerevisiae* ABYS1 [15]. A cell-free extract was prepared as described by Rothblatt and Meyer [16] and centrifuged at 15 000 × *g* for 15 min. The supernatant (1.5 ml) was layered over a 1 ml cushion of 1 M sucrose, 25 mM KCl, 5 mM $MgCl_2$, 25 mM Tris-HCl, pH 7.6, in a 3 ml centrifuge tube and the ribosomes pelleted at 500 000 × *g* and 4°C for 40 min in a Beckman TLA-100.3 rotor. The pellet was resuspended and stored as for *E. coli* ribosomes.

2.3. *Depurination assay*

Reaction mixtures (final volume 20 μl) containing 30 μg of yeast or *E. coli* ribosomes and the amounts of various RIPs indicated in the figure legends in 25 mM KCl, 5 mM $MgCl_2$, 25 mM Tris-HCl, pH 7.6, were incubated for 1 h at 30°C. Control reactions lacking RIPs were similarly incubated. RNA was extracted [17] and dissolved in sterile distilled water at 3 μg/ml. Aniline treatment of the rRNA and electrophoresis in agarose/formamide gels was carried out as described previously [18].

2.4. *Primer extension on* E. coli *23S rRNA*

Oligonucleotide (100 ng) was end-labelled and annealed with 4 μg of RIP-modified or control *E. coli* rRNA in a final volume of 7.5 μl of 50 mM HEPES-KOH, pH 7.0, 5 mM sodium borate and 0.1 M KCl [18]. An aliquot (1 μl) of the annealed mixture was incubated in a final volume of 5 μl containing 50 mM Tris. HCl, pH 8.5, 50 mM KCl, 10 mM dithiothreitol, 10 mM $MgCl_2$, 50 μM of each dNTP and 1 U of AMV reverse transcriptase for 30 min at 37°C [19]. Dideoxy sequencing reactions were performed by primer extension of control rRNA exactly as described by Moazed et al. [19]. Extension reactions were stopped by ethanol precipitation and DNA was electrophoresed on 8% acrylamide, 7.5 M urea TBE gels [20].

3. RESULTS AND DISCUSSION

Recently several single-chain RIPs have been cloned and attempts made to express the recombinant proteins in *E. coli*. We encountered difficulty in expressing PAP (Z. Chen, R.F. White and J.F. Antoniw, unpublished observations) and noted that attempts to express saproin 6 [21] had also been problematical (M. Soria, personal communication). These difficulties were reminiscent of those described earlier in the case of MAP [11]. This prompted us to investigate whether single-chain RIPs could specifically depurinate prokaryotic 23S rRNA in addition to their well established ability to depurinate 26S or 28S rRNA from eukaryotic 80S ribosomes. The specific RIP-mediated depurination of the large ribosomal subunit RNA renders it susceptible to amine-catalysed hydrolysis of the sugar-phosphate backbone at the depurination site, releasing a small fragment of 130–400 nucleotides from the 3' end of the rRNA. This fragment is diagnostic for RIP-catalysed depurination and is readily observed following agarose/formamide gel electrophoresis [18].

Fig. 1 shows an ethidium bromide-stained gel of RNA extracted from yeast and *E. coli* ribosomes incubated with either ricin A chain or dianthin 32. Aniline treatment of rRNA from yeast ribosomes incubated with either RIP at 0.5 μg/ml (corresponding to a ribosome/RIP molar ratio of 22:1) caused the release of a fragment of about 370 nucleotides (Fig. 1, lanes 4 and 6). Stirpe et al. [22] have shown that this fragment, of 367 nucleotides, arises from the removal of A_{3024} in yeast 26S rRNA. rRNA from *E. coli* ribosomes incubated with a very high concentration of ricin A chain (500 μg/ml, corresponding to a ribosome/RIP molar ratio of 0.029:1) showed no diagnostic fragment on aniline treatment (Fig. 1, lane 10). In contrast, the incubation of *E. coli* ribosomes with 5 μg/ml of dianthin 32 gave rise to ca. 240 nucleotide fragment (Fig. 1, lane 12).

Further experiments were carried out to determine the relative activities of ricin A chain and dianthin 32 in depurinating yeast and *E. coli* ribosomes. Under standard assay conditions, 50% of the 26S rRNA in yeast ribosomes was depurinated by 100 pg of both ricin A chain and dianthin 32. In contrast, 50 ng of dianthin 32 was required to cause 50% depurination of 23S rRNA in *E. coli* ribosomes (data not shown). Thus dianthin 32 is some 500-fold less active on *E. coli* ribosomes than on yeast ribosomes.

The removal of A_{2660} from *E. coli* 23S rRNA (corresponding to A_{3024} in yeast 26S rRNA) should result in

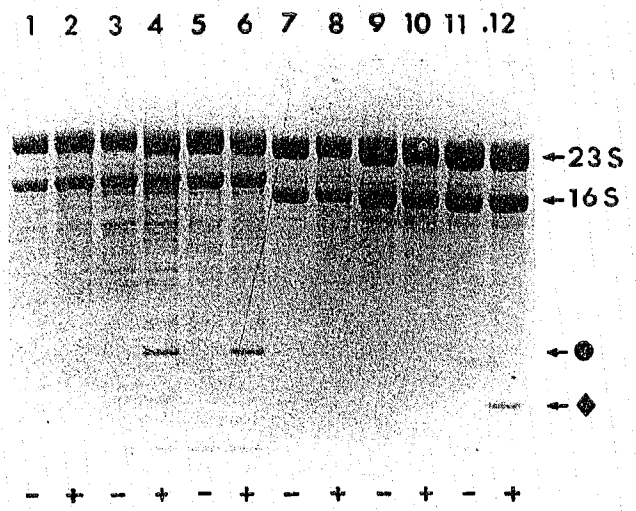

Fig. 1. Effect of ricin A chain and dianthin 32 on RNA from yeast and *E. coli* ribosomes. Ribosomes were incubated without additions or with ricin A chain or dianthin 32 as described in section 2. Following incubation, rRNA was extracted and 3 μg aliquots treated with aniline. These samples together with non-aniline-treated controls were fractionated on a 2.2% agarose/formamide gel and the bands were visualized by ethidium bromide staining. Lanes 1 and 2, untreated yeast ribosomes; lanes 3 and 4, yeast ribosomes incubated with 10 ng ricin A chain; lanes 5 and 6, yeast ribosomes incubated with 10 ng dianthin 32; lanes 7 and 8, untreated *E. coli* ribosomes; lanes 9 and 10, *E. coli* ribosomes incubated with 10 μg ricin A chain; lanes 11 and 12, *E. coli* ribosomes incubated with 100 ng dianthin 32. + indicates aniline treatment; − indicates no aniline treatment; ● and ◆ indicate the fragments released from yeast and *E. coli* ribosomes, respectively, by aniline treatment of modified rRNA.

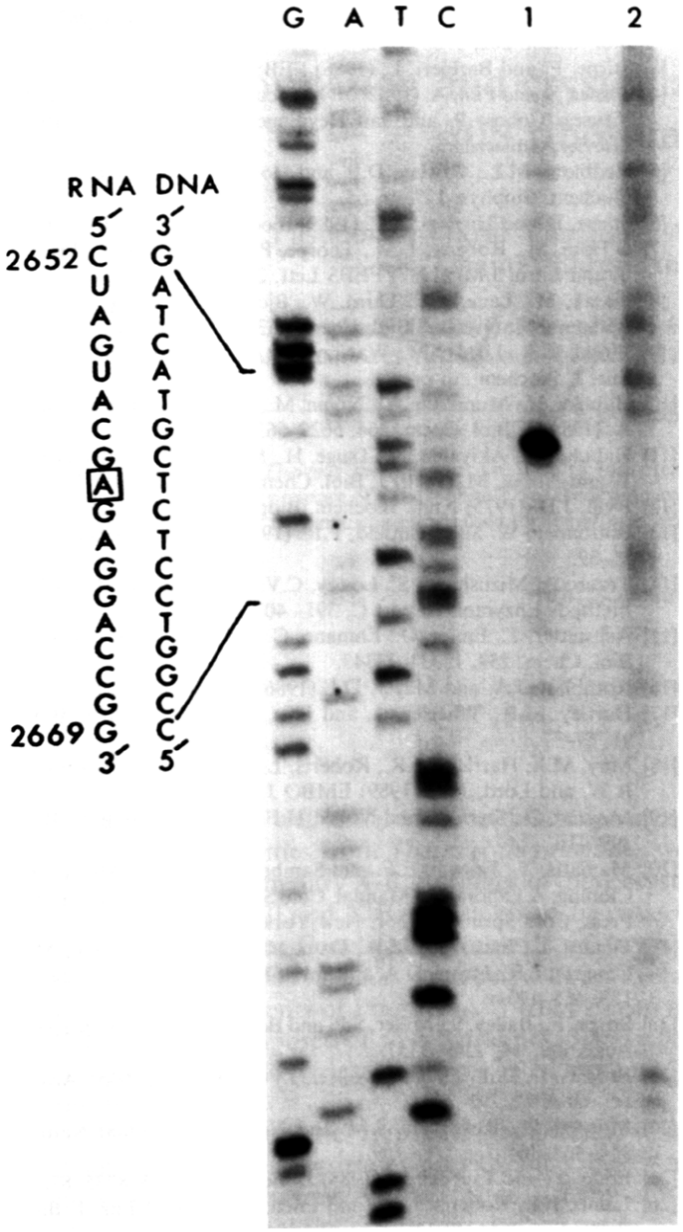

Fig. 2. Location of the dianthin 32 modification site in *E. coli* 23S rRNA by primer extension. *E. coli* ribosomes were incubated with and without the addition of dianthin 32, as described in Fig. 1. The rRNA was extracted, annealed with the oligonucleotide primer and the primer was extended with reverse transcriptase as described in section 2. Lane 1, dianthin 32 modified rRNA; lane 2, untreated rRNA; lanes labelled G, A, T and C refer to sequencing reactions carried out on the unmodified rRNA template in the presence of the respective dideoxynucleotide at a final concentration of 25 μM. The boxed nucleotide in the RNA sequence shows the position of the adenine removed by dianthin 32.

the generation of a fragment of 243 nucleotides on aniline cleavage [23]. The precise position of the modification to *E. coli* 23S rRNA caused by dianthin 32 was determined by primer extension. The radionale for this is that reverse transcriptase is unable to read through the depurination site, generating a band corresponding to the length of the cDNA from the 5' end of the primer to the nucleotide immediately preceeding the modified position. The primer used here was 5'- TGCTTTCAG-CACTTATC-3' which is complementary with the 23S rRNA sequence from G_{2737} to A_{2753} [23]. Fig. 2 shows a sequencing gel of the products of primer extension from unmodified 23S rRNA (lane 2) and dianthin-modified rRNA (lane 1), together with dideoxy sequencing lanes from unmodified rRNA extended from the same primer. The product from the dianthin-modified rRNA shows a strong termination site corresponding to G_{2661} in the RNA sequence, i.e. one nucleotide to the 3' side of A_{2660}, the base in *E. coli* 23S rRNA homologous to A_{3024} in yeast 26S rRNA which is removed by ricin A chain. This fragment is absent when unmodified 23S rRNA is used as template (Fig. 2, lane 2).

The finding that dianthin 32, but not ricin A chain is active in depurinating 23S rRNA in *E. coli* ribosomes prompted us to ask whether such activity is a general feature of single chain RIPs, but not of heterodimeric RIPs. Fig. 3 shows the action of reduced abrin holotoxin (at 300 μg/ml) and PAP, PAP-S and dianthin 30 (each at 5 μg/ml) on *E. coli* rRNA. Abrin was without effect (Fig. 3, lane 1) even though it is around 10-fold more active than ricin on reticulocyte ribosomes (K.A. Wood, personal communication). PAP (Fig. 3, lane 2), PAP-S (Fig. 3, lane 3) and dianthin 30 (Fig. 3, lane 4) all produced diagnostic fragments, although PAP was more active than PAP-S and dianthin 30.

It seems likely that many, possibly most, single chain RIPs can modify *E. coli* and presumably other eubacterial ribosomes. The reason why *E. coli* ribosomes are sensitive to these single chain RIPs but are completely insensitive to the A chains of ricin and abrin is unclear. The active site regions of all these proteins are very

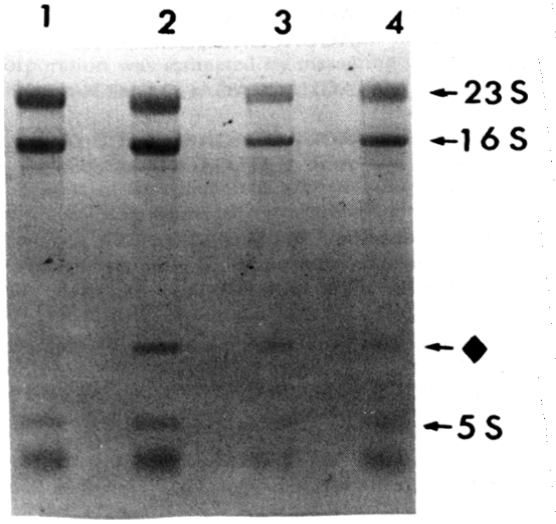

Fig. 3. Effect of abrin, PAP, PAP-S and dianthin 30 on *E. coli* ribosomes. *E. coli* ribosomes were incubated with 4 μg reduced abrin holotoxin (lane 1), 100 ng PAP (lane 2), 100 ng PAP-S (lane 3) or 100 ng dianthin 30 (lane 4) as described in section 2. Following incubation rRNA was extracted and 3 μg aliquots were treated with aniline and then fractionated on an agarose/formamide gel. ◆ indicates the fragment released.

Table I

Active site region comparison of selected RIPs

RIP	Residue positions	Amino acid sequence	Ref.
Ricin A chain	172–185	CIQM I SEAARFQYI	[26]
Abrin A chain	158–171	I I QMVSEAARFRYI	[9]
PAP	170–183	AIQMVSEAARFKYI	[27]
MAP	163–176	AIQMVSEAARFKYI	[10]
Saporin 6	171–184	AIQMTAEAARFRYI	[21]
Dianthian 30	152–165	AIQMTAEAARFRYI	[28]
Trichosanthin	154–167	LI Q STSEAARYK FI	[29]

similar and key active site residues such as E_{177} or R_{180} of ricin A chain are absolutely conserved (Table I). Likewise the nucleotide sequence around the target adenine is highly conserved in 28S, 26S, 23S and chloroplast rRNA (Table II). The adenine residue *N*-glycosidically removed from *E. coli* 23S rRNA by the single chain RIPs is known to be a key binding residue for EF-G and EF-Tu [24]. Although intact *E. coli* ribosomes are resistant to ricin A chain, the target adenine is released from isolated, deproteinized *E. coli* 23S rRNA by high concentrations of ricin A chain [25]. This suggests that ribosomal protein(s) affect the conformation of ribosomes in ways that renders the rRNA either sensitive of refractory to a given RNA *N*-glycosidase.

Table II

Nucleotide sequence in rRNA surrounding the adenine removed by RIPs

Ribosomal RNA	Sequence	Ref.
	↓	
Escherichia coli 23S	AGUACGAGAGGACC 244	[23]
Nicotiana tabacum chloroplast 23S	AGUACGAGAGGACC 133	[30]
Saccharomyces cerevisiae 26S	AGUACGAGAGGAAC 368	[31]
Citrus limon 26S	AGUACGAGAGGAAC 360	[32]
Rattus norvegicus 28S	AGUACGAGAGGAAC 393	[33]

The target site for dianthin 32 on *E. coli* 23S rRNA is shown by the arrow. The number after each sequence gives the distance, in nucleotides, from the target adenine to the 3' end of the rRNA.

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