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RESEARCH COMMUNICATION

Some ribosome-inactivating proteins depurinate ribosomal RNA at multiple sites

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Saporin-S6, a ribosome-inactivating protein (RIP) from *Saponaria officinalis* released more than 1 mol of adenine/mol of ribosomes from house fly (*Musca domestica*) larvae and from rat liver. The release of adenine from rat liver ribosomes by several RIPs (plant enzymes with RNA *N*-glycosidase activity) was examined. Saporins, pokeweed antiviral protein from roots of *Phytolacca americana* (PAP-R), and trichokirin from *Trichosanthes kirilowii* seeds depurinated rat liver ribosomes at more than one site. Up to 33 mol of adenine were released from 1 mol of ribosomes. This property is not common to all RIPS.

INTRODUCTION

Ribosome-inactivating proteins (RIPs) from plants (reviewed in [1]), of either type 1 (single-chain proteins) or type 2 (two-chain proteins, ricin and related toxins), damage ribosomes from eukaryotes and, at higher concentrations, ribosomes from prokaryotes [2–4]. They are RNA *N*-glycosidases which depurinate the major ribosomal RNA at a position corresponding to adenine-4324 of rat liver 28 S rRNA [5,6]. This site of action is a highly conserved loop-and-stem structure, and the conditions necessary for its identification by ricin have been determined by Endo and co-workers [7]. This is the only known site of action of RIPs on eukaryotic ribosomes, although ricin [7] and *Mirabilis* antiviral protein [3] also depurinated naked 16 S *Escherichia coli* rRNA at another site, A-1014.

We report here that saporins (RIPs from seeds, leaves and roots of *Saponaria officinalis*), pokeweed antiviral protein from *Phytolacca americana* roots (PAP-R), and trichokirin from *Trichosanthes kirilowii* seeds depurinate rat liver ribosomes more extensively than do other known RIPs.

MATERIALS AND METHODS

All reagents were of analytical grade and, when possible, RNAase-free. Saporins were purified from seeds (saporins-S), roots (saporins-R) and leaves (saporins-L) of *Saponaria officinalis* according to J. M. Ferreras, L. Barbieri & F. Stirpe (unpublished work; details available on request from the authors), and volkensin was purified according to [8]. All other RIPs were purified as described in [9]. Ribosomes were prepared according to [10] (rat liver) and [11] [*Musca domestica* (housefly) larvae]. Incubation conditions are reported in the legends to Fig. 1 (*Musca domestica* larvae) and Table 1 (rat liver).

Adenine release was determined as described in [12], with minor modifications for rat liver [10] and *Musca domestica* larvae [11], utilizing a reverse-phase Spherisorb C_{18} column (10 μm particle size; 25 cm × 0.46 cm). The h.p.l.c. apparatus was from Kontron Instruments, and was equipped with an SFM 25 spectrophotofluorimeter and an MT 450 data system for chromatography control and data acquisition and analysis.

Analysis of rRNA was performed essentially by the method of Endo *et al.* [5], as in [13]. Ribosomes (15 pmol) were incubated for 10 min at 37 °C in 100 μl of 20 mM-Tris/HCl, pH 7.8, 7.0 mM-magnesium acetate, 100 mM-NH_4Cl and 1 mM-dithiothreithol. RNA was extracted and treated with aniline as described previously [13]. Electrophoresis was carried out on a

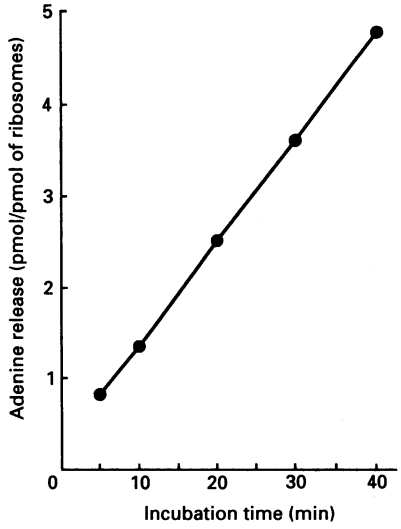

Fig. 1. Time course of adenine release from *Musca domestica* ribosomes

Each sample contained, in a final volume of 100 μl, 55.4 pmol of ribosomes and an equimolar amount of saporin-S6 in 80 mM-Tris/HCl, pH 7.4, 9.9 mM-magnesium acetate, 135 mM-KCl and 2.1 mM-dithiothreitol. Incubation was at 24 °C. The reaction was arrested in ice by addition of 100 μl of 10% (w/v) trichloroacetic acid. Controls were run without ribosomes and with added adenine. Values were calculated by linear regression analysis using a standard curve of adenine added to non-incubated ribosomes.

Abbreviations used: RIP, ribosome-inactivating protein; PAP, pokewood antiviral protein; saporins-S, -R and -L, saporins from seeds, roots and leaves respectively of *Saponaria officinalis*.

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Table 1. Depurination of rat liver ribosomes by RIPs

Adenine release was measured by h.p.l.c. as described in the Materials and methods section. Each sample contained, in a final volume of 100 μl, 25 pmol of ribosomes and the appropriate amount of RIP. Incubation was for 10 min at 37 °C in 20 mM-Tris/HCl, pH 7.8, 7.0 mM-magnesium acetate, 100 mM-NH_4Cl and 1 mM-dithiothreitol. Controls were run without ribosomes and with added adenine. Values were calculated by linear regression analysis using standard curves by adenine added to non-incubated ribosomes. The following RIPs released $\leqslant$ 1 mol of adenine/mol of ribosomes in 10 min at a 1:1 molar ratio of RIP to ribosomes: ricin*, volkensin*†, asparin 2†, barley seed inhibitor†, bryodin†, dianthin 32†, crotins from *Croton tiglium* seeds, curcins from *Jatropha curcas* seeds, gelonin†, RIPs from *Hura crepitans* latex, lychnin†, momordin†, RIPs from *Manihot esculenta*, petroglaucins, and PAP-S†.

RIP	RIP/ribosomes...	Adenine released (pmol/pmol of ribosomes) 1:10	1:1
Saporin-L1		9.2	36.3‡
Saporin-L2		9.2	26.1‡
Saporin-R1		1.3	2.8
Saporin-R2		12.6‡	32.0‡
Saporin-R3		2.4	11.9
Saporin-S5		0.9	1.5‡
Saporin-S6		0.9	2.5‡
Saporin-S8		0.8	1.3§
Saporin-S9		1.2	5.6
PAP-R		1.2	2.7
Trichokirin		1.9	2.0

* Reduced prior to incubation with 50 mM-dithiothreitol, 30 min, 37 °C.
† Incubation time 30 min.
‡ Mean of replicate experiments.
§ Release with an incubation time of 30 min was 2.33 pmol/pmol.

polyacrylamide gel (5 %) using a Sequencing Gel Electrophoresis System Model S2 (Bethesda Research Laboratories). The gel size was 0.4 mm (thickness) × 38.5 cm (height) × 31 cm (width). The gel was pre-electrophoresed for 100 min at 65 W. Samples loaded contained approx. 1 μg of RNA, and electrophoresis was at 65 W for 3 h. A separate gel was run for 1 h to examine low-molecular-mass components. The gels were fixed for 30 min in acetic acid/methanol and stained with silver according to [14].

RESULTS AND DISCUSSION

In the course of previous studies, it was observed that the major form of saporin from seeds (saporin 6; referred to as saporin-S6) released more than equimolar amounts of adenine from the ribosomes of *Musca domestica* if the reaction was continued for more than 10 min. The reaction was linear with time for at least 40 min, indicating that the substrate and other necessary components were not limiting (Fig. 1).

These preliminary observations prompted us to study the effects of RIPs on rat liver ribosomes, a much more well known substrate for RIP action. Among a number of RIPs tested under the present experimental conditions (Table 1), only saporins, PAP-R and trichokirin released $\geqslant$ 2 mol of adenine/mol of ribosomes in 30 min. These RIPs seem to act at different rates in the rat liver ribosome system used in the present experiments. When tested at a 1:10 molar ratio with rat liver ribosomes, the saporins from leaves and one from roots (saporin-R2) released from 1 mol to $\geqslant$ 10 mol of adenine/mol of ribosomes in 10 min. At a 1:1 molar ratio the same RIPs released even more adenine (Table 1). Other saporins, PAP-R and trichokirin released more than 1 mol of adenine/mol of ribosomes in 10 min when tested at 1:1 molar ratio with ribosomes. All other type 1 and type 2 RIPs tested released $\leqslant$ 1 mol of adenine/mol of ribosomes at an RIP/ribosome molar ratio of 1:1. This last finding was consistent with results reported in the literature, i.e. all RIPs tested to date [12,14,15] released not more than 1 mol of adenine/mol of ribosomes.

The RIPs releasing more than 1 mol of adenine/mol of ribosomes were then analysed in more detail. A time-course study at a 1:1 molar ratio of RIP to ribosomes showed the examined RIPs to display different behaviours. The release of adenine by saporin-S6 continued at a linear rate for at least 40 min, whereas in the case of PAP-R and trichokirin the release of adenine was arrested before 20 min had elapsed, after approx. 3 and 2 mol of adenine respectively had been released/mol of ribosomes (Fig. 2). When dianthin 32 was used as a representative RIP releasing less than 1 mol of adenine, the reaction reached a plateau at 10 min, at which time 0.8 mol of adenine had been released/mol of ribosome, and did not proceed any further (Fig. 2).

The concentration-dependence of adenine release by saporin-S6 and saporin-R2 is shown in Fig. 3. These curves are completely different from those obtained with ricin [15], which reached a plateau below 1 mol of adenine/mol of ribosomes; this was not altered by concentrations of ricin several orders of magnitude larger.

The electrophoretic pattern of the RNA extracted from ribosomes incubated for 10 min with various RIPs and treated with aniline showed the appearance of the 'aniline fragment' (Endo's band) observed previously [5,13] (results not shown). RNA extracted from ribosomes treated with saporin-R2 showed four additional fainter bands with higher molecular mass, indicating breaks at multiple points, which appeared stronger after aniline treatment (Fig. 4). These bands were not present in the RNA from ribosomes treated with saporin-S6, which, under the experimental conditions used, releases slightly more than 1 mol of adenine/mol of ribosomes. The confirmed presence in the latter of the Endo's band suggests that adenine at position 4324 is apparently more sensitive to saporin-S6 than other sites. Trichokirin- and PAP-R-treated ribosomes showed an additional band with apparent mobility between 5.8 S and 5.0 S. This band

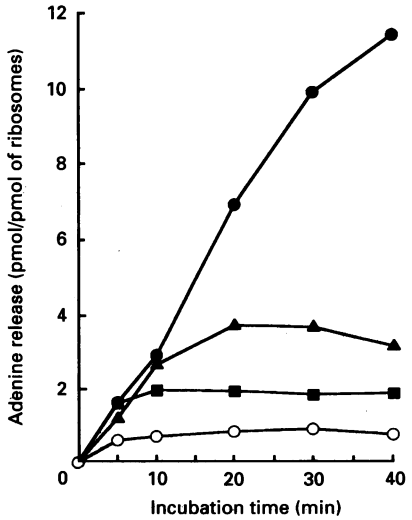

Fig. 2. Time course of adenine release from rat liver ribosomes

Incubation and other experimental conditions are described in the legend to Table 1. ●, Saporin-S6; ▲, PAP-R; ■, trichokirin; ○, dianthin 32.

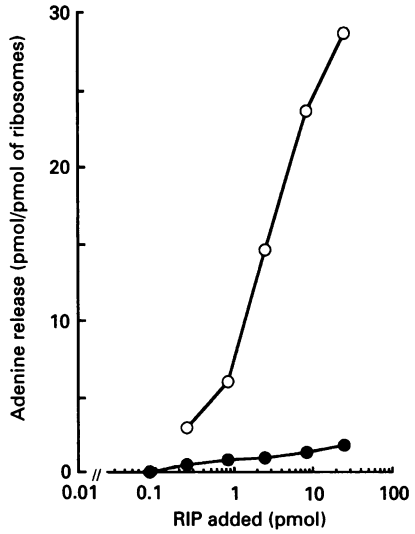

Fig. 3. Dose–response curve of adenine release from rat liver ribosomes

Incubation and other experimental conditions are described in the legend to Table 1. ●, Saporin-S6; ○, saporin-R2.

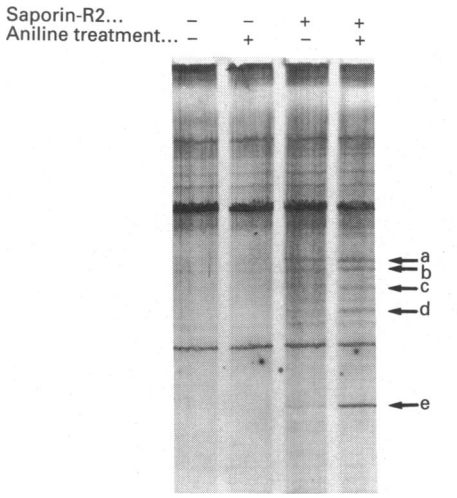

Fig. 4. Electrophoresis of rat liver ribosome RNA

Incubation and other experimental conditions are described in the text. Only the upper part of gel is shown; no appreciable differences were present in the lower parts between treated and untreated samples. a–d are additional high-molecular-mass fragments; e is Endo's band.

was absent from samples treated with saporins or with PAP-S run as control in the same experiment (results not shown).

The differences in the adenine-releasing activity of the examined RIPs were not correlated with their inhibitory activity on cell-free protein synthesis ([1]; J. M. Ferreras, L. Barbieri & T. Girbés, unpublished work). For example, PAP-S and PAP-R had similar activities in a rabbit reticulocyte lysate system, whereas they released 1 and 4 mol of adenine respectively/mol of ribosomes.

Several control experiments were run to check the possibility that the results obtained were due to artifacts. (i) The possibility of a non-specific ribonuclease activity owing to contaminants or to the saporins themselves could be excluded by the pattern of RNA obtained on electrophoresis (a control sample with RNAase A was run). (ii) The experiments were repeated with three different preparations of rat liver ribosomes with comparable results. (iii) Saporin-S6 was further chromatographed by f.p.l.c. on a Superose 12 gel filtration column. The RIP was eluted as a single peak apparently completely free from contaminants, and the activity of the protein in the peak was identical to that of the original preparation (results not shown). (iv) Finally, the chromatographic peak attributed to adenine was further checked and it was confirmed that nucleotides and nucleosides of adenine had different retention times, as reported in the original methodology paper by McCann *et al.* [16]. The techniques used would not allow us to detect the presence, if any, of other bases.

The present results indicate that most type 1 and type 2 RIPs depurinate ribosomes at one site, whereas saporins, PAP-R and trichokirin (and possibly other as yet unknown or untested RIPs) depurinate at more than one site ribosomes from organisms taxonomically distant from each other, such as the rat and *Musca domestica*. The depurinating activity seems to be greater on the adenine at the site originating the fragment identified by Endo rather than at other sites, as shown by the more rapid appearance of Endo's fragment. The capacity for multiple depurination has the following properties: (i) it is not related to the inhibitory activity of the various RIPs on cell-free protein synthesis; (ii) it is not related to the plant species or to the plant tissue from which the RIPs were purified; and (iii) it is exerted on ribosomes from organisms as phylogenetically distant from each other as an insect and a mammal.

The differences in the depurinating activities of RIPs do not coincide with differences in the requirement for cofactor(s) of some RIPs [17]. These functional characteristics, and the peculiar structure of an RIP from maize [18], support the notion [19] that RIPs, initially considered to be functionally identical, are actually different.

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