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Molecular action of the type 1 ribosome-inactivating protein saporin 5 on *Vicia sativa* ribosomes

Rosario Iglesias[a], F. Javier Arias[a], M. Angeles Rojo[a], Cristina Escarmis[b], J. Miguel Ferreras[a] and Tomás Girbés[a]

[a]*Departamento de Bioquímica y Biología Molecular, Facultad de Ciencias, Universidad de Valladolid, E-47005 Valladolid, Spain* and [b]*Centro de Biología Molecular (CSIC-UAM), Canto Blanco, Madrid, Spain*

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The type 1 ribosome-inactivating protein (RIP) saporin 5 isolated from seeds of *Saponaria officinalis* L. strongly inhibited translation carried out by *Vicia sativa* L. purified ribosomes. The toxin multidepurinated *V. sativa* rRNA, which upon treatment with acid aniline releases several RNA fragments including an RNA fragment of approximately 370 nucleotides the 5'-end sequence of which was 5'-GAGGAACG-3'.

Ribosome-inactivating protein; Saporin 5; *Vicia sativa*; Protein synthesis

1. INTRODUCTION

Ribosome-inactivating proteins (RIPs) are plant *N*-glycosidases of unknown biological role that arrest protein synthesis at the level of polypeptide chain elongation [1]. Their toxicity against ribosomes has been demonstrated in mammals, bacteria and fungi and, very recently, in plants ([2–7], unpublished results). The common target for RIPs in mammalian, fungal, protozoan and bacterial ribosomes is an adenine located in a highly conserved rRNA loop [6–9] that is in part responsible for the interaction of both the eukaryotic and prokaryotic elongation factors with the ribosome [1,10]. Very recently it has been shown that the pokeweed antiviral protein (PAP), a RIP from *Phytolacca americana* [11], also modifies plant ribosomes, the rRNA of which releases an RNA fragment of about 330 nucleotides upon treatment with acid aniline [12].

In this work we investigated for the first time in plant ribosomes the molecular action of saporin 5, a type 1 RIP that is a very strong inhibitor of translation carried out by purified cytosolic ribosomes of *Vicia sativa* L. (Leguminoseae), which has been shown to be lacking in endogenous RIP activities [13].

2. MATERIALS AND METHODS

2.1. *Biological materials*

V sativa ribosomes were isolated from a postmitochondrial extract active in translation [13], essentially as described elsewhere [14]. Bacterial ribosomes were purified as described previously [15]. Saporin 5 (SO-5), PAP-S and crotin 2 were prepared from *Saponaria officinalis* L., *Phytolacca americana* and *Croton tiglium* seeds, by a modification of the procedure of Barbieri et al. [16], and were generous gifts from Prof. F Stirpe.

Correspondence address. T. Girbés, Departamento de Bioquímica y Biología Molecular, Facultad de Ciencias, Universidad de Valladolid, E-47005 Valladolid, Spain. Fax: (34) (83) 423 082.

2.2. *Polypeptide synthesis*

Polypeptide synthesis was carried out in reaction mixtures of 50 μl essentially as described previously [13]. Incubation was for 30 min at 30°C. Assessment of the radioactivity incorporated into proteins was performed as described elsewhere [13].

2.3. *Generation of the RNA fragments and electrophoretic analysis of the rRNA*

200 μg of ribosomes from *V. sativa* were incubated with either 5 (SO-5) or 2 (PAP-S) μg of the corresponding RIP for 15 min at 37°C in a reaction mixture of 50 μl of buffer that contained 25 mM Tris-HCl (pH 7.6), 50 mM KCl, 8 mM $Mg(acetate)_2$ and 5 mM DTT. The reaction was stopped by the addition of 2 μl of 0.5 M EDTA (pH 8.0) and 500 μl of 0.5% SDS containing 50 mM Tris-HCl (pH 7.6). RNA extraction, aniline treatment and electrophoresis of rRNA were carried out as described elsewhere [17].

2.4 *5'-End labelling and sequencing of the RNA fragments*

The RNA fragments from *V. sativa* and *E. coli* were isolated by electrophoresis as described above and extracted for 12 h from the crushed gel with a solution containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 5 mM EDTA and 50% phenol. The fragments were then treated with alkaline phosphatase (Boehringer Mannheim) following the supplier's instructions. After elimination of the phosphatase, the RNA was labelled with [γ-^{32}P]ATP and polynucleotide kinase and sequenced as described in [18].

3. RESULTS AND DISCUSSION

V. sativa is an RIP$^-$ plant that displays high sensitivity to type 1 RIPs such as PAP-S from *Phytolacca americana* [13] and petroglaucin from *Petrocoptis glaucifolia* [5]. We investigated the molecular action of saporin 5,

an isoform of the type 1 RIP saporins, on *V. sativa* ribosomes as a model of plant ribosomes.

As illustrated in Fig. 1, saporin 5 was able to inhibit protein synthesis carried out by *V. sativa* ribosomes and encoded by endogenous messengers. The relative IC_{50} was 10 ng/ml (0.34 nM), within the range of values reported previously for other RIPs acting on mammalian translation systems [1]. As a control of ribosomal sensitivity to a type 1 RIP, we assayed the effect of PAP-S and found the same inhibition of translation as was observed for saporin 5 (Fig. 1).

Treatment of the rRNA isolated from RIP-treated ribosomes with acid aniline splits as many RNA fragments from rRNA as depurination sites [9]. In contrast to other RIPs, some of them isolated from *S. officinalis*, saporin 5 promoted the single depurination of mammalian ribosomes [19]. We investigated the effects of saporin 5 on a plant ribosome. Fig. 2A illustrates how the treatment of *V. sativa* ribosomes with saporin 5 and subsequent isolation and treatment of the rRNA with acid aniline generated a collection of RNA fragments, including an approximately 370-mer RNA coincident with the 370-mer RNA released from *V. sativa* rRNA when subjected to the action of pokeweed antiviral protein (PAP-S) (Fig. 2B). This RNA fragment (presumably the almost 365-mer fragment) was released from

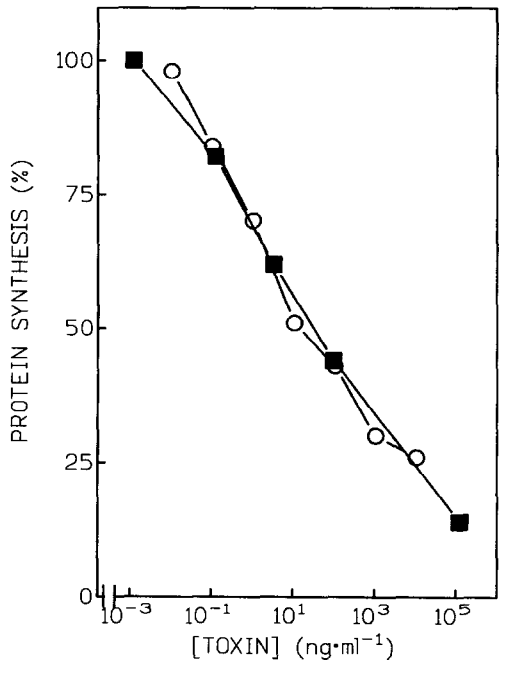

Fig. 1. Effect of saporin 5 and PAP-S on protein synthesis carried out by *V. sativa* ribosomes. Varying concentrations of either saporin 5 (■) or PAP-S (○) were added to *V. sativa* translation mixtures coded by endogenous messengers using 224 nM L-[^{3}H]valine (sp.act. 33 Ci/mmol) as label. Protein synthesis is expressed as the percentage of label incorporated into proteins referred to controls run in the absence of RIP. Control mixtures incorporated 50,500 dpm per mg of protein of cell-free extract.

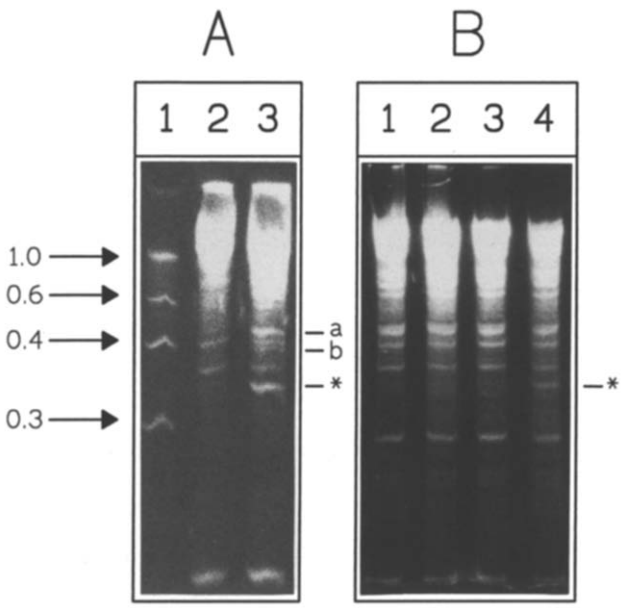

Fig. 2. Effect of saporin 5 and PAP-S on the electrophoretic profile of the rRNA from *V. sativa*. (A) Samples of 6 μg of rRNA either from control (lane 2) or saporin 5-treated (lane 3) ribosomes were subjected to RNA fragment release analysis and then electrophoresed in 5% polyacrylamide gel Lane 1: markers. Large arrows on the left indicate the RNA fragments split by the treatment with acid aniline of isolated rRNA from saporin-treated ribosomes. (B) Samples of 6 μg of rRNA from either control (lanes 1 and 2) or PAP-S-treated (lanes 3 and 4) ribosomes were subjected (lanes 2 and 4) or not (lanes 1 and 3) to the acid aniline treatment and then electrophoresed in 5% polyacrylamide gel Lane 5: markers. In both panels the asterisks indicate the RNA fragment which is diagnostic for RIP action and the small arrows indicate the size of the markers in kb (from top to bottom: 1, 0.6, 0.4, 0.3).

wheat germ and pokeweed rRNA by acid aniline treatment after depurination of wheat germ and pokeweed ribosomes with PAP [12]. The other two major fragments readily visible in the gels were of approximately 450 and 390 nucleotides (Fig. 2A).

We also isolated the 370-mer RNA fragment which, after 5′-dephosphorylation with alkaline phosphatase and 5′-end labelling with [γ-^{32}P]ATP and polynucleotide kinase, was 5′-sequenced with base-specific RNases [18]. As shown in Fig. 3, the 5′-end nucleotide sequence of the RNA fragment is 5′-GAGGAAC-3′. As a control, we also included the diagnostic RNA fragment, 243 nucleotides, released from *E. coli* rRNA by the action of the type 1 RIP crotin 2 from *Croton tiglium* [10]. As also shown in Fig. 3, the *E. coli* RNA terminal sequence (5′-GAGGACC-3′) was the same as that reported very recently using both primer extension [6], and sequencing with RNases [7].

The terminal sequence 5′-GAGGA-3′ is common to all the rRNA fragments from single rRNA targets for known RIPs [1]. Table I shows the sequences of some rRNAs that contain this universal RNA sequence target for RIPs. The sequence is located in the highly conserved rRNA loop which interacts with either the elon-

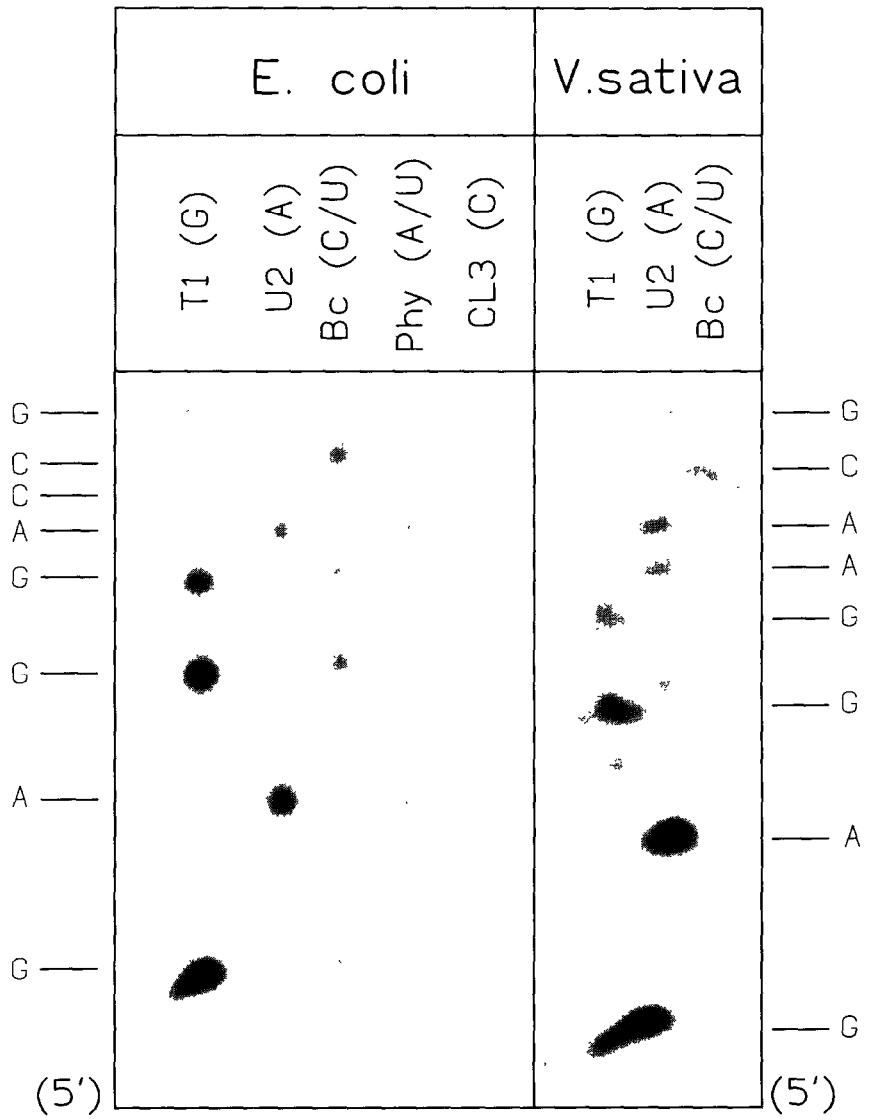

Fig. 3. 5'-end nucleotide sequence of the 370 nucleotide RNA fragment released from *V. sativa* rRNA by the action of saporin 5 and acid aniline. The 370-nucleotide RNA fragment produced by cleavage of *V. sativa* saporin 5-depurinated rRNA with acid aniline was isolated, 5'-labelled and sequenced by the RNases procedure [18]. As a control, we also sequenced the diagnostic RNA fragment released from *E. coli* crotin 2-depurinated rRNA after treatment with acid aniline [10]. RNase T1 (G), RNase U2 (A), RNase from *B. cereus* (C/U), RNase *Physarium* M (A/U), RNase CL3 (C). The digests were separated by electrophoresis on denaturating polyacrylamide gels [18].

Table I

Comparison of the sequence at the site of depurination of plant rRNA by saporin 5 (370-mer RNA), bacterial rRNA by crotin 2 and other rRNAs from different species

Ribosomal RNA	Nucleotide sequence	Ref.
	↓	
E coli fragment (243-mer)	G A G G A C C	
Vicia sativa fragment (370-mer)	G A G G A A C	
E. coli 23 S	A G U A C G A G A G G A C C	[6]
Rat 28 S	A G U A C G A G A G G A A C	[8]
Xenopus 28 S	A G U A C G A A A G G A C C	[21]
Saccharomyces cerevisiae 26 S	A G U A C G A G A G G A A C	[9]
Nicotiana tabacum Chloroplast 23 S	A G U A C G A G A G G A C C	[22]
Citrus limon 26 S	A G U A C G A G A G G A A C	[23]
Rice 25 S	A G U A C G A G A G G A A C	[24]

The arrow indicates the depurination site.

gation factor 2 [1] or G [10]. This is also supported by the fact that the binding of the elongation factor G to *E. coli* ribosomes protected them from the single chain RIP crotin 2-catalyzed depurination of A_{2660} [10].

Our results indicate for the first time that the basic molecular mechanism of ribosome deactivation by RIPs, namely depurination at the highly conserved elongation factor-binding rRNA loop [1], also occurs in the plant kingdom and seems to be a universal feature. Further experiments will be carried out to answer the key question of why, in contrast to mammalian ribosomes [1,2], plant ribosomes display such great variability in their sensitivity to RIPs [5,20]. It is likely that the differences in ribosomal proteins would lead to differential folding and accessibility of the rRNA, in particular the highly conserved loop containing the RNA sequence target for RIPs.

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