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Effects of Ribosome-Inactivating Proteins on *Escherichia coli* and *Agrobacterium tumefaciens* Translation Systems

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The effects of 30 type 1 and of 2 (ricin and volkensin) type 2 ribosome-inactivating proteins (RIPs) on *Escherichia coli* and *Agrobacterium tumefaciens* cell-free translation systems were compared with the effects on a rabbit reticulocyte translation system. The depurinating activity of RIPs on *E. coli* ribosomes was also evaluated. Only six type 1 RIPs inhibited endogenous mRNA-directed translational activity of *E. coli* lysates, with submicromolar 50% inhibitory concentrations. Four RIPs had similar activities on poly(U)-directed phenylalanine polymerization by *E. coli* ribosomes, and three RIPs inhibited poly(U)-directed polyphenylalanine synthesis by *A. tumefaciens* ribosomes, with submicromolar 50% inhibitory concentrations.

Ribosome-inactivating proteins (RIPs) from plants (reviewed in reference 34), either single-chain (type 1 RIPs) or two-chain (type 2 RIPs, i.e., ricin and related toxins), are *N*-glycosidases which hydrolyze the *N*-glycosidic bond of adenine in a highly conserved region of rRNA (7). As a result of this alteration, eukaryotic ribosomes become unable to bind elongation factors, with the consequent arrest of protein synthesis. On the other hand, protein synthesis by prokaryotic ribosomes is not significantly affected by ricin or other RIPs (4, 5, 12, 18, 25, 29), and fully functional transgenic RIPs could be expressed in *Escherichia coli* (10, 24, 31). Nevertheless, at least ricin (8) and *Mirabilis jalapa* antiviral protein (MAP) (14) depurinate naked 23S and 16S *E. coli* rRNAs at A-2660 and at A-1014, respectively, and MAP could not be expressed at high levels in *E. coli* because of the toxicity of the protein to this organism (15). It was then found that MAP inhibited protein synthesis by *E. coli* ribosomes, although at concentrations some 100-fold higher than those affecting eukaryotic ribosomes (13). More recently it was reported that other type 1 RIPs, although at high concentrations, depurinate 23S rRNA of *E. coli* ribosomes (16, 17, 26).

These results prompted us to study in a quantitative manner the activities of several RIPs on three prokaryote translation systems.

Materials. *E. coli* MRE 600 was supplied by J. P. Ballesta, Madrid, Spain. *Agrobacterium tumefaciens* was obtained from the Departamento de Microbiologia, University of Valencia, Valencia, Spain.

RIPs were purified from members of various plant families as follows: Caryophyllaceae, *Dianthus caryophyllus* leaves (dianthins), *Lychnis chalcedonica* seeds (lychnin), and *Saponaria officinalis* leaves (saporin-L proteins), roots (saporin-R), and seeds (saporin-S proteins); Cucurbitaceae, *Bryonia dioica* roots (bryodin-R), *Citrullus colocynthis* seeds (colocins), *Momordica cochinchinensis* seeds (momorcochin-S), *Momordica charantia* seeds (momordin 1), and *Trichosanthes kirilowii* seeds (trichokirin); Euphorbiaceae, *Gelonium multiflorum* seeds (gelonin), *Hura crepitans* latex (*H. crepitans* RIP 5), *Croton tiglium* seeds (crotins), *Jatropha curcas* seeds (curcins), *Manihot utilissima* seeds (manutin 1), and *Ricinus communis* seeds (ricin 60); Poaceae, *Hordeum vulgare* seeds (barley RIP 1); Asparagaceae, *Asparagus officinalis* seeds (asparins); Passifloraceae, *Adenia volkensii* (volkensin); Phytolaccaceae, *Phytolacca americana* in vitro cell cultures (PAP-C), roots (PAP-R), and seeds (PAP-S). Ricin was purified as described by Nicolson et al. (23), volkensin was purified as described by Barbieri et al. (1), and other RIPs were purified essentially as described by Barbieri et al. (3).

L-[^{14}C]Leucine (specific activity, 11.4 GBq/mmol) was from Amersham International, Amersham, United Kingdom, and L-[^{3}H]valine (specific activity, 1.22 TBq/mmol) was from New England Nuclear, Chicago, Ill.

Chloroacetaldehyde was prepared as described by McCann et al. (19). All other reagents were of analytical or molecular biology grade and, when possible, RNase free.

Translation systems. Protein synthesis was determined with a reticulocyte lysate as described previously (35) and with a cell-free system from *E. coli*. From *E. coli* cultures in logarithmic growth polysomes were isolated as described previously (11), and high-speed (100,000 × *g*) supernatant (S-100) was prepared (22) and stored in small portions in liquid nitrogen until used. Reaction mixtures contained the following in a final volume of 50 μl: 50 mM Tris-HCl buffer (pH 7.8), 80 mM NH_4Cl, 10 mM magnesium acetate, 0.05 mM amino acid mixture (minus valine), 1 mM ATP, 0.02 mM GTP, 0.02 mM CTP, 5 mM phosphoenolpyruvate, 1.5 μg of pyruvate kinase, 1 mM dithiothreitol, 81 nM L-[^{3}H]valine, 5 μg of tRNA mixture from *E. coli*, 60 μl of S-100, 40.5 μg of *E. coli* ribosomes. After incubation for 15 min at 37°C, 100 μg of bovine serum albumin and 1 ml of 10% (wt/vol) cold trichloroacetic acid were added and the acid-insoluble radioactivity was evaluated as described previously (11).

The concentration giving 50% inhibition (IC_{50}) was calculated by linear-regression analysis.

Determination of the *N*-glycosidase activity. Adenine released from RIP-treated *E. coli* ribosomes was determined by high-pressure liquid chromatography (20, 37) as described by Barbieri et al. (2). The position of the adenine removed was determined by acid aniline cleavage of depurinated rRNA. To this purpose, *E. coli* or *A. tumefaciens* ribosomes (200 μg) were incubated with 0.6 μg of crotin 2 for 15 min at 37°C in 50 μl of

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TABLE 1. Inhibition of cell-free translation[a]

RIP	IC_{50} (nM) in translation system			
	Rabbit reticulocyte, mRNA directed	*E. coli*		*A. tumefaciens*, poly(U) directed
		mRNA directed	Poly(U) directed	
Crotin 2	0.48	63	80	192
Crotin 3	0.20	13	40	ND[b]
Curcin 1	0.19	>1,000	550	ND
Dianthin 32	0.12	>1,000	>1,000	991
Hura crepitans RIP 5	0.17	450	>1,000	>1,000
Momordin I	0.06	857	190	190
Saporin-R1	0.86	927	>1,000	>1,000
Saporin-R2	0.47	423	>1,000	>1,000

[a] The following RIPs had IC_{50}s of >1,000 nM in all three systems tested: type 1 RIPs, asparin 1, asparin 2, barley RIP 1, bryodin-R, colocin 1, colocin 3, curcin 2, curcin 3, dianthin 30, lychnin, manutin 1, momorchochin-S, momordin I, PAP-C, PAP-R, PAP-S, saporin-L1, saporin-L2, saporin-R3, saporin-S5, saporin-S6, and trichokirin; type 2 RIPs (reduced with 50 mM dithiothreitol for 30 min at 37°C), ricin 60 and volkensin.

[b] ND, not determined.

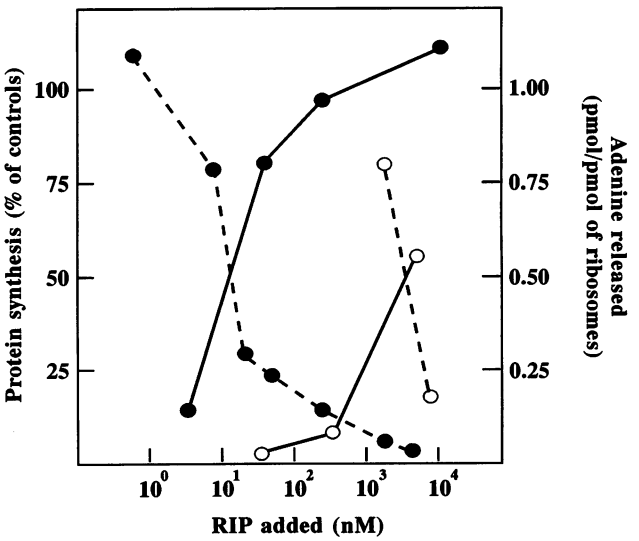

FIG. 1. Effects of dianthin 32 and crotin 3 on *E. coli* ribosomes: adenine release and protein synthesis inhibition. Adenine released (solid lines) and protein synthesis (broken lines) were measured in the presence of crotin 3 (●) and dianthin 32 (○).

40 mM Tris-HCl buffer (pH 7.6) containing 60 mM NH_4Cl and 10 mM magnesium acetate. The reaction was arrested by adding 2 μl of 0.5 M EDTA (pH 8.0) and 500 μl of 50 mM Tris-HCl buffer (pH 7.6) containing 0.5% sodium dodecyl sulfate. The RNA was extracted with phenol and precipitated with ethanol (28), and 6 μg was dissolved in 10 μl of water and incubated in darkness for 10 min at 0°C with 1 volume of 2 M aniline (pH 4.5). The reaction was arrested by dilution with 200 μl of water, aniline was removed by two extractions with ether, and RNA was recovered by precipitation with ethanol. Electrophoresis of rRNA was carried out in 5% acrylamide gels at 21 mA for 40 min as described elsewhere (27).

Sequencing of the 23S rRNA fragment. The rRNA fragments generated from the action of crotin 2 on *E. coli* and *A. tumefaciens* ribosomes were isolated by electrophoresis in a 5% polyacrylamide gel as described above. The RNA fragments (approximately 1 μg of RNA) were extracted by immersion of the crushed gel pieces in a mixture of 1 volume of 50 mM Tris-HCl buffer (pH 7.5) containing 100 mM NaCl and 5 mM EDTA and 1 volume of phenol for 15 h at 4°C, and then the fragments were precipitated with 2 volumes of ethanol at −90°C for 2 h. The RNA was 5′ dephosphorylated with alkaline phosphatase according to the supplier's instructions. After inactivation of the enzyme at 65°C in the presence of 20 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), the RNA was deproteinized with two extractions with phenol and 5′ phosphorylated with polynucleotide phosphorylase and [γ-^{32}P]ATP. The labelled RNA was extracted with phenol, the unincorporated radioactivity was separated by gel filtration through a Sephadex G-50 column, and the 5′-labelled RNA fragment was purified by electrophoresis in an 8 M urea–4% polyacrylamide gel. The largest RNA fragment was eluted as described above and sequenced by partial digestion with RNases as described by Escarmis et al. (9).

Effects on eukaryotic translation systems. Previous reports on the effects of type 1 RIPs on *E. coli* ribosomes led to the hypothesis that possibly most type 1 RIPs can modify *E. coli* and presumably other eubacterial ribosomes (16). Our results indicate that only some RIPs are active on bacterial ribosomes. The activities of RIPs on *E. coli* translation systems varied greatly from one protein to another, the most active being crotin 2 and crotin 3 from *Croton tiglium* seeds (Table 1). There was no correlation between the activities of RIPs on *E. coli* translation systems and those on a mammalian (rabbit reticulocyte) endogenous mRNA-directed translation system, and the effects were not modified by the addition of rabbit reticulocyte lysate supernatant (results not shown) which could sensitize *Artemia salina* ribosomes to several RIPs (32).

It is noteworthy that RIPs which inhibited *E. coli* ribosomes had IC_{50}s lower than those of well-known inhibitors of protein synthesis run as positive controls. Thus, crotin 3 was 2,000-fold more active, on a molar basis, than fusidic acid; 600-fold more than tetracycline; and 15,000-fold more than streptomycin in inhibiting poly(U)-directed phenylalanine polymerization by *E. coli* ribosomes.

The RNA *N*-glycosidase activities of RIPs on *E. coli* ribosomes were consistent with the inhibitory effect on protein synthesis, as indicated by the rate of adenine release. Representative results obtained with RIPs with different activities are given in Fig. 1. In both cases, 50% inhibition of protein synthesis was obtained with the same concentration of RIP which induced release of 0.5 mol of adenine per mol of ribosomes. Saporin-S6 at a 1:1 molar ratio with ribosomes released 3.5 mol of adenine per mol of ribosomes, in this differing from dianthin 32 and crotin 2, which released 1 mol/mol or less. The same phenomenon was observed with rat liver and *Musca domestica* ribosomes (2). Poly(U)-directed polyphenylalanine synthesis by *A. tumefaciens* ribosomes was inhibited only by momordin 1, crotin 2, and, less efficiently, by dianthin 32, with submicromolar IC_{50}s (Table 1).

These results are consistent with those reported by Hartley et al. (16); however, the quantitative measurements of protein synthesis and of adenine released showed that RIPs acted on *E. coli* ribosomes at concentrations much higher than those effective on eukaryotic ribosomes (Table 1). This difference does not seem to be due to rRNA itself since (i) the affected region is highly conserved and (ii) it has been shown that ricin depurinates at the same rate purified rRNA from rat liver or from *E. coli* and an artificial 35-mer (6, 8). In all cases, the concentrations of ricin required to depurinate purified rRNA are much higher than those effective on rat liver ribosomes.

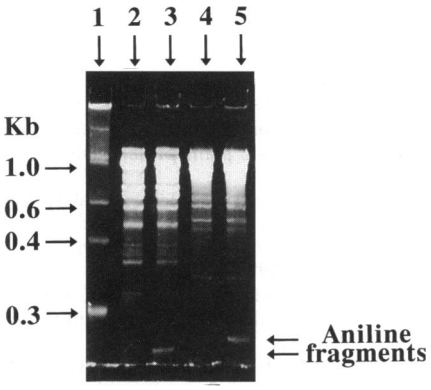

FIG. 2. RNA *N*-glycosidase activity of crotin 2 on ribosomes from *E. coli* and *A. tumefaciens*. rRNA samples were prepared and analyzed by electrophoresis as described in Materials and Methods. Lanes: 1, RNA markers; 2 and 3, ribosomes from *A. tumefaciens* incubated with crotin 2; 4 and 5, ribosomes from *E. coli* incubated with crotin 2. Samples in lanes 3 and 5 were treated with aniline as described in Materials and Methods. Arrows indicate the fragment split by aniline.

Thus the high activity on whole ribosomes seems to be due to ribosomal proteins, which either interact with RIPs in some way or keep rRNA in a conformation which allows the RIPs to act.

Consistent with previous observations with ricin and abrin (25), the type 2 RIPs ricin and volkensin at the highest concentration tested (3.3 μM) did not significantly affect either endogenous RNA-directed or poly(U)-directed reactions by both *E. coli* and *A. tumefaciens* ribosomes.

The rRNA of *E. coli* and *A. tumefaciens* ribosomes was treated with crotin 3 or dianthin 32 and acid aniline to promote the cleavage of the depurinated RNA (7). Treatment with crotin 2 (taken as an example of an active RIP) and acid aniline led to the release of a fragment of approximately 235 nucleotides (Fig. 2). The first 16 nucleotides at the 5′ end of the fragments of *A. tumefaciens* and *E. coli* were determined and compared with those obtained from other RIP-treated ribosomes (Table 2).

The first 9 nucleotides in the fragments released by crotin 2 from both *A. tumefaciens* and *E. coli* rRNA coincided with a nucleotide sequence of the helix 90 of domain VI of the 23S rRNA of *E. coli*. This suggests that the depurination site was the A-2660 of the 23S rRNA of *E. coli* and that the target of crotin 2 on *A. tumefaciens* rRNA was the equivalent of the target in *E. coli*. The 5′-GAGGACC sequence belongs to the highly conserved loop that interacts with elongation factors G and Tu in *E. coli* (21). Moreover, it was shown recently that the binding of elongation factor G to *E. coli* ribosomes as a stable complex with fusidic acid protected ribosomes against the action of crotin 2 (17).

TABLE 2. Comparison of the nucleotide sequence at the site of depurination of rRNA from different species by RIPs

rRNA	Nucleotide sequence[a]	Reference
	*	
A. tumefaciens fragment	GAGGACC	This study
E. coli fragment	GAGGACC	This study
E. coli 23S	AGUACGAGAGGACC	16
Saccharomyces cerevisiae 26S	AGUACGAGAGGAAC	33
Rat liver 28S	AGUACGAGAGGAAC	7
Xenopus oocyte 28S	AGUACGAAAGGACC	30

[a] The asterisk indicates the depurination site.

Although RIPs from the same source can be considered to be isoenzymes in that they catalyze the same reaction, the marked differences in the activities of various RIPs on the same *E. coli* system confirm that these proteins are different from each other (36). These differences exist among RIPs from taxonomically related plants (e.g., crotins and gelonin from members of the family Euphorbiaceae), from the same plant (e.g., saporins from seeds and roots), and even from the same tissue (e.g., dianthins). The observed variations in their effects on *E. coli* ribosomes can be due either to a sort of specificity of the various RIPs for different ribosomes or to the requirement by certain RIPs of some cofactor(s) which may not be present in the *E. coli* system used in the experiments and which must be different from those present in rabbit reticulocyte lysate (32).

Why and how RIPs which have little effect on *E. coli* protein synthesis are toxic to this organism when expressed in it remain to be solved. It is possible that (i) the intracellular concentration in microcompartments builds up to a level sufficient to alter *E. coli* ribosomes (and indeed all RIPs showed some activity at concentrations above 5,000 nM; results not shown) or (ii) that some component(s) of this organism may potentiate the effects of RIPs.

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