Volume 318, number 2, 189–192 FEBS 12153 March 1993

Fusidic acid-dependent ribosomal complexes protect *Escherichia coli* ribosomes from the action of the type 1 ribosome-inactivating protein crotin 2

Rosario Iglesias[a], Cristina Escarmis[b], Carlos Alegre[a], J. Miguel Ferreras[a] and Tomás Girbés[a]

[a]*Departamento de Bioquimica 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*

Received 29 December 1992

The type 1 ribosome-inactivating protein crotin 2 depurinated *Escherichia coli* ribosomes which, upon treatment of the isolated rRNA with acid aniline, released a fragment of around 240 nucleotides whose 5'-end sequence was 5'-GAGGACCGGAGUGGAC-3'. The formation of fusidic acid-dependent ribosomal complexes completely prevented release of the fragment. Ribosomes from crotin 2-pretreated fusidic acid complexes were insensitive to acid aniline. They released the RNA fragment only after a second treatment with crotin 2 and acid aniline whereas unprotected ribosomes released the fragment directly after acid aniline.

Ribosome-inactivating protein; *Escherichia coli*; Protein synthesis; Fusidic acid; 23 S rRNA from *E. coli*

1. INTRODUCTION

Ribosome-inactivating proteins (RIPs) are *N*-glycosidases [1] that depurinate rRNA; they may be classified as single chain (type 1) RIPs and two chain (type 2) RIPs [2]. Old and more recent reports indicate that RIPs inhibit protein synthesis by mammalian, plant, protozoal, fungal and bacterial ribosomes ([2–8], and unpublished results). This was the reason why attempts to clone and express some RIPs in *E. coli* were unsuccessful [6,8]. Inhibitory RIPs depurinate the 23 S rRNA of *E. coli* which, upon treatment with acid aniline, release a fragment of around 240 nucleotides [6,7]. The depurination site was A_{2660} in the helix 90 of domain VI 23 S rRNA [6,7], which is a highly conserved loop [9]. A_{2660} has been shown to be one of the bases that are protected from the attack of dimethylsulphate by fusidic acid-dependent ribosomal complexes containing elongation factor G and GDP [10].

In this work we approached the question of whether fusidic acid-dependent ribosomal complexes might also protect ribosomes from the action of inhibitory RIPs. It is reported for the first time that these complexes afford a stable protection of *E. coli* ribosomes towards crotin 2, a type 1 RIP isolated from *Croton tiglium* which, at low concentration, strongly inhibits translation in *E. coli* ribosomes.

Correspondence address: T. Girbés, Departamento de Bioquimica y Biología Molecular, Facultad de Ciencias, Universidad de Valladolid, E-47005 Valladolid, Spain.

2. MATERIALS AND METHODS

2.1. *Biological materials*

E. coli MRE 600 cell growth and harvesting, grinding with alumina and the preparation of the 30,000 × *g* (S30), 100,000 × *g* (S100) supernatants and purified ribosomes were performed exactly as described elsewhere [11]. Crotin 2 was prepared from *Croton tiglium* seeds by a modification (manuscript in preparation) of the procedure of Barbieri et al. [12].

2.2. *Fusidic acid-dependent complex formation*

For complex formation, *E. coli* ribosomes were incubated at 37°C for 15 min with S100, fusidic acid and GDP in a buffer solution that contained 10 mM Mg_2Cl, 125 mM NH_4Cl, 40 mM Tris-HCl (pH 7.8) and 5 mM dithiothreitol [13].

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

200 μg of ribosomes were incubated with 0.3 μg of crotin 2 for 1 min at 37°C in a reaction mixture of 0.05 ml of buffer that contained 40 mM Tris-HCl (pH 7.6), 125 mM NH_4Cl, 10 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 [14].

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

RNA fragment generated by the action of crotin 2 was isolated by electrophoresis as described above, 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. Thereafter, the fragment was 5'-dephosphorylated with alkaline phosphatase and 5'-end phosphorylated with [^{32}P]ATP and polynucleotide kinase as described previously [15]. The sequence of the 5'-end was performed as indicated elsewhere [15].

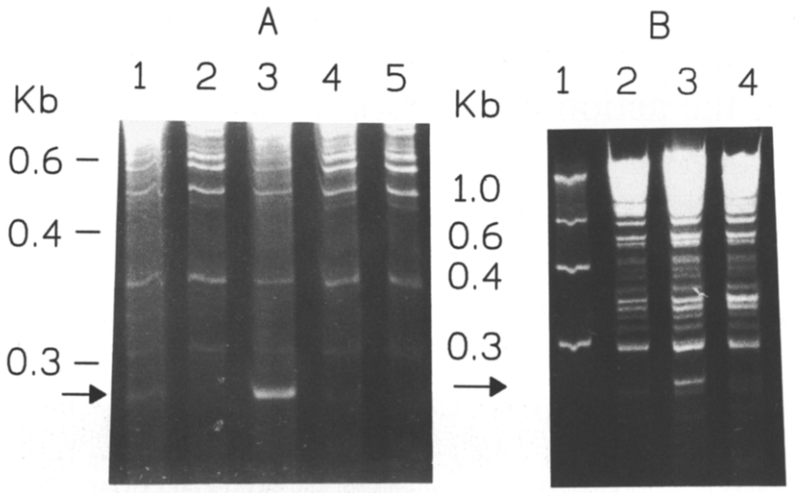
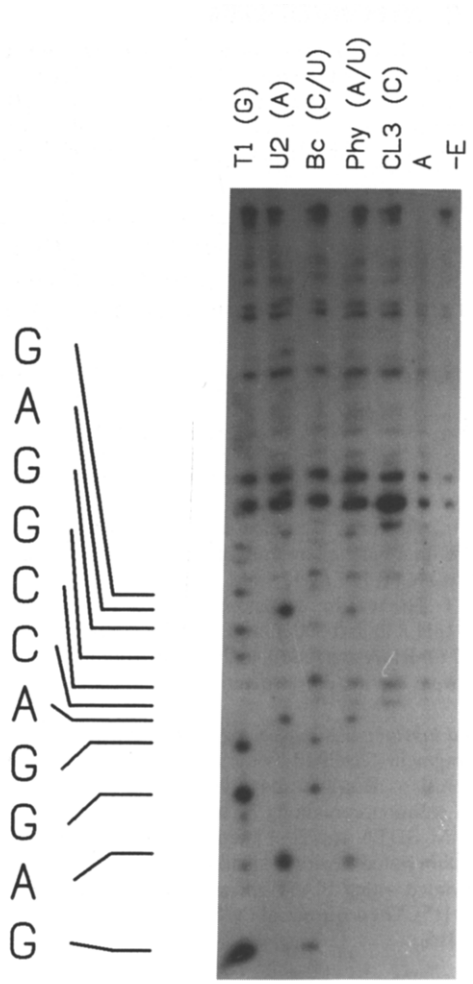


Fig. 1. Effects of crotin 2 and dianthin 32 on the electrophoretic profile of the rRNA from *Escherichia coli*. A: samples of 6 μg of rRNA either from toxin-treated ribosomes (dianthin 32, lanes 1 and 2; crotin 2, lanes 3 and 4) or control (lane 5) were subjected to RNA fragment analysis [14] either without any treatment (lanes 2, 4 and 5) or with acid aniline treatment (lanes 1 and 2). B. lane 1, RNA markers; lane 2, control; lane 3, incubation with crotin 2 and after treatment with acid aniline; lane 4, incubation with 6 mM of fusidic acid and crotin 2 and after treatment with aniline. Pictures in A and B belong to different experiments carried out with different ribosome batches. Arrows indicate the fragment split by the treatment with aniline.



3. RESULTS AND DISCUSSION

Fusidic acid forms ribosomal complexes with elongation factor G (EF-G) and GDP [13], that partially protected several bases of the 23 S rRNA (i.e. G_{2655}, A_{2660}, G_{2661} and A_{2665} in the helix 90 in domain VI) towards dimethyl sulphate [10]. These fusidic-dependent complexes were more stable in the presence of S100 than in its absence [16]. Therefore, since we needed to work with complexes as stable as possible, all experiments were performed with a concentrated S100 as a source of EF-G.

Type 1 RIPs dianthin 32 [6] and crotin 2 (Fig. 1A) promotes the release of a 240 nucleotide RNA fragment from *E. coli* ribosomes. Crotin 2 action was so strong that it was chosen to investigate protection of ribosomes by fusidic acid complex formation against RIPs. By itself, fusidic acid was unable to prevent the release of the fragment from purified *E. coli* ribosomes but when added together with S100 and GDP under conditions of complex formation it completely prevented the release of the fragment (Fig. 1B). Neither GDP nor the S100 alone were able to prevent the release. As also shown

←

Fig. 2. Electrophoretic analysis of partial digestions of the RNA fragment produced by the cleavage of RNA by crotin 2 and acid aniline. RNA labeled at the 5′ end with polynucleotide kinase was partially digested with RNases or with an alkaline buffer [$NaHCO_3/Na_2CO_3$ (pH 9.0)] as detailed elsewhere [15]. Lanes indicate treatment with RNase T1 (G), RNase U2 (A), RNase from *B. cereus* (C/U), RNase Physarum M (A/U), RNase CL3 (C), alkaline buffer and without enzyme, respectively.

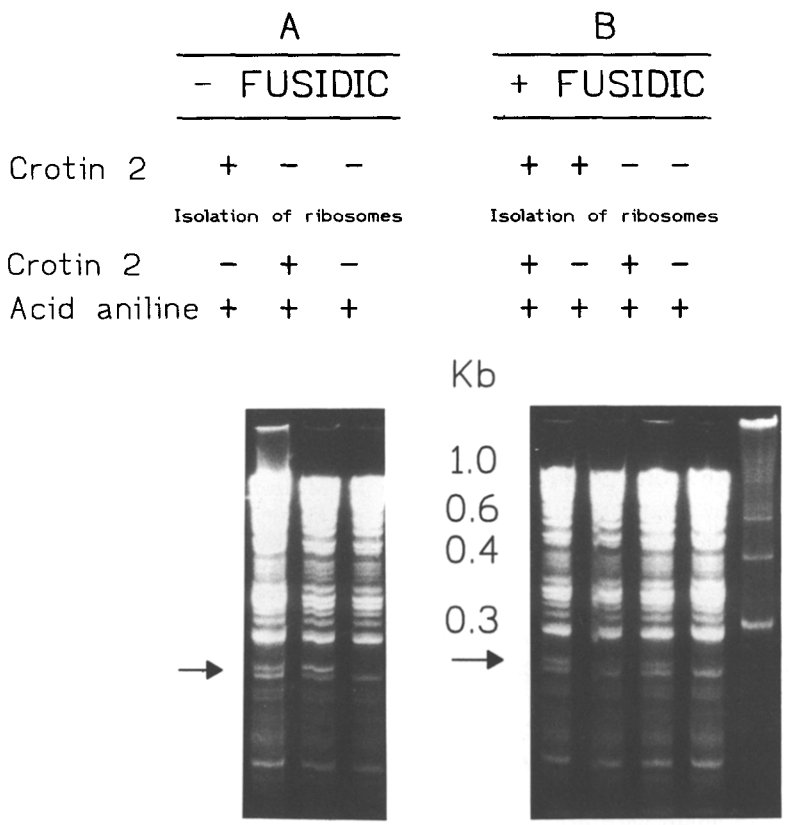


Fig. 3. Prevention by fusidic acid of *E. coli* ribosome depurination by crotin 2 analyzed by RNA electrophoresis. The experiment was performed in two steps. In the first the ribosomes were incubated either without (A) or with (B) fusidic acid with or without crotin 2 as indicated. Thereafter the ribosomes were isolated by centrifugation in the absence of fusidic acid and GDP to promote complex dissociation. In the second step the isolated ribosomes were treated with acid aniline with prior treatment either with or without crotin 2. RNA electrophoresis was carried out as in Fig. 1. In panel B markers are on the right-most lane and numbers indicate the size in kb. The arrow indicates the position of the RNA fragment.

in Fig. 1, this RNA fragment ran in 5% acrylamide electrophoresis as a band coinciding with the band of the RNA fragment released from dianthin 32-treated ribosomes under the same conditions [6], Fig. 1A). This suggested that the modes of action of crotin 2 and dianthin 32 were the same or equivalent. To prove this the diagnostic RNA fragment was isolated, 5′-end labelled and sequenced by the RNases procedure. As shown in

Table I

Comparison of the nucleotide sequence at the site of depurination of RNA from different species by RIPs

Ribosomal RNA	Nucleotide sequence	Ref.
	↓	
Bacteria		
Escherichia coli 23 S	A G U A C G A G A G G A C C	[6]
E. coli fragment isolated here	G A G G A C C	
Plants		
Nicotiana tabacum chloroplast 23 S	A G U A C G A G A G G A C C	[17]
Citrus lemon 26 S	A G U A C G A G A G G A A C	[18]
Rice 25 S RNA	A G U A C G A G A G G A A C	[19]
Yeast		
Saccharomyces cerevisiae 26 S	A G U A C G A G A G G A A C	[20]
Animals		
Rat 28 S	A G U A C G A G A G G A A C	[21]
Xenopus 28 S	A G U A C G A A A G G A C C	[22]

The arrow indicates the depurination site.

Fig. 2, the nucleotide sequence of the fragment's 5'- end is 5'-GAGGACCGGAGUGGAC-3'. Table I shows that this sequence coincides with the highly conserved sequence reported to be the target for RIP action [1]. Therefore, the RNA target for crotin 2 was the A_{2660} of 23 S rRNA and fusidic prevented its release. In order to better assess the protection of ribosomes in the fusidic complexes we incubated ribosomes either complexed or not with fusidic acid with crotin 2. Following this, the ribosomes were isolated by ultracentrifugation under conditions that would favour the dissociation of ribosomal complexes. Next, these ribosomes were subjected to acid aniline treatment with or without a new incubation with crotin 2. As shown in Fig. 3A, crotin-untreated ribosomes were insensitive to acid aniline treatment; when incubated with crotin 2 and acid aniline these ribosomes released the fragment (Fig. 3A). By contrast, crotin-treated ribosomes were sensitive to acid aniline and therefore released the RNA fragment (Fig. 3A). On the other hand, ribosomes complexed with fusidic acid, when reisolated, were unsensitive to acid aniline (Fig. 3B) but sensitive to a new treatment with crotin 2 and subsequent treatment with acid aniline (Fig. 3B). The ribosomes derived from crotin 2-treated ribosomal complexes remained intact since they were insensitive to acid aniline in the second incubation (Fig. 3B). However, when treated again with crotin 2 they released the RNA fragment upon treatment with acid aniline (Fig. 3B). All this indicated that the ribosomes were protected from attack by crotin 2 through the formation of fusidic acid-dependent complexes.

Acknowledgements. This work was supported by grants from CICYT (BI092-0234) and Junta de Castilla y Léon to T.G. The technical assistance of J.E. Basterrechea is greatly appreciated.

REFERENCES

[1] Stirpe, F., Barbieri, L., Battelli, M.G., Soria, M. and Lappi, D.A. (1992) Bio/technology 10, 405–412.
[2] Barbieri, L. and Stirpe, F. (1982) Cancer Surveys 1, 489–520.
[3] Cenini, P., Battelli, M.G., Bolognesi, A., Stirpe, F. and Villemez, C.L. (1987) Biochem. Biophys. Res. Commun. 148, 521–527.
[4] Batelli, M.G., Lorenzoni, E., Stirpe, F., Cella, R. and Parisi, B. (1984) J. Exp. Bot. 35, 882–889.
[5] Arias, F.J., Rojo, M.A., Ferreras, J.M., Iglesias, R., Muñoz, R., Rocher, A., Méndez, E., Barbieri, L. and Girbés, T. (1992) Planta 186, 532–540.
[6] Hartley, M.R., Legname, G., Osborn, R., Chen, Z. and Lord, J.M. (1991) FEBS Lett. 290, 65–68.
[7] Prestle, J., Hornung, E., Schönfelder, M. and Mundry, K. (1992) FEBS Lett. 297, 250–252.
[8] Habuka, N., Akiyama, K., Tsuge, H., Miyano, M., Matsumoto, T. and Noma, M. (1990) J. Biol. Chem. 265, 10988–10992.
[9] Noller, H.F. (1984) Annu. Rev. Biochem. 53, 119–162.
[10] Moazed, D., Robertson, J.M. and Noller, H.F. (1988) Nature 334, 362–364.
[11] Girbés, T., Cabrer, B. and Modolell, J. (1979) Methods Enzymol. 59, 353–362.
[12] Barbieri, L., Stoppa, C. and Bolognesi, A. (1987) J. Chromatogr. 408, 235–243.
[13] Girbés, T., Campuzano, S., Vázquez, D. and Modolell, J. (1977) Eur. J. Biochem. 81, 483–490.
[14] Sallustio, S. and Stanley, P. (1990) J. Biol. Chem. 265, 582–588.
[15] Escarmís, C., Toja, M., Medina, M. and Domingo, E. (1992) Virus Res. 26, 113–125.
[16] Girbés, T., Vázquez, D. and Modolell, J. (1976) Eur. J. Biochem. 67, 257–265.
[17] Takaiwa, F. and Sugiura, M. (1982) Eur. J. Biochem. 124, 13–19.
[18] Kolosha, V.O. and Fodor, I. (1990) Plant Mol. Biol. 14, 147–161.
[19] Takaiwa, F., Oono, K., Iida, Y. and Sugiura, M. (1985) Gene 31, 255–259.
[20] Stirpe, F., Bailey, S., Miller, S.P. and Bodley, J.W. (1988) Nucleic Acids Res. 16, 1349–1357.
[21] Endo, Y., Mitsui, K., Motizuki, M. and Tsurugi, K. (1987) J. Biol. Chem. 262, 5908–5912.
[22] Saxena, S.K., O'Brien, A.D. and Ackerman, E.J. (1989) J. Biol. Chem. 264, 596–601.