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Ribosomal core-particles as the target of ricin

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Core-particles and split-proteins were prepared by treatment with ethanol and NH_4Cl of control and ricin-treated *Artemia salina* ribosomes. No modification of the ricin-treated split-proteins was detected by polyacrylamide-gel electrophoresis. Moreover, the split-proteins from ricin-treated ribosomes complemented control core-particles in poly(U)-directed phenylalanine polymerization. Conversely, ricin-treated core-particles remained totally inactive when supplemented with control split-proteins.

Ricin is a highly toxic protein which inhibits protein synthesis in intact cells and in cell-free systems. It has long been known that the site of action of ricin is the 60S ribosomal subunit (Sperti *et al.*, 1973), which is catalytically and irreversibly inactivated by the toxin (for a review, see Vázquez, 1979). In spite of several efforts, the precise substrate and the nature of the enzymic action of ricin have not yet been clarified. The available data, however, indicate that ricin inhibits protein synthesis by affecting the elongation-factor-dependent GTPase activity of ribosomes (Benson *et al.*, 1975; Sperti *et al.*, 1975).

In this reaction of the elongation cycle, two proteins of the larger ribosomal subunit are clearly involved (Möller, 1974). Treatment with 1M-NH_4Cl and 50% (v/v) ethanol at 0°C selectively removes these two proteins (L7 and L12) from prokaryotic ribosomes (Hamel *et al.*, 1972). The same treatment applied to eukaryotic ribosomes splits a larger number of proteins (Möller *et al.*, 1975; Reyes *et al.*, 1977; van Agthoven *et al.*, 1978), the most acidic of which, coming from the larger subunit, are the eukaryotic counterparts of bacterial proteins L7 and L12 (Möller *et al.*, 1975; Howard *et al.*, 1976; van Agthoven *et al.*, 1977). Reassociation of the split-protein fraction with the residual inactive core-particles restores to a significant extent poly(U)-directed phenylalanine polymerization (Hamel *et al.*, 1972; Reyes *et al.*, 1977; Cox & Greenwell, 1980).

The present paper presents the results of cross-recombination experiments between core-particles and split-proteins obtained from control and ricin-treated ribosomes from *Artemia salina* (brine shrimp). Evidence is presented that the split-proteins are both functionally and structurally unaffected by ricin and that the core-particles are the target of the toxin.

Experimental

Unless otherwise stated, the buffer used was 80mM-Tris/HCl, pH 7.4, containing 120mM-KCl, 7mM-magnesium acetate and 2mM-dithiothreitol (medium A). Ricin was prepared as described by Nicolson & Blaustein (1972) and Nicolson *et al.* (1974). KCl-washed *Artemia salina* 80S ribosomes from undeveloped cysts were prepared as described by Sierra *et al.* (1974). Control ribosomes and ricin-treated ribosomes were prepared by incubating 8nmol of ribosomes in 9ml of medium A in the absence (control ribosomes) or in the presence (ricin-treated ribosomes) of 8nmol of ricin. After 5min at 24°C, the samples were centrifuged at 120000*g* for 45min. The pellets were resuspended with 0.2ml of medium A and passed through Sephacryl S-200 columns (1cm × 3.5cm) to remove any residual ricin. The effluent fractions (0.1ml) were monitored at 260nm and the fractions containing ribosomes were pooled.

Core-particles and split-proteins were prepared by extracting ribosomes with 1M-NH_4Cl and 50% ethanol as described by Hamel *et al.* (1972). A single extraction was performed, at 0°C for 25min. After extraction and centrifugation at 25000*g* for 15min, the supernatants (split-proteins) were equilibrated with 2% (v/v) acetic acid by filtration through Sephadex G-25M columns PD-10 (1.5cm × 5cm) (Pharmacia Fine Chemicals AB, Uppsala, Sweden) and freeze-dried. The precipitates (core-particles) were resuspended in buffer D of Sierra *et al.* (1974) containing 50% (v/v) glycerol, and stored at −20°C. Before protein-synthesis assays glycerol was removed by filtration through Sephadex G-25M columns PD-2 (1cm × 2.5cm) (Pharmacia) equilibrated with medium A. Freeze-dried split-proteins were dissolved in medium A.

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Poly(U)-directed phenylalanine polymerization was carried out as previously described (Montanaro *et al.*, 1978). After 25 min at 24°C, the reaction was stopped and the hot-acid-insoluble radioactivity was measured.

One-dimensional polyacrylamide-gel electrophoresis of split-proteins was carried out at pH 4.5 by the method of Gesteland & Staehelin (1967) on gel slabs (14 cm × 10 cm × 1.5 cm thick) in the model-220 Vertical Slab Electrophoresis cell (Bio-Rad Laboratories, Richmond, CA, U.S.A.).

The NH_4Cl/ethanol-extractable proteins which were eluted unretained from CM-cellulose columns at pH 6.0 were prepared as described by van Agthoven *et al.* (1978) and analysed for charge and molecular weight by two-dimensional polyacrylamide-gel electrophoresis as suggested by O'Farrell (1975). The two-dimensional electrophoresis was performed as described by Barbieri *et al.* (1980), except that Ampholines pH range 4–6 were used in the isoelectric-focusing gel of the first dimension.

An enzyme fraction containing elongation factors ('S-105 supernatant') was obtained by precipitating the *A. salina* postribosomal supernatant in 75%-satd. $(NH_4)_2SO_4$ (Sierra *et al.*, 1974). The concentration of ribosomes was calculated from the A_{260} with the following assumptions (Montanaro *et al.*, 1978): $A^{1\%}_{1cm} = 125$; 1 mg of ribosomes = 250 pmol. The same values were applied to estimate the amount of core-particles. Protein was determined as described by Lowry *et al.* (1951), with bovine serum albumin as standard.

Results and discussion

The core-particles obtained from control ribosomes showed some residual activity when tested for poly(U)-directed polyphenylalanine synthesis. As shown in Table 1, this residual activity was further decreased when ricin was present in the reaction mixture. Recombination of the core-particles with the extracted proteins led to an increase of the activity which was of the same magnitude when the split-proteins came either from control or from ricin-treated ribosomes. The activity of the reconstituted system relative to unextracted ribosomes was in both cases approx. 10%.

The core-particles isolated from the ricin-treated ribosomes displayed a very low activity, which was not further decreased by the presence of ricin in the reaction mixture (Table 1). Moreover, these core-particles could not reconstitute functionally active ribosomes when supplemented with split-proteins isolated from either control or ricin-treated ribosomes. These results clearly indicate that pretreatment of ribosomes with ricin does not affect the ability of split-proteins to co-operate with control core-particles, but it makes the core-particles unable to support the activity of split-proteins.

Polyacrylamide-gel electrophoresis at pH 4.5 separated the split-protein fractions from both control and ricin-treated ribosomes into ten discrete bands, the relative positions and intensities of which were the same in both samples (results not shown). Two-dimensional gel electrophoresis of the acidic proteins of the NH_4Cl/ethanol extract revealed, in both control and ricin-treated samples, two major proteins, with no modification induced by ricin in either isoelectric point (isoelectric focusing in the first dimension) or molecular weight (sodium dodecyl sulphate/polyacrylamide-gel electrophoresis in the second dimension). These results, which indicate the structural integrity of the split-proteins from ricin-treated ribosomes, are in agreement with the finding of Lugnier *et al.* (1976) that ricin does not affect the electrophoretic pattern of total ribosomal proteins. Since prokaryotic ribosomes are resistant to ricin (Olsnes *et al.*, 1973; Greco *et al.*, 1974), the finding that eukaryotic acidic proteins are not the target of the toxin is consistent with data which indicate the prokaryotic proteins L7 and L12 and their equivalent from eukaryotic

Table 1. *Poly(U)-directed phenylalanine polymerization catalysed by core-particles and split-proteins from control and ricin-treated ribosomes*

The reaction mixture contained, in a final volume of 0.25 ml of medium A: 2 mM-GTP, 50 pmol of [^{14}C]phenylalanyl-tRNA, 200 μg of poly(U) and 250 μg of 'S-105 supernatant'. Core-particles (100 μg, equivalent to 25 pmol of ribosomes), split-proteins (9 μg) and ricin (25 pmol) were present where indicated. The results are means of two experiments. Control (3.12 pmol) and ricin-treated ribosomes (3.12 pmol), tested in the same assay system before the ethanol/NH_4Cl extraction procedure, gave 6137 and 52 d.p.m. respectively.

	Core-particles from control ribosomes		Core-particles from ricin-treated ribosomes	
Addition	Ricin absent (d.p.m.)	Ricin present (d.p.m.)	Ricin absent (d.p.m.)	Ricin present (d.p.m.)
None	353	166	108	131
Split-proteins from control ribosomes	639	168	164	101
Split-proteins from ricin-treated ribosomes	609	105	156	106

ribosomes are immunologically cross-reactive (Stöffler *et al.*, 1974; Howard *et al.*, 1976; Leader & Coia, 1978; Wool, 1979) and functionally partially interchangeable (Wool & Stöffler, 1974; Möller *et al.*, 1975). Taken together, these results indicate that, if some change is induced by ricin, it must be looked for in the core-particles and in some component which has undergone a more extensive evolutionary divergence from prokaryotic ribosomes.

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References

Barbieri, L., Zamboni, M., Montanaro, L., Sperti, S. & Stirpe, F. (1980) *Biochem. J.* **185**, 203–210

Benson, S., Olsnes, S., Pihl, A., Skorve, J. & Abraham, K. A. (1975) *Eur. J. Biochem.* **59**, 573–580

Cox, R. A. & Greenwell, P. (1980) *Biochem. J.* **186**, 861–872

Gesteland, R. F. & Staehelin, T. (1967) *J. Mol. Biol.* **24**, 149–155

Greco, M., Montanaro, L., Novello, F., Saccone, C., Sperti, S. & Stirpe, F. (1974) *Biochem. J.* **142**, 695–697

Hamel, E., Koka, M. & Nakamoto, T. (1972) *J. Biol. Chem.* **247**, 805–814

Howard, G. A., Smith, R. L. & Gordon, J. (1976) *J. Mol. Biol.* **106**, 623–637

Leader, D. P. & Coia, A. A. (1978) *Biochem. J.* **176**, 569–572

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Lugnier, A. A. J., Dirheimer, G., Madjar, J. J., Reboud, J. P., Gordon, J. & Howard, G. A. (1976) *FEBS Lett.* **67**, 343–347

Möller, W. (1974) in *Ribosomes* (Nomura, M., Tissières, A. & Lengyel, P., eds.), pp. 711–731, Cold Spring Harbor Laboratory, Cold Spring Harbor

Möller, W., Slobin, L. I., Amons, R. & Richter, D. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 4744–4748

Montanaro, L., Sperti, S., Zamboni, M., Denaro, M., Testoni, G., Gasperi-Campani, A. & Stirpe, F. (1978) *Biochem. J.* **176**, 371–379

Nicolson, G. L. & Blaustein, J. (1972) *Biochim. Biophys. Acta* **266**, 543–547

Nicolson, G. L., Blaustein, J. & Etzler, M. E. (1974) *Biochemistry* **13**, 196–204

O'Farrell, P. H. (1975) *J. Biol. Chem.* **250**, 4007–4021

Olsnes, S., Heilberg, R. & Pihl, A. (1973) *Mol. Biol. Rep.* **1**, 15–20

Reyes, R., Vázquez, D. & Ballesta, J. P. G. (1977) *Eur. J. Biochem.* **73**, 25–31

Sierra, J. M., Meier, D. & Ochoa, S. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 2693–2697

Sperti, S., Montanaro, L., Mattioli, A. & Stirpe, F. (1973) *Biochem. J.* **136**, 813–815

Sperti, S., Montanaro, L., Mattioli, A. & Testoni, G. (1975) *Biochem. J.* **148**, 447–451

Stöffler, G., Wool, I. G., Lin, A. & Rak, K. H. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 4723–4726

van Agthoven, A. J., Maassen, J. A. & Möller, W. (1977) *Biochem. Biophys. Res. Commun.* **77**, 989–998

van Agthoven, A., Kriek, J., Amons, R. & Möller, W. (1978) *Eur. J. Biochem.* **91**, 553–565

Vázquez, D. (1979) *Mol. Biol. Biochem. Biophys.* **30**, 67–78

Wool, I. G. (1979) *Annu. Rev. Biochem.* **48**, 719–754

Wool, I. G. & Stöffler, G. (1974) in *Ribosomes* (Nomura, M., Tissières, A. & Lengyel, P., eds.), pp. 417–460, Cold Spring Harbor Laboratory, Cold Spring Harbor