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Mechanism and site of action of a ribosome-inactivating protein type 1 from *Dianthus barbatus* which inactivates *Escherichia coli* ribosomes

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A single chain ribosome-inactivating protein with RNA *N*-glycosidase activity, here named Dianthin 29, was isolated from leaves of *Dianthus barbatus* L. Incubation of intact *Escherichia coli* ribosomes with Dianthin 29 and subsequent aniline treatment of the isolated rRNA releases a rRNA fragment of 243 nucleotides from 23 S rRNA. Nucleotide sequence studies showed that the site of *N*-glycosidic bond cleavage is at A-2660 within the universally conserved sequence 5'-AGUACGAGAGGA-3' near the 3'-end of 23/28 S rRNAs. To our knowledge, Dianthin 29 is the first ribosome-inactivating protein which is shown to inactivate intact prokaryotic ribosomes in the same manner as eukaryotic ribosomes.

Escherichia coli 23 S rRNA; Ribosome-inactivating protein; RNA *N*-glycosidase; Dianthin 29

1. INTRODUCTION

Ribosome-inactivating proteins (RIPs) from plants are well known inhibitors of protein synthesis [1,2]. They act as highly specific RNA *N*-glycosidases by cleaving a single *N*-glycosidic bond between adenine and ribose within a highly conserved target domain in 25/28 S RNAs of eukaryotic ribosomes [3,4]. As a consequence translation is blocked at the step of GTP-dependent binding of elongation factor 1 and/or 2 [5,6] to ribosomes. RIPs can be devided into two groups: single chain type 1 RIPs like PAP (Phytolacca Antiviral Protein) from *Phytolacca americana* L. [7], or type 2 RIPs, consisting of an A (active) chain and a B (binding) chain with lectine properties, like Ricin from seeds of *Ricinus communis* L. [8]. Until recently it seemed to be a general characteristic of RIPs that they fail to inactivate intact *E. coli* ribosomes [2]. However, Endo and Tsurugi [9] demonstrated that Ricin A acts on naked *E. coli* 23 S rRNA with the same specificity as it does on intact rat liver ribosomes. It was concluded that ribosomal protein(s) might protect the target domain in 23 S rRNA from RIP-activity [10]. Additionally, Habuka et al. showed that a RIP type 1 isolated from roots of *Mirabilis jalapa* L. was able to inhibit cell-free protein synthesis in an *E. coli* system at high concentration [10], though the precise mechanism of inhibition was not elucidated. In the present study, the inactivation of eukaryotic ribosomes by Dianthin 29 as well as the mode of action on intact *E. coli* ribosomes is shown.

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2. MATERIALS AND METHODS

Dianthin 29 was isolated and purified from frozen leaf material by affinity chromatography on Blue 2 S-Sepharose [11] (Pharmacia LKB) and subsequent cation exchange chromatography on MONO S HR5/5 (Pharmacia LKB) with a NaCl gradient elution. Purity and M_r of the isolated protein were analysed by SDS-PAGE. *E. coli* ribosomes were isolated from strain B [12]. Ricin A was purchased from Sigma. Inhibition of cell-free protein synthesis was measured in a rabbit reticulocyte lysate (Boehringer, Germany) as recommended by the suppliers. Incubation of *E. coli* ribosomes and rabbit reticulocyte lysate with Dianthin 29 and Ricin A, extraction of the rRNA and treatment with aniline at acidic pH to induce chain scission at any modified site was done as described elsewhere [13]. The rRNA was analysed on 3.5% polyacrylamide–7 M urea gels. The rRNA fragment obtained after incubation of *E. coli* ribosomes with Dianthin 29 and aniline treatment of the isolated rRNA was eluted from the gel by diffusion. The 5'-end of the rRNA fragment was labeled with [^{32}P]γ-ATP and T4-polynucleotidkinase [14]. Sequence analysis of the labeled fragment was done by the enzymatic method [15–17].

3. RESULTS AND DISCUSSION

Dianthin 29 was obtained by the final chromatography step on MONO S HR5/5 as a single protein as judged by SDS-PAGE (Fig. 1). From comparison to marker proteins its M_r is ca. 29,000. With reference to the already known RIPs Dianthin 30 and Dianthin 32 from *Dianthus caryophyllus* L. [18], the name Dianthin 29 is proposed for the described protein.

Cell-free protein synthesis in a rabbit reticulocyte lysate is inhibited by Dianthin 29 with an IC_{50} value (concentration at which protein synthesis is reduced by 50%) of 1.5 nM (Fig. 2). In order to test Dianthin 29 for RNA *N*-glycosidase activity rabbit reticulocyte lysate was incubated with the protein. Subsequent aniline treatment of the isolated rRNA revealed a fragment of approximately 400 nucleotides in length (Fig. 3a, lane 4). The same result was obtained with Ricin A as a

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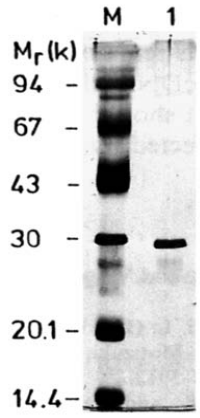

Fig. 1. Sodium dodecylsulfate gel electrophoresis of Dianthin 29. The gel was loaded with 1 μg Dianthin 29 (lane 1) and electrophoresis run at 25 mA for 1.5 h followed by silver staining. (Lane M) molecular size markers.

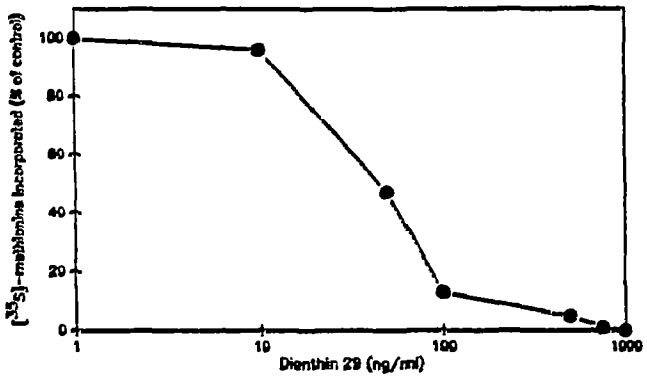

Fig. 2. Inhibition of in vitro translation by Dianthin 29. To reticulocyte lysate translation mixtures with TMV-RNA as messenger and L-[^{35}S]methionine, varying amounts of Dianthin 29 were added. After incubation (1 h, 37°C) aliquots were spotted on filter paper (3MM) and TCA-precipitated. Protein synthesis is expressed in % of [^{35}S] incorporation against a control (100%) without Dianthin 29.

control, indicating that both proteins act by the same enzymatic mechanism (Fig. 3a, lane 2).

From those examined so far, an adenosine residue in a conserved domain of 25/28 S rRNAs is the target of all RIPs from plants. This conserved sequence is also present in *E. coli* 23 S rRNA, where A-2660 corresponds in terms of neighbouring sequences to the RIP-sensitive A-4324 in the 28 S rRNA of rat. Provided that modification of *E. coli* 23 S rRNA by Dianthin 29 occurs at A-2660, aniline treatment of isolated rRNA should yield a fragment of 243 nucleotides in length from Dianthin 29 treated *E. coli* ribosomes, because the full length of *E. coli* 23 S rRNA is 2904 nucleotides. As expected, Ricin A has no effect in vitro on complete *E. coli* ribosomes (Fig. 3b, lanes 1 and 2), while Dianthin 29 gives raise to a new rRNA fragment of about 250 nucleotides in length by aniline treatment of the rRNA (Fig. 3b, lanes 3 and 4). To confirm the supposition described above, the newly formed fragment was iso-

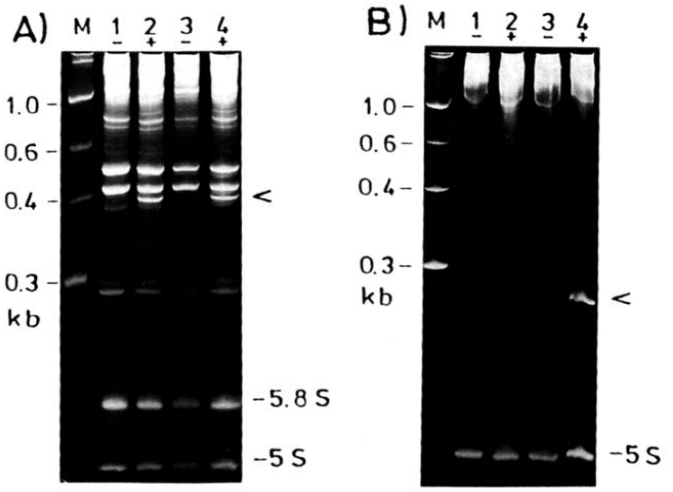

Fig. 3. Comparison of RNA *N*-glycosidase activity of Dianthin 29 and Ricin A on ribosomes of (A) rabbit and (B) *E. coli*. 10 μl rabbit reticulocyte lysate or 0.5 A_{260} units *E. coli* ribosomes were incubated with either Ricin A (lanes 1 and 2) or Dianthin 29 (lanes 3 and 4) for 10 min at 37°C (concentration of RIPs, 1 μg/ml). rRNA was extracted and treated with aniline (+) or not (–) and analysed by PAGE (3.5% gel, 7 M urea, 40 V/cm, 1.5 h). Bands were visualized with ethidium bromide. Arrows denote the position of rRNA fragments resulting from aniline-catalyzed hydrolysis at the RIP-modified site.

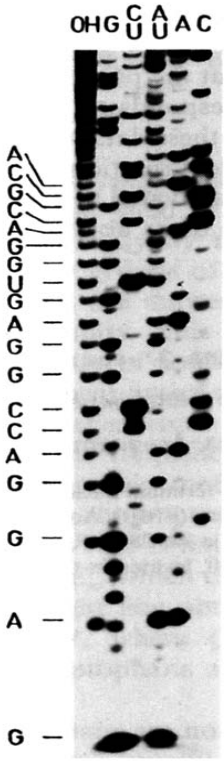

Fig. 4. 5′-terminal nucleotide sequence of the 23 S rRNA fragment (Fig. 3B) derived from Dianthin 29-treated *E. coli* ribosomes. Radioautograph of a 20% polyacrylamide sequencing gel with partial digests of the 5′ ^{32}P-labelled fragment obtained with either alkali (lane OH), ribonuclease T1 (lane G), B.cer (lane CU), Phy M (lane AU), U2 (lane A) or Cl3 (lane C).

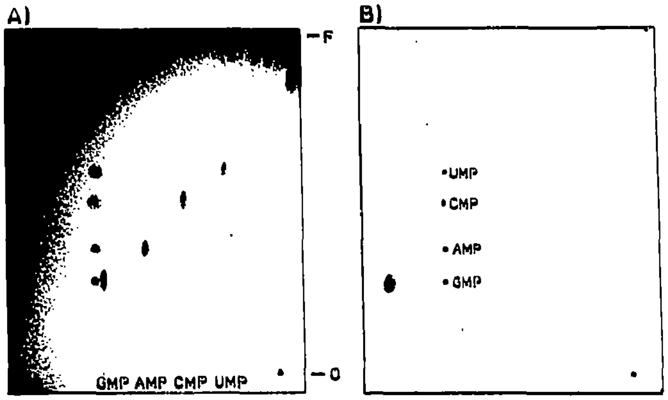

Fig. 5. Identification of the 5′-terminal nucleotide of the 23 S rRNA fragment derived from Dianthin 29-treated *E. coli* ribosomes. The 5′-^{32}P-labelled fragment was digested with nuclease P1 for 4 h at 37°C followed by PEI-cellulose thin-layer chromatography with 1 M LiCl. Lanes for sample and 5′nucleoside monophosphate standards are as indicated; positions of the standards, chromatographic origin (O) and solvent front at the end of the run (F) were marked by radioactive dye. (A) UV photograph; (B) corresponding radioautograph.

lated and its 5′-terminal nucleotide sequence was determined by the enzymatic method (Fig. 4). The pG 5′-terminus of this fragment was further verified by PEI-cellulose thin-layer chromatography of a complete hydrolysate with nuclease P1 (Fig. 5). Comparison of the sequence obtained with the sequence of *E. coli* 23 S rRNA gene shows that the pG at the 5′-terminus of the rRNA fragment corresponds to G-2661 in *E. coli* 23 S rRNA (Fig. 6). From these data it can be concluded that A-2660 is the site of depurination when complete *E. coli* ribosomes become inactivated by Dianthin 29. Whether Dianthin 29, because of its ability to inactivate *E. coli* ribosomes, takes an exceptional position in the growing list of RIPs from plants remains to be seen. In any case, before expression of cDNA clones from RIP genes in *E. coli* is anticipated it should be certified that the host ribosomes are not affected by the gene product.

REFERENCES

[1] Fernandez-Puentes, C. and Vasquez, D. (1977) FEBS Lett. 78, 143–146.
[2] Stirpe, F. and Barbieri, L. (1986) FEBS Lett. 195, 1–8.
[3] Endo, Y., Mitsui, K., Motizuki, M. and Tsurugi, K. (1987) J. Biol. Chem. 262, 5908–5912.
[4] Endo, Y., Tsurugi, K. and Lambert, J.M. (1988) Biochem. Biophys. Res. Commun. 150, 1032–1036.
[5] Nilsson, L., Asano, K., Svensson, B., Poulsen, F.M. and Nygard, O. (1986) Biochim. Biophys. Acta 868, 62–70.
[6] Brigotti, M., Rambelli, F., Zamboni, M., Montanaro, L. and Sperti, S. (1989) Biochem. J. 257, 723–727.
[7] Irvin, J.D. (1975) Arch. Biochem. Biophys. 169, 522–528.
[8] Olsnes, S. and Pihl, A. (1972) FEBS Lett. 28, 48–50.
[9] Endo, Y. and Tsurugi, K. (1988) J. Biol. Chem. 262, 8128–8130.
[10] Habuka, N., Akiyama, K., Tsuge, H., Miyano, M., Matsumoto, T. and Noma, M. (1989) J. Biol. Chem. 264, 6629–6637.
[11] Sperti, S., Montanaro, L., Rambelli, F. and Zamboni, M. (1986) Biosci. Rep. 6, 901–908.
[12] Noll, M., Hapke, B., Schreier, M.H. and Noll, H. (1973) J. Mol. Biol. 75, 281–294.
[13] Stirpe, F., Bailey, S., Miller, S.P. and Bodley, J.W. (1988) Nucleic Acids Res. 16, 1349–1357.
[14] Boseley, P.G., Tuyns, A. and Birnstiel, M.L. (1978) Nucleic Acids Res. 5, 1121–1137.
[15] Donis-Keller, H., Maxam, A.M. and Gilbert, W. (1977) Nucleic Acids Res. 4, 2527–2538.
[16] Donis-Keller, H. (1980) Nucleic Acids Res. 8, 3133–3142.
[17] Lockard, R.E., Alzner-Deweerd, B., Heckman, J.E., McGee, J., Tabor, M.W. and Rajbhandary, U.L. (1978) Nucleic Acids Res. 5, 37–56.
[18] Stirpe, F., Williams, D.G., Onyon, L.J., Legg, R.F. and Stevens, W.A. (1981) Biochem. J. 195, 399–405.

		Ricin A ↓	
	4301	4324	4350
Rat 28S rRNA 3'-end:	5'...CCAUGGUAAUCCUGCUC	AGUACGAGAGGA	ACCGCAGGUUCAGACAUUUGG...3'
	2637	2660	2686
E.coli 23S rRNA gene:	5'...TGAGGGGGGCTGCTCCT	AGTACGAGAGGA	CCGGAGTGGACGCATCACTGG...3'
		1	26
E.coli rRNA-fragment:	5'	GAGGA	CCGGAGUGGACGCAUCACUGG...3'

Fig. 6. Alignment of the 5′-terminal nucleotide sequence of the 23 S rRNA fragment derived from Dianthin 29-treated *E. coli* ribosomes with that of the *E. coli* 23 S rRNA gene (EMBL Acc. #J01695) and comparison with a homologous domain in rat 28 S rRNA 3′-end (EMBL Acc. #M11120). The RIP-sensitive A-4324 in rat 28 S rRNA is designated by an arrow. The site of action of Dianthin 29 in *E. coli* 23 S rRNA can be identified as A-2660, located in the universally conserved sequence in 23/28 S rRNA (framed) as described in the text.