Modification of ribosomal RNA by ribosome-inactivating proteins from plants

Fiorenzo Stirpe*, Suzanne Bailey, Stephen P. Miller and James W. Bodley

Department of Biochemistry, University of Minnesota, Minneapolis, MN 55455, USA

Received November 12, 1987; Revised and Accepted January 20, 1988

ABSTRACT:

We have surveyed 14 different toxic and nontoxic ribosome-inactivating proteins from plants for the ability to act on the RNA of the eucaryotic 60 S ribosomal subunit. All of these proteins act to introduce a specific modification into 26-28 S RNA which renders the RNA sensitive to cleavage by aniline. Sequence analysis of the 5'-termini of the fragments produced by ricin and saporin following aniline cleavage indicate that both proteins possess identical specificity. Our observations support the conclusion of Endo and Tsurugi (J. Biol. Chem. 262, 8128-8130, 1987) that ricin is a specific N-glycosidase and we have located the site of this cleavage by direct sequence analysis. Our results further suggest that all plant ribosome-inactivating proteins function as specific N-glycosidases with the same specificity.

INTRODUCTION:

Proteins capable of inactivating the eucaryotic ribosome, RIPs (ribosome-inactivating proteins), are widely distributed in the plant kingdom (reviewed in refs. 1-3). These proteins exist either as single chain (Type 1 RIPs) or as two chain (Type 2 RIPs) proteins. The latter proteins are potent toxins (4), the best known example of which is ricin. The B-chains of these toxins bind to galactose-containing receptors on the cell surface and allow the A-chains to enter the cytoplasm where they inactivate ribosomes. The more numerous Type 1 RIPs are much less toxic to intact cells because they lack the surface binding component.

All known Type 1 RIPs and the A-chains of Type 2 RIPs act catalytically to inactivate the 60 S ribosomal subunit. This inactivation occurs in the absence of cofactors and it is thus presumed that RIPs are hydrolytic enzymes (5). However, despite a great deal of interest and investigation, the target(s) of the RIPs within the ribosome and the mechanism(s) of RIP action have until recently eluded discovery. A major breakthrough in this area was recently reported by Endo et al. (6,7). These investigators observed that the

toxins ricin, abrin and modeccin introduce a lesion at a specific location in 28 S RNA of rat liver ribosomes concomitant with the release of adenine. This lesion renders the phosphodiester backbone of RNA susceptible to aniline-catalyzed hydrolysis. The properties of this "aniline-labile site" and the release of adenine suggested that ricin is a specific N-glycosidase. The observations we report in this paper extend the findings of Endo and coworkers and further suggest that N-glycosidase activity is the general mechanism of plant RIP action.

MATERIALS AND METHODS:

The proteins dodecandrin, cloned ricin A-chain, α -sarcin and viscumin were gifts from J.D. Robertus (University of Texas), L.L. Houston (Cetus Corp.), I.G. Wool (University of Chicago) and P. Ziska (Berlin, DDR), respectively. Pokeweed antiviral protein was purchased from Worthington. All other RIPs were prepared as described in the references cited in Table 1. Rabbit reticulocyte lysate was prepared by the method of Pelham and Jackson (18). Yeast lysates were prepared by mechanical breakage (19). Both types of lysates were stored frozen at -70°C .

In order to analyze the effect of α -sarcin and RIPs on rRNA, lysates were incubated with these proteins for 20 min at 37°C . The reactions were terminated by the addition of sodium dodecylsulfate to a final concentration of 0.5% and RNA was prepared by phenol extraction. In order to cleave the "aniline-labile site", RNA was dissolved in 1 M aniline-acetate (pH 4.5) and the solution was incubated for 10 min at 0°C . At the conclusion of the incubation, the solution was extracted twice with ether and the RNA was precipitated with ethanol. For both analysis and purification of fragments, the RNA was electrophoresed (20) on 5% acrylamide gels (4.85% acrylamide, 0.15% bis-acrylamide, 7 M urea, 90 mM Tris-Borate pH 8.3). Electrophoresis was at 1200 V until the bromophenol dye had migrated 30 cm.

For the results shown in Fig. 1, the gel described above was stained with silver (21). In order to prepare fragments for 5'-terminal labeling, the RNA was visualized by U.V. shadowing (22) and the fragments were eluted by diffusion. Labeling of the 5'-termini of the fragments with ^{32}P was performed with T_4 polynucleotide kinase (23). Terminal base analysis of labeled RNA was performed by P_1 nuclease digestion (24) followed by PEI cellulose thinlayer chromatography with 1 M LiCl. Enzymatic sequence analysis (23) was performed on 20% acrylamide-7M urea gels. The thinlayer chromatograms and sequencing gels were autoradiographed overnight.

RESULTS AND DISCUSSION:

The presence of the "aniline-labile site" created in rRNA by ricin action on the ribosome can be readily visualized by polyacrylamide gel electrophoresis of total rRNA (Fig. 1). Aniline-catalyzed hydrolysis of RNA extracted from ricin-inactivated reticulocyte ribosomes generates an RNA fragment (designated by the arrows) of approximately 400 bases (lane 3+). This fragment is not observed in RNA from ricin inactivated ribosomes which has not been subjected to aniline-catalyzed hydrolysis (lane 3-) or in RNA from control ribosomes (lanes 1+ and 1-). We have assayed all of the RIPs listed in Table 1 for the ability to create this "aniline-labile site". The results of this test were positive for all RIPs and representative examples of these results are shown in Fig. 1, lanes 4-6. In each case, a fragment was observed only after

Figure 1: Gel electrophoretic analysis of rRNA from reticulocyte lysates inactivated by RIPs. The proteins were present at a concentration of 1 $\mu\text{g}/\text{ml}$ as follows: lane 1, none; lane 2, α -sarcin; lane 3, cloned ricin A-chain; lane 4, tritin; lane 5, momordin; lane 6, saporin; lane 7, the lysate was treated sequentially with ricin A-chain and then α -sarcin. Samples in the lanes designated with a minus were not treated with aniline while those designated by a plus were treated with aniline.

Table 1: Ribosome-Inactivating Proteins Surveyed for Their Effect on Ribosomal RNA.

PROTEIN	SOURCE	REFERENCE
Barley inhibitor	<u>Hordeum vulgare</u>	8
Bryodin	<u>Bryonia dioica</u>	9
Dianthin 30	<u>Dianthus caryophyllus</u>	10
Dianthin 32	<u>Dianthus caryophyllus</u>	10
Dodecandrin	<u>Phytolacca dodecandra</u>	11
Gelonin	<u>Gelonium multiflorum</u>	12
Momordin	<u>Momordica charantia</u>	13
Pokeweed antiviral protein	<u>Phytolacca americana</u>	a
Saporin	<u>Saponaria officinalis</u>	14
Saporin 6	<u>Saponaria officinalis</u>	14
Trichokirin	<u>Trichosanthes kirilowii</u>	b
Tritin	<u>Triticum aestivum</u>	15
Viscumin	<u>Viscum album</u>	16
Volkensin	<u>Adenia volkensii</u>	17

^apurchased from Worthington.

^bCasellas, p., Dussosoy, D., Falasca, A.D., Barbieri, L., Bolognesi, A., Genini, P. and Stirpe, F. (manuscript submitted for publication).

aniline treatment and in all cases this fragment was indistinguishable in size from that produced by ricin A-chain.

It has been suggested by Obrig et al. (25) that preparations of RIPs contain ribonuclease activity which may be relevant to the mechanism of ribosome inactivation. In our experiments, nuclease cleavage of rRNA was observed with several RIP preparations. An example of this result, obtained with the RIP momordin, is shown in Fig. 1, lane 5. Under the conditions of this experiment several discrete RNA fragments were produced. (Note, for example, the species between 5 and 5.8 S RNA.) However, two observations indicate that this nuclease cleavage is not responsible for ribosome inactivation. First, the RNAs from both subunits were degraded (data not shown), while inactivation is specific to the 60 S subunit. Second,

significant RNA cleavage was only observed at high RIP concentrations (1 $\mu\text{g/ml}$) while only the fragment corresponding to the "aniline-labile site" was produced at the low concentrations of RIP (1 ng/ml) which inactivate the ribosome (data not shown). Several fragments were seen in lane 5+, but not in lane 5-, which presumably resulted from nuclease cleavage in the vicinity of the "aniline-labile site". These fragments were not seen at low RIP

Figure 2: Sequence analysis of 5'-terminally labeled fragments resulting from the action of α -sarcin (lanes 1-4) or saporin followed by treatment with aniline (lanes 5-8) on the 26 S RNA of yeast ribosomes. Lanes 1 and 5 are controls in which the RNA fragments were incubated without enzymes. Lanes 3 and 7 are base hydrolysis ladders. Lanes 2 and 6 are digests with the G-specific enzyme T_1 . Lanes 4 and 8 are digests with the A-specific enzyme U_2 . The sequences and numbering on the left and right are in agreement with the known sequence of yeast 26 S RNA (27). The purine residues were read from this gel while the pyrimidine bases were inferred from the known sequence. The 5'-terminal residues were identified by the experiment shown in Fig 3. The arrows in lane 5 designate the single base off-set between the two sequences (see text).

concentrations (data not shown) and are thus not related to ribosome inactivation.

The fungal toxin α -sarcin is well known to inactivate the ribosome by introducing a single nuclease cleavage in 26-28 S RNA (reviewed in ref. 25). This cleavage occurs in a highly conserved, purine-rich sequence about 400 residues from the 3' end of 28 S RNA. The fragment of RNA produced by the action of α -sarcin on reticulocyte ribosomes is shown in Fig. 1, lane 2 for comparative purposes. As noted above, none of the plant RIPs yielded the characteristic RNA fragment unless the RNA was first treated with aniline, thus α -sarcin remains the sole example of a 60 S ribosomal subunit-specific endonuclease. It should also be noted that the fragments produced from reticulocyte ribosomes by α -sarcin and all of the plant RIPs are of indistinguishable length as analyzed on these gels.

A curious feature of the action of α -sarcin and plant RIPs on this region of 28 S RNA in ribosomes is illustrated in Fig. 1, lane 7. Here, ribosomes were acted on sequentially, first by ricin and then by α -sarcin. Electrophoretic analysis shows that the prior glycosidic action of ricin is without detectable effect on the ability of α -sarcin to hydrolyze the phosphodiester backbone of the adjacent sequence. Ricin-catalyzed deglycosylation was without effect on either the rate or extent of α -sarcin cleavage when the latter enzyme was present at limiting concentrations (data not shown). Thus, despite the fact that the adenine residue removed by RIPs is both functionally essential and near the α -sarcin cleavage site, this residue is not essential to the recognition of RNA by α -sarcin.

The RIPs listed in Table 1 were also tested on yeast ribosomes. In each case an RNA fragment was observed only after aniline-catalyzed hydrolysis. These fragments, by the criterion of polyacrylamide gel electrophoresis, were also indistinguishable in length from each other and from that produced by α -sarcin (data not shown). In yeast ribosomes, the conserved sequence at which α -sarcin cleaves is nearer to the 3'-terminus of 26 S RNA and the resulting fragment is only about 300 bases in length (26).

Endo and coworkers (6) demonstrated that following ricin modification of rat liver ribosomes the two phosphodiester bonds to the 5'-side of the α -sarcin site become resistant to cleavage by a number of nucleases used in sequence analysis. This finding, and the observed release of adenine (7) lead to the prediction that the fragment produced by aniline-catalyzed hydrolysis following the action of ricin is one residue longer than the fragment produced by α -sarcin. We have tested this prediction by direct 5'-terminal sequence analysis of the fragments derived from yeast ribosomes (Fig. 2). The location

of the α -sarcin cleavage site is established in lanes 1-4. Lanes 1 and 3 are the control and alkaline hydrolysis ladders, respectively, and lanes 2 and 4 are the cleavages introduced by G- and A-specific nucleases, respectively. The numbering on the left and right identifies the residues in the known sequence of yeast 26 S RNA (27). The identification of the terminating residue as adenine (corresponding to residue 3026) was established by analysis of the 5'-terminal residue (Fig. 3, lane 1). The location of the aniline cleavage site following the action of saporin is shown in Fig. 2, lanes 5 to 8 (identical results were obtained with ricin A-chain, data not shown). Lanes 5 and 7 are the control and alkaline hydrolysis ladders, respectively, and lanes 6 and 8 are the cleavages introduced by the G- and A-specific enzymes, respectively. The identity of the terminal residue as guanine (corresponding

Figure 3: Analysis of the 5'-terminal base on yeast 26 S RNA fragments produced by α -sarcin (lane 1) or saporin following treatment with aniline (lane 2). The locations of nucleoside monophosphate standards run in parallel are designated on the right. The chromatographic origin and front are designated by the O and F, respectively.

to residue 3025) was established by analysis of the 5'-terminal residue (Fig. 3, lane 2). Thus, the fragment produced by saporin (and ricin A-chain) is (as emphasized by the arrows in lane 5 of Fig. 2) one residue longer than that produced by α -sarcin.

On the basis of the foregoing, we conclude that α -sarcin cleaves the phosphodiester bond of yeast 26 S RNA between the guanine residue at position 3025 and the adenine residue at position 3026 and that saporin (and ricin A-chain) acts at adenine residue 3024, presumably by removing the base. It thus seems probable that all Type 1 and Type 2 RIPs act by the same mechanism with exactly the same specificity.

ACKNOWLEDGMENTS:

This work was supported by grants from the NIH (GM-26832), the Graduate School of the University of Minnesota and the Minnesota Medical Foundation.

*Permanent address: Department of Experimental Pathology, University of Bologna, I-40126 Bologna, Italy

REFERENCES

1. Barbieri, L., and Stirpe, F. (1982) *Cancer Survey* 1, 489-520.
2. Roberts, W.K., and Selitrennikoff, C.P. (1986) *Biosci. Rep.* 6, 19-29.
3. Stirpe, F., and Barbieri, L. (1986) *FEBS Lett.* 195, 1-8.
4. Thorpe, P.E. (1985) In *Monoclonal Antibodies '84: Biological and Clinical Applications* (A. Pinchera, G. Doria, F. Dammacco, and A. Bargellesi, Eds.), pp. 475-506. Kurtis, Milan.
5. Olsnes, S., Fernandez-Puentes, C., Carrasco, L., and Vazquez, D. (1975) *Eur. J. Biochem.* 60, 281-288.
6. Endo, Y., Mitsui, K., Motizuki, M., and Tsurugi, K. (1987) *J. Biol. Chem.* 262, 5908-5912.
7. Endo, Y., and Tsurugi, K. (1987) *J. Biol. Chem.* 262, 8128-8130.
8. Barbieri, L., Bolognesi, A., and Stoppa, C. (1987) *J. Chromat.* (in press).
9. Stirpe, F., Barbieri, L., Battelli, M.G., Falasca, A.I., Abbondanza, A., Lorenzoni, E., and Stevens, W.A. (1986) *Biochem. J.* 240, 659-665.
10. Stirpe, F., Williams, D.G., Onyon, L.J., Legg, R.R., and Stevens, W.A. (1981) *Biochem. J.* 195, 399-405.
11. Ready, M.P., Adams, R.P., and Robertus, J.D. (1984) *Biochem. Biophys. Acta* 791, 314-319.
12. Stirpe, F., Olsnes, S., and Pihl, A. (1980) *J. Biol. Chem.* 255, 6947-6953.
13. Barbieri, L., Zamboni, M., Lorenzoni, E., Montanaro, L., Sperti, S., and Stirpe, F. (1980) *Biochem. J.* 186, 443-452.
14. Stirpe, F., Gasperi-Campani, A., Barbieri, L., Falasca, A., Abbondanza, A., and Stevens, W.A. (1983) *Biochem. J.* 216, 617-625.
15. Roberts, W.K., and Stewart, R.S. (1979) *Biochem.* 18, 2615-2621.
16. Ziska, P., Franz, H., and Kindt, A. (1978) *Experientia* 34, 123-1245.
17. Barbieri, L., Falasca, A.I., and Stirpe, F. (1984) *FEBS Lett.* 171, 277-279.

18. Pelham, R.B., and Jackson, R.J. (1976) *Eur. J. Biochem.* 67, 247-256.
19. Hofbauer, R., Fessler, F., Hamilton, B., and Ruis, H. (1982) *Eur. J. Biochem.* 122, 199-203.
20. Rickwood, D., and Hames, B.D. (1985) *In Gel Electrophoresis of Nucleic Acids: A Practical Approach*, IRL Press, pp. 179-180.
21. Berry, M.J., and Samuel, C.E. (1982) *Anal. Biochem.* 124, 180-184.
22. Hassur, S.M. and Whitlock, H.W. Jr. (1974) *Anal. Biochem.* 59, 162-164.
23. Donis-Keller, H., Maxam, A.M., and Gilbert, W. (1977) *Nuc. Acids Res.* 4, 2527-2538.
24. Banerjee, A.K. (1980) *Microbiol. Rev.* 44, 175-205.
25. Obrig, T.G., Moran, T.P., and Colinas, R.J. (1985) *Biochem. Biophys. Res. Commun.* 130, 879-884.
26. Wool, I.G. (1984) *TIBS* 9, 14-17.
27. Veldman, G.M., Klootwijk, J., de Regt, V.C.H.F., and Planta, R.J. (1981) *Nuc. Acids Res.* 9, 6935-6952.