Differential up-regulation by tRNAs of ribosome-inactivating proteins

Maurizio Brigotti, Domenica Carnicelli, Paola Alvergna, Alessandra Pallanca, Simonetta Sperti, Lucio Montanaro*

Dipartimento di Patologia sperimentale dell'Università di Bologna, Via San Giacomo 14, I-40126 Bologna, Italy

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Abstract Some plant ribosome-inactivating proteins (RIPs) with RNA-*N*-glycosidase activity on 28S RNA require, for the inactivation of ribosomes, the presence of macromolecular cofactors present in post-ribosomal supernatants. In the case of gelonin one of the cofactors is $tRNA^{Trp}$ lacking one or two nucleotides at the 3'-CCA end [Brigotti, M., Carnicelli, D., Alvergna, P., Pallanca, A., Lorenzetti, R., Denaro, M., Sperti, S. and Montanaro, L. (1995) Biochem. J. 310, 249–253]. In the present study it is shown that tRNAs are involved in the up-regulation of all the cofactor-requiring RIPs up to now identified (agrostin, barley RIP, PAP and tritin, besides gelonin). Polyacrylamide gel electrophoresis shows that tRNA fractions with different mobilities stimulate different RIPs. With the identification of agrostin, the cofactor-requiring RIPs (italics) add to five out of a total of thirteen investigated: *barley RIP*, bryodin-R, *gelonin*, lychnin, momordin, momorcochin-S, *PAP*, saporin-6, *tritin* [Carnicelli, D., Brigotti, M., Montanaro, L. and Sperti, S. (1992) Biochem. Biophys. Res. Commun. 182, 579–582], *agrostin*, luffin, trichokirin and trichosanthin (present study).

Key words: Ribosome-inactivating protein; Gelonin; Agrostin; Barley RIP; PAP; Tritin; Luffin; Trichokirin; Trichosanthin; tRNA

1. Introduction

Gelonin, the RIP (ribosome-inactivating protein) from *Gelonium multiflorum* with RNA-*N*-glycosidase activity on 28S RNA, acts very poorly on isolated ribosomes unless ATP and macromolecular cofactors from a post-ribosomal supernatant are also present [1]. This observation is consistent with the 10,000-fold divergence in the IC_{50} values reported [2] for the RIP acting on the unfractionated rabbit reticulocyte lysate translating endogenous mRNA (IC_{50} 0.4 nM) and on the translation of poly(U) in a system reconstituted from isolated ribosomes and elongation factors (IC_{50} 3–12 μM).

One of the cofactors responsible for the up-regulation of gelonin has been recently isolated from rat liver post-ribosomal supernatant and identified as an RNA [3] about 70-nt long showing a high level of similarity with mammalian $tRNA^{Trp}$ truncated by one or two nucleotides at the 3'-CCA end [4].

The initial observations with gelonin [1] prompted a survey of other RIPs to identify a demand for cofactors [2]. Out of eight RIPs, three (tritin, PAP and barley RIP) required, as gelonin, the presence of ATP and of a post-ribosomal supernatant for an efficient inactivation of ribosomes.

In the present paper the survey is extended to other RIPs. Agrostin shows up as a novel cofactor-requiring RIP, while no demand for cofactors was observed with luffin, trichokirin and trichosantin. It is shown that the up-regulation of all the cofactor-requiring RIPs depends on the presence of tRNA, but that only in the case of gelonin $tRNA^{Trp}$ is the active species involved. Moreover, different electrophoretic fractions of tRNA act as cofactors for the different RIPs, as if a specific tRNA were involved in the up-regulation of each cofactor-requiring RIP. The identification of the specific cofactor tRNA is a promising goal, since it will allow to select, in the use of RIPs as immunotoxins and antiviral drugs, the most appropriate RIP according to which tRNA is particularly expressed in the undesired target cell. It is well known that specific tRNAs act as primers for the reverse transcriptase of retroviruses [5] and that, as shown in the case of Rous sarcoma virus, the primer $tRNA^{Trp}$ [6] accumulates in virus infected cells [7].

2. Materials and methods

2.1. RIPs

Gelonin, the RIP from *Gelonium multiflorum*, agrostin, the RIP from *Agrostemma githago* and luffin, the RIP from *Luffa aegyptiaca*, were purchased from Sigma. Tritin, from *Triticum aestivum*, was purified as previously described [2]. Other RIPs listed by Carnicelli et al. [2], trichokirin and trichosanthin were generous gifts of Prof. F. Stirpe of this Department and had been prepared by previously described methods (refs. in [8]).

2.2. Chemicals

Micrococcal nuclease was from Boehringer-Mannheim and RNAase H (875 units/ml) was from Pharmacia. An oligodeoxynucleotide complementary to positions 55–72 of mammalian (bovine) $tRNA^{Trp}$ was synthesized with Applied Biosystems model 391 PCR MATE synthesizer and purified by gel filtration [9]. The 55–72 region was chosen since it is peculiar to mammalian $tRNA^{Trp}$ and does not contain, except one, modified bases [10]. This complementary oligodeoxynucleotide had been shown, by gel mobility shift and RNAase H digestion, to specifically hybridize with $tRNA^{Trp}$ [4].

2.3. tRNA

Rat liver post-ribosomal supernatant, obtained by centrifugation at $100,000 \times g$ for 2 h, is denoted as S-100. An RNA fraction (crude tRNA) consisting mostly of species migrating in gel electrophoresis in the region of tRNAs was isolated from S-100 by Fast Flow Q Sepharose chromatography [3]. A tRNA highly homologous to $tRNA^{Trp}$ was purified from this fraction by sequential polyacrylamide gel electrophoresis [4]. Total RNA was extracted from the same post-ribosomal supernatant by the Ultraspec RNA isolation system (Biotecx Laboratories, Houston, TX, USA) according to the protocol for biological fluids.

2.4. Assays

Experimental details of the standard two-step assay [2,11] used to test the cofactor requirement of RIPs for the inactivation of ribosomes are given in the legend to Table 1. IC_{50} (concentration of RIP giving 50%

*Corresponding author. Fax: (39) (51) 25-1315.

Abbreviations: PAP, pokeweed antiviral protein; RIP, ribosome-inactivating protein; S-100, rat liver post-ribosomal supernatant.

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inhibition of protein synthesis) was calculated by the least-squares method applied to the linear regression between fractional activity and log of RIP concentration. A kDa of 30 was assumed for all RIPs [8]. For micrococcal nuclease treatment, 1.5 μl of S-100 or 150 ng of crude tRNA were incubated for 10 min at 28°C in a final volume of 6 ml containing 5 mM $CaCl_2$ and 150 units of enzyme. At the end of incubation the nuclease was inactivated by adding 1 μl of 100 mM EGTA and the mixtures were assayed for residual cofactor activity in the standard assay.

2.5. Oligo-directed RNAase H digestion

The digestion of the crude tRNA was performed by incubating for 15 min at 40°C 800 ng of RNA and 300 pmol of the $tRNA^{Trp}$ complementary oligo in 8 μl of hybridization buffer [12]. RNAase H (2 μl, 1.75 units) was then added and incubation was allowed to proceed for 20 min at 40°C. Residual cofactor activity was assayed on the RNA precipitated from the samples with ethanol. Control crude tRNA was processed through the same steps omitting the oligonucleotide.

3. Results and discussion

In the experiments of Table 1 RIPs were preincubated with ribosomes in the absence and in the presence of the different cofactors added singularly or together. Ribosomes were then tested for poly(U) translation in a system in which RIPs and other components from the first step were made ineffective by extensive dilution (40-fold). Inhibition of translation thus depended only on the extent of ribosome inactivation which had occurred during preincubation.

The results obtained with rat liver post-ribosomal supernatant (S-100) and ATP are quite similar to those previously reported with rabbit reticulocyte post-ribosomal supernatant and ATP [2], the simultaneous presence of the two cofactors lowering the IC_{50} values from a minimum of 80-fold in the case of PAP to a maximum of 24,000-fold in the case of barley RIP. However, the effects of crude tRNA and ATP, added either singularly or together, differed largely among RIPs. As shown in Table 1, agrostin behaves as gelonin; in fact they are both highly tRNA-dependent and the simultaneous presence of ATP has only a moderate effect, just as moderate is the effect of ATP alone. On the contrary, barley RIP is strongly stimulated by ATP, which is strictly required for tRNA to have any effect at all. Both PAP and tritin are almost unaffected by either ATP or tRNA, but the two cofactors added together strikingly lower the IC_{50} in the case of PAP while they have a poor effect in the case of tritin. This behaviour of tritin was not modified when crude tRNA was substituted with total RNA extracted from S-100 (see legend to Table 1).

Table 1
IC_{50} of RIPs assayed for the inactivation of ribosomes in the absence and in the presence of cofactors

Cofactor	IC_{50} (nM)				
	Gelonin	Agrostin	Barley RIP	PAP	Tritin
None	145	7	733	10	1181
S-100 + ATP	1	0.03	0.03	0.12	3
ATP	82	4	20	6	428
Crude tRNA	8	0.40	616	6	630*
Crude tRNA + ATP	5	0.32	4	0.40	248*

Artemia salina ribosomes (10 pmol) were preincubated with RIPs for 10 min at 28°C in 10 μl of 10 mM Tris-HCl, pH 7.4, containing 100 mM ammonium acetate and 2 mM magnesium acetate. ATP (1 mM), S-100 (1.5 μl) and crude tRNA (150 ng) were present where indicated. The amount of crude tRNA is approximately equal to that present in 1.5 μl of S-100 [3]. At the end of preincubation, samples containing 2.5 pmol of ribosomes were withdrawn and assayed for poly(U) translation in a 100-μl system containing 80 mM Tris-HCl, pH 7.4, 120 mM KCl, 7 mM magnesium acetate, 2 mM dithiothreitol, 2 mM GTP, 22 pmol of L-[^{14}C]phenylalanyl-tRNA, 80 μg of poly(U) and 80 μg (as protein) of a crude preparation of *A. salina* elongation factors. Preincubation with cofactors, in the absence of RIPs, had no effect on control phenylalanine incorporation (10.5 pmol).
*Substitution of crude tRNA by total RNA extracted from S-100 gave very similar results (IC_{50} = 620 nM in the absence and 250 nM in the presence of ATP).

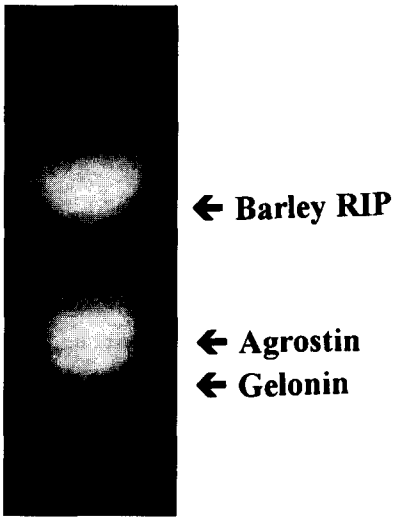

Fig. 1. Ability of tRNA fractions to stimulate the activity of RIPs. Crude tRNA was separated on a 40-cm 10% polyacrylamide/4 M urea gel and bands were visualised with ethidium bromide. From a parallel preparative lane loaded with 5 mg of crude tRNA, the gel was sliced at 5 mm intervals. RNA was extracted from the slices, precipitated with ethanol and separated from gel impurities by chromatography on Mono-Q [4] before assay for RIP stimulating activity. The peaks of activity for gelonin, agrostin and barley RIP are indicated by the arrows; the activities for PAP and tritin were spread throughout the fractions.

In spite of the low sensitivity to isolated RNA of the system containing tritin, RNA is an essential component of the cofactor activity displayed by S-100. In fact, as previously shown for gelonin [3], treatment of S-100 with micrococcal nuclease completely abolished its ability to stimulate the inactivation of ribosomes by tritin (Table 2). The nuclease treatment of S-100 was very effective also in the case of barley RIP, but less effective in the case of PAP and, particularly, of agrostin (Table 2). These results might indicate that different tRNAs with different intrinsic sensitivity to micrococcal nuclease act as cofactors for the different RIPs. However treatment of crude tRNA with the nuclease completely abolished its stimulating activity for all RIPs, including agrostin (Table 2). More likely, the level of tRNAs sensitivity to the nuclease reflects a different protective effect afforded by S-100 protein(s) associated to tRNAs. It must be recalled that proteins are involved in the stimulation of gelonin by S-100 [3,4] and that S-100 is much more active than isolated tRNA in stimulating all the cofactor-requiring RIPs (Table 1). In addition to other hypothesis [4], it may be postulated that bound proteins are required to maintain the tRNAs in an active conformation [13].

The RIPs listed in Table 1 are the only ones for which a cofactor requirement was observed. All the RIPs previously

Table 2
Effect of micrococcal nuclease treatment of S-100 and crude tRNA on the inactivation of ribosomes by RIPs

Addition	Poly(U) translation — [^{14}C]Phenylalanine incorporated (dpm)					
	RIP absent	Gelonin	Agrostin	Barley RIP	PAP	Tritin
None	9704 ± 305	9810 ± 315	9629 ± 311	9740 ± 312	9980 ± 301	9619 ± 319
S-100	10191 ± 314	2674 ± 298	4379 ± 300	1793 ± 289	3823 ± 298	1467 ± 301
Nuclease-treated S-100	10318 ± 320	9673 ± 318	5658 ± 297	8294 ± 303	7878 ± 315	9256 ± 320
Crude tRNA	10220 ± 317	4970 ± 299	4060 ± 289	3760 ± 296	3840 ± 300	4906 ± 302
Nuclease-treated crude tRNA	10190 ± 319	9850 ± 300	9607 ± 305	9007 ± 318	9807 ± 311	9630 ± 310

S-100 (1.5 μl) or crude tRNA (150 ng) were preincubated in the absence and in the presence of micrococcal nuclease as described in section 2. The concentrations of RIPs were: 5 nM gelonin, 0.05 nM agrostin, 0.08 nM barley RIP, 0.3 nM PAP and 7 nM tritin when assayed with S-100; 5 nM gelonin, 0.5 nM agrostin, 10 nM barley RIP, 1 nM PAP and 250 nM tritin when assayed with crude tRNA. ATP (1 mM) was present in all samples. Data are means of three independent experiments ± S.E.M.

Table 3
$tRNA^{Trp}$ is not a cofactor for agrostin, barley RIP, PAP and tritin

RNA	Poly(U) translation — [^{14}C]Phenylalanine incorporated (dpm)					
	RIP absent	Gelonin	Agrostin	Barley RIP	PAP	Tritin
None	10885 ± 320	10904 ± 330	10464 ± 347	10624 ± 338	10442 ± 342	1014 ± 327
Crude tRNA	10675 ± 344	5950 ± 296	5597 ± 305	4693 ± 320	4458 ± 307	6117 ± 301
Crude tRNA digested with RNAase H	10500 ± 351	10005 ± 328	5131 ± 308	4147 ± 300	4066 ± 301	5476 ± 299
Purified $tRNA^{Trp}$	10900 ± 389	3547 ± 295	10002 ± 335	10966 ± 390	10003 ± 320	10885 ± 388

Crude tRNA and crude tRNA digested with RNAase H in the presence of the oligonucleotide complementary to $tRNA^{Trp}$ were obtained as described in section 2. The amount of purified $tRNA^{Trp}$ was 40 ng. ATP (1 mM) was present in all samples. The concentrations of RIPs were: 5 nM gelonin, 0.4 nM agrostin, 4 nM barley RIP, 0.4 nM PAP and 250 nM tritin. Data are means of three independent experiments ± S.E.M.

shown to be unaffected by the presence of post-ribosomal supernatant and ATP [2] were also unaffected by crude tRNA. Also luffin, trichosanthin and trichokirin showed no cofactor requirement for the inactivation of ribosomes.

The notion that different tRNAs are involved in the stimulation of the different cofactor-requiring RIPs is supported by the following observations. Digestion of crude tRNA with RNAase H in the presence of an oligonucleotide complementary to the $tRNA^{Trp}$ molecule which up-regulates gelonin [4] does not destroy the ability of the tRNA to support the activity of agrostin, barley RIP, PAP and tritin (Table 3). Consistently, these RIPs are not stimulated by purified $tRNA^{Trp}$, which is thus a gelonin specific cofactor (Table 3). Moreover, electrophoresis of crude tRNA on a 10% polyacrylamide/4 M urea gel, followed by elution and assay of the RNA [4], showed that the stimulating activities for gelonin, agrostin and barley RIP peaked in fractions with different electrophoretic mobility (Fig. 1). In the case of PAP and tritin, the activities were not recovered in any particular fraction, but appeared spread throughout the tRNA-containing fractions.

The knowledge that different tRNAs are involved as cofactors for different RIPs may have theoretical and practical implications.

The biological function of RIPs in plants is still a matter of debate. An antiviral role has been proposed for some RIPs, in particular for PAP [14]. The antiviral mechanism would be based on a selective entering of PAP, which is localized outside the cell membrane, in virus-infected cells. Block of protein synthesis, leading to suicide of the cell, would prevent the replication of the virus and the spreading of infection. The postulated mechanism requires that ribosomes are susceptible to the 'conspecific' RIP, from which they are protected only by spatial segregation in uninfected cells [15–17]. In the case of other RIPs, such as tritin, which does not damage 'conspecific' ribosomes [15], a different mechanism may be postulated in which ribosomes become susceptible to the 'conspecific' RIP if associated with a particular viral RNA. Interestingly, many viral RNA possess 3'-terminal tRNA-like structures with amino acid accepting activity [18]. The observation that some RIPs are unable to inactivate isolated ribosomes unless a particular tRNA is present may suggest that this tRNA mimics a crucial part of the viral RNA, thus rendering ribosomes susceptible to the RNA-*N*-glycosidase activity of the RIP.

In the last decade gelonin and other RIPs have gained widespread interest because of their use in the preparation of immunotoxins directed against undesired cells [8]. The possible onset of immunological reactions towards a RIP in prolonged therapy is claimed as the need to search, characterize and employ a variety of RIPs. The demand for a different cofactor by different RIPs and the different level of expression of the required cofactor in target cells may be useful parameters in selecting the RIP of choice to optimize the therapeutic index of an immunotoxin.

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