RALyase; a terminator of elongation function of depurinated ribosomes

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Abstract Plant ribosomal RNA apurinic site specific lyase (RALyase) cleaves the phosphodiester bond at the depurinated site produced by ribosome-inactivating protein, while the biological role of this enzyme is not clear. As the depurinated ribosomes retain weak translation elongation activities, it was suggested that RALyase completes the ribosome inactivation. To confirm this point, we measured the effects of the phosphodiester cleavage using a fusion of wheat RALyase produced with a cell-free protein synthesis system from wheat germ. The results indicated that RALyase diminishes the residual elongation activities of the depurinated ribosomes.

Key words: RALyase; Cell-free protein synthesis system; Poly(Phe) synthesis; GTPase; Sarcin–ricin loop; Ribosome-inactivating protein

1. Introduction

The sarcin–ricin loop (SRL) in the large ribosomal RNA plays central roles in peptide-chain elongation [1–7] and is the most common target of ribotoxins that inactivate the ribosomes. Two different mechanisms for these enzymes are known: (1) fungal ribotoxins, such as α-sarcin, are ribonucleases that cleave a phosphodiester bond within SRL resulting in complete inactivation of the ribosomes [3,4]; (2) ribosome-inactivating proteins (RIPs) found in higher plants inhibit polypeptide chain elongation by hydrolyzing the *N*-glycosyl bond of a specific adenylate residue adjacent to the α-sarcin site in SRL [6–8]. Most commonly, these toxins are single-chain proteins. Although the biological role of RIPs is as yet unclear, it is generally believed that they participate in a self-defense mechanism against pathogens such as viruses [6]. Analyses at the molecular level revealed that the depurination, however, does not completely inactivate the ribosomes: the depurination leads to an enhancement of their elongation factor-2 (eEF-2)-dependent GTPase activity, and those ribosomes retain a significant level of polyphenylalanine synthesis activity when tested under a high magnesium concentration without eEF-2 [9–12]. Therefore, the inactivation by RIPs is incomplete, by contrast to the complete inactivation by fungal ribotoxins.

Recently, we found a new class of enzyme in wheat embryo that cleaves the phosphodiester backbone of the apurinic site introduced by an RIP, such as tritin from *Triticum aestivamu*. The enzyme, ribosomal RNA apurinic site specific lyase (RALyase), was purified, and the cDNA was cloned [13]. As we also reported the same enzymatic activity in other RIP-producing plants, and as a homologous gene could be found in the genome of rice, RALyase, as well as RIP, may be widely distributed among plants [13]. We have suggested that the cleavage of the phosphodiester backbone, as in the case of fungal ribotoxins, may terminate the activity of the deadenylated ribosomes, although the site of the cleavage by RALyase is different from that by fungal ribotoxins. To understand structural and functional involvement of SRL in the ribosomes as well as to see a biological significance of these unique enzymes, it is important to confirm this point experimentally.

To this end, we needed a purified sample of RALyase. Since the content of the enzyme in wheat embryo is very low, as described in the previous paper, the purification would be a very laborious work requiring a large amount of wheat seeds and many steps of fractionation [13]. Recently, we have developed a highly productive cell-free protein synthesis system based on an extract of wheat embryos [14–16]. As the system could produce milligrams of proteins per milliliter reaction without being affected by the cell physiology, it is expected that the cell-free system could be useful for many research applications.

In this paper, we report a method for the preparation of active-form RALyase through our cell-free system and the effects of the cleavage of the phosphodiester backbone of the deadenylated ribosomes on the eEF-2-dependent GTPase and on the eEF-2-independent polyphenylalanine synthesis. We also discuss on a possible biological role of RALyase in the context of the roles of RIPs.

2. Materials and methods

2.1. General

Isolation of wheat embryos, preparation of the wheat extract, extraction of RNA with phenol and SDS, and separation of the ribosome by sucrose density gradient centrifugation were performed as described [8,13,14].

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2.2. Protein synthesis with the wheat cell-free translation system

The coding region for GST from pGEX-6P-1 (Amersham Biosciences, Uppsala, Sweden) was amplified by PCR and was inserted into a pEU vector [16] with an *Nco*I site at the start codon (pEU-GST). An *Xho*I site was introduced just 5′ to the RALyase coding region in pGEM-RALyase [13] by polymerase chain reaction (PCR), and its *Xho*I–*Not*I fragment was inserted into pEU-3b [16] (pEU-RALyase) and pEU-GST (pEU-GST-RALyase). The produced plasmids were confirmed by DNA sequencing. Preparation of mRNA was essentially as described [16]. Cell-free protein synthesis was performed at 26°C for 24 h with the dialysis mode [14–16], with 300 μl of the translation reaction mixture containing 72 μl of the extract, 24 mM HEPES–KOH, pH 7.8, 1.2 mM ATP, 0.25 mM GTP, 16 mM creatine phosphate, 0.45 mg/ml creatine phosphokinase, 2 mM DTT, 0.3 mM spermidine, 0.3 mM 20 amino acids, 2.5 mM $Mg(OAc)_2$, 100 mM KOAc, 0.005% NaN_3, 50 μg/ml of tRNA prepared from wheat embryo, 1 μM E64 (a protease inhibitor from Roche Diagnostics), and 20 nmol mRNA. The outer solution was the same as the reaction mixture except that it lacked the extract, mRNA, E64, and tRNA.

2.3. Purification of GST and GST-RALyase

Following steps were carried out at 4°C. After the protein synthesis with the wheat cell-free system, the reaction mixture was centrifuged at 100 000 × *g* for 1 h, and the supernatant was loaded onto a glutathione Sepharose 4B (Amersham Biosciences) column with a solution of 25 mM Tris–HCl, pH 7.6, 50 mM KCl, 5 mM $MgCl_2$, 0.006 mM E64. The column was extensively washed with this solution, and the bound proteins were eluted with 20 mM reduced glutathione in the same buffer [17]. Each fraction was analyzed by SDS–PAGE. Aliquots were stored at −80°C until use.

2.4. Determination of the RALyase activity

To prepare the substrate for RALyase, the wheat ribosomes were treated with gypsophilin, an RIP from *Gypsophila elegans*, for 15 min at 30°C in 30 μl of a buffer (25 mM Tris–HCl, pH 7.6, 50 mM KCl, 1 mM $MgCl_2$) [18]. In these conditions, 100% of the ribosomes were depurinated as shown by the aniline-induced cleavage of the phosphodiester backbone [8]. To these substrate ribosomes, GST or GST-RALyase fraction were added and incubated for 1 h at 30°C in a 50-μl reaction mixture containing 25 mM Tris–HCl, pH 7.6, 50 mM KCl, and 0.5 mM $MgCl_2$. To determine the RALyase activity, RNA was extracted from the reaction mixture by phenol/SDS and separated on a 4% polyacrylamide gel.

2.5. Poly(U)-directed polyphenylalanine synthesis

The wheat extract was treated with gypsophilin in the presence or absence of GST-RALyase. Each extract (17 μl) was incubated at 26°C for 60 min in a 35-μl reaction mixture containing 24 mM HEPES–KOH, pH 7.8, 1.2 mM ATP, 0.25 mM GTP, 16 mM creatine phosphate, 0.45 mg/ml creatine phosphokinase, 2 mM DTT, 0.3 mM spermidine, 0.3 mM each of the 19 amino acids except phenylalanine, 0.02 μM [^{14}C]phenylalanine, 7.5 mM magnesium acetate, 100 mM potassium acetate, and 3.5 μg poly(U). To determine the amount of the synthesized polyphenylalanine, [^{14}C]phenylalanine incorporation into hot TCA-insoluble fraction was quantified with a liquid scintillation counter.

2.6. Preparation of the wheat ribosome and the depurinated ribosome

To prepare substrate for the assay of the eEF-2-dependent GTPase activity, the ribosomes from the wheat extract were separated by sucrose density gradient centrifugation with 10–40% sucrose, 25 mM Tris–HCl, pH 7.6, 50 mM KCl, and 5 mM $MgCl_2$. The isolated monosomes were concentrated with a Centricon (Millipore Corp.). To this substrate solution, α-sarcin (0.4 μg), gypsophilin (0.2 μg) with the GST-RALyase fraction (20 μl), or gypsophilin (0.2 μg) with the GST-RALyase fraction (20 μl) were added in a buffer containing 25 mM Tris–HCl, pH 7.6, 50 mM KCl, and 0.5 mM $MgCl_2$. The reaction was incubated for 1 h at 30°C. After the reaction, the mixture was centrifuged at 100 000 × *g* for 16 h, and the isolated ribosomes were then suspended with a buffer containing 25 mM Tris–HCl, pH 7.6, 50 mM KCl, and 5 mM $MgCl_2$. Aliquots were stored at −80°C until use.

2.7. Determination of the eEF-2-dependent GTPase activity

eEF-2 from pig liver was prepared as described [19]. 20 mM Tris–HCl, pH 7.6, 50 mM KCl, 3 mM $MgCl_2$, 2.5 pmol of the RIP-treated or untreated ribosomes, 3000 pmol [γ-^{32}P]GTP (100–300 μCi/pmol, Amersham Biosciences), and 5 pmol eEF-2 were incubated at 30°C for 10 min. After the incubation, released [^{32}P]phosphate was extracted as phosphomolybdate with benzene/isopropanol (1:1) [20]. The radioactivity was measured by using a liquid scintillation counter.

3. Results

3.1. Cell-free synthesis of fused and non-fused RALyases

In order to obtain the RALyase protein in a large amount enough to treat the depurinated ribosomes, the gene for either the GST-fused or the non-fused form of RALyase was inserted into the pEU vector and the proteins were synthesized in vitro. The analysis of the crude translation mixture showed that the GST-fused RALyase produced by this method is mostly soluble (Fig. 1, lane 5), whereas the non-fusion protein is insoluble (Fig. 1, lane 3). The amount of the GST-fused RALyase produced per 1-ml reaction was about 500 μg and the solubility was about 70% as determined by densitometric scanning of the CBB-stained gel (Fig. 1, lanes 7–9).

3.2. Purification and activity of GST-RALyase

Then, we purified the soluble GST-RALyase (Fig. 2A, lane 8), and the activity was tested on the depurinated ribosomes (Fig. 2B). The large fragment of 28S rRNA produced by the cleavage at SRL could be observed on the lanes of the agarose gel separating the aniline-treated, depurinated rRNA (Fig. 2B, lane 3) and the RNA from the depurinated ribosomes treated with the soluble GST-RALyase (Fig. 2B, lane 5). A GST fraction obtained by the same methods as the GST-RALyase (Fig. 2A, lane 4) did not have any activity (Fig. 2B, lane 4), which showed that the activity in the purified GST-RALyase was not originated from the contamination by natural RALyase from the extract of wheat embryo. Therefore, the soluble GST-RALyase has the activity of RALyase.

3.3. Effects of GST-RALyase treatment of the depurinated ribosomes on poly(Phe) synthesis

In order to clarify the functional differences between the ribosomes with the cleaved SRL chain and the depurinated,

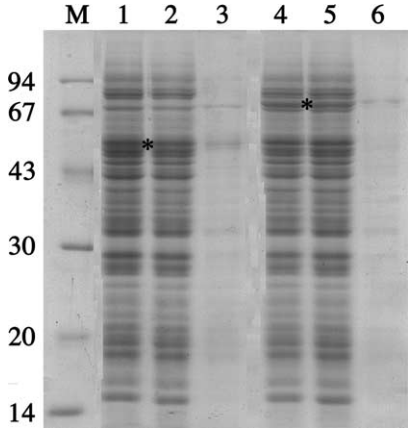

Fig. 1. An SDS polyacrylamide gel separating the products from cell-free protein synthesis. Total protein (lanes 1 and 4), the soluble fraction (lanes 2 and 5), and the insoluble fraction (lanes 3 and 6) from the wheat germ cell-free protein synthesis system with the non-fusion RALyase mRNA (lanes 1–3) or from that with the GST-RALyase mRNA (lanes 4–6) were separated on a standard SDS–polyacrylamide gel, and were stained with CBB. The triangles show the positions of RALyase and GST-RALyase.

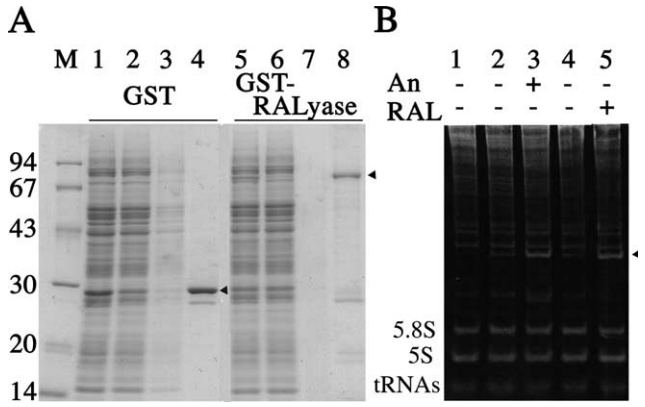

Fig. 2. Proteins in each fraction during purification of GST and GST-RALyase (A) and the ribosomal RNAs treated with different fractions and reagents (B). (A) GST (lanes 1–4) and GST-RALyase (lanes 5–8) were purified as described in Section 2, and the supernatants from the ultracentrifugation at 100 000 × *g* (lanes 1 and 5), the flow-throughs of the affinity chromatography (lanes 2 and 6), the eluents without glutathione (lanes 3 and 7), and the eluents with the glutathione-containing buffer (lane 4 and 8) were separated by SDS–PAGE and stained with CBB. The triangles show the mobilities of GST and GST-RALyase. (B) The 80S ribosomes were treated with nothing (lane 1), gypsophilin (lanes 2 and 3), gypsophilin and then with the purified GST (lane 4), gypsophilin and then with the purified GST-RALyase (lane 5), and the rRNAs were extracted. The sample for lane 3 was then treated with aniline. These samples were separated on a 2% agarose gel and stained with ethidium. The triangle marks the mobility of the aniline-induced specific rRNA fragment.

uncleaved ribosomes, we tested the poly(Phe) synthesis activity by using the gypsophilin-treated wheat extract in the presence or absence of GST-RALyase. As reported before [11], the RIP-treated extract had a low poly(Phe) synthesis activity (Fig. 3, squares). By contrast, the extract treated with the RIP and the soluble GST-RALyase had almost no activity (Fig. 3, triangles).

3.4. Effects on the eEF-2-independent GTPase activity

Then, we measured the eEF-2-dependent GTPase activities of the purified ribosomes after various treatments (Fig. 4).

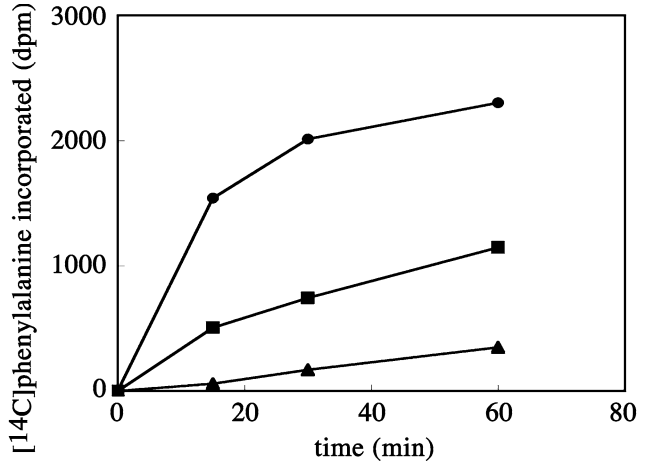

Fig. 3. Poly(U)-dependent poly(Phe) synthesis activities of differently treated wheat-germ extracts. The reaction was carried out with the extract treated with nothing (closed circles), gypsophilin (closed square), or gypsophilin and GST-RALyase (closed triangle). The figure shows the result from one of the two independent experiments, both of which gave essentially the same results.

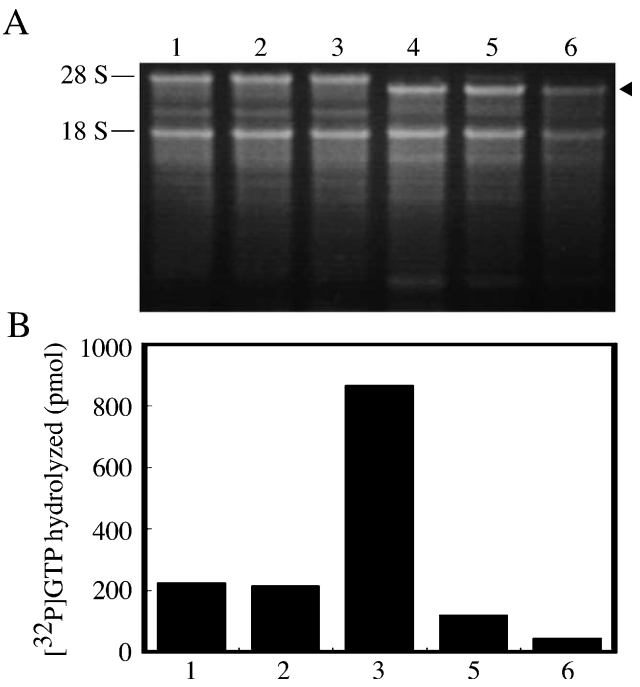

Fig. 4. The ribosomal RNAs (A) and the eEF-2-dependent GTPase activities (B) of differently treated 80S ribosomes. A: The rRNAs were extracted from the 80S ribosomes treated with nothing (lane 1), the purified GST-RALyase (lane 2), gypsophilin (lanes 3 and 4), gypsophilin and then with the GST-RALyase (lane 5), and α-sarcin (lane 6). The sample for lane 4 was then treated with aniline. These samples were separated on a 2% agarose gel and stained with ethidium. B: eEF-2-dependent hydrolysis of GTP catalyzed by the ribosomes treated as in (A). The figure shows the result from one of the two independent experiments, both of which gave essentially the same results.

After the treatment with an RIP, the rRNA chain was not cleaved (Fig. 4A, lane 3), and the GTPase activity was enhanced as compared with that of the untreated ribosomes (Fig. 4B, column 3). If the ribosomes were treated with an RIP and GST-RALyase, SRL was cleaved (Fig. 4A, lane 5), and the GTPase activity dropped below that of the untreated ribosomes (Fig. 4B, column 5). Because the SRL cleavage with GST-RALyase was not complete (Fig. 4A, lane 5), and because the GTPase activity of the depurinated, uncleaved ribosomes is higher than that of the untreated ribosomes, the observed low GTPase activity after the treatment could be ascribed to the uncleaved, depurinated ribosomes. The treatment with the α-sarcin cleaved SRL almost completely (Fig. 4A, lane 6) and eliminated the GTPase activity (Fig. 4B, column 6).

4. Discussion

In the present study, we could obtain a soluble form of a protein that has the RALyase activity in a practical amount. The fusion with GST dramatically improved the solubility of the protein during and after cell-free synthesis. We could purify the soluble GST-RALyase by a standard one-step affinity chromatography, and this fraction had the RALyase activity. Though the SDS–PAGE analysis of the purified fraction (Fig. 2A, lane 8) showed a weak band in addition to the GST-RALyase at 25 kDa, this band does not seem to contribute to the RALyase activity because the GST fraction obtained by

the same way as the soluble GST-RALyase contains the same protein but had no activity (Fig. 2A, lane 4, and Fig. 2B, lane 2). Anyway, the GST-RALyase fraction was obtained easily and was used to cleave the rRNA chain at the apurinic site produced by an RIP for the subsequent use in biochemical analyses.

It was confirmed, in the present study, that the depurinated ribosomes have the eEF-2-independent poly(Phe) synthesis activity and the eEF-2-dependent GTPase activity. The GTPase activity was higher than that of the intact ribosomes. These activities were found to be depressed almost completely by the cleavage of the rRNA chain by GST-RALyase, although the product of the cleavage is different from that produced by the cleavage by fungal toxins. These results strongly suggest that a role of natural RALyases is to terminate the elongation activity of the depurinated ribosomes. However, it is still unclear why the complete inactivation is necessary, as it seems that the depurination alone could arrest the elongation activity for the most part and could kill the cell. And, if the complete inactivation is necessary by some reason, why do plants separate the ribosome inactivation into the two steps instead of killing the ribosomes in one step as in the case of fungal toxins?

In a mammal system, 28S rRNA could be cleaved in response to ER stress, which could lead to apoptosis [21]. By analogy, it might be possible that the cleavage of the plant SRL chain induces some apoptosis-like response including the degradation of the ribosomes (depurinated or not) and/or down-regulation of other cellular functions, which could block infecting virus, if any, to grow. In fact, the translation system in the plant cells is quite robust by itself, as indicated by the fact that our wheat-germ extract could retain its translation activity for more than 2 weeks [14–16]. Therefore, it may be necessary, in some cases, to inactivate the translation system completely by a positive mechanism.

As the eEF-2-dependent GTPase activity of the depurinated ribosomes is quite high, it is possible that it is uncontrolled. In fact, some mutations within SRL cause a high level of mistranslation such as frameshifting [22–25], which may indicate that the mutant ribosomes may have lost control of the eEF-2 related activities, such as translocation. Therefore, it is possible that the depurination, which is a kind of a mutation within the same rRNA region, also cause such mistranslation. The mistranslation products, if any, might induce heat shock-like responses that might lead to the controlled cell death. If so, RALyase might purge the initial signal and restore accurate translation depending on the undamaged ribosomes so that the following responses could proceed properly. It is also possible that RALyase might be required for minimizing the possibility of virulent mutation in the infecting virus, if any, by preventing mistranslation. We are currently testing several hypotheses for biological roles of RALyase.

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