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The role of SmpB protein in *trans*-translation

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Abstract The function of SmpB protein in the *trans*-translation system was evaluated using the well-defined cell-free translation system consisting of purified ribosome, alanyl-tRNA synthetase and elongation factors. The analysis showed that SmpB protein enhances alanine-accepting activity of tmRNA and that SmpB protein and tmRNA are sufficient to complete the *trans*-translation process in the presence of translational components. Moreover, SmpB is indispensable in the addition of tag-peptide onto ribosomes by tmRNA. In particular, the A-site binding of tmRNA is inhibited in the absence of SmpB.

Key words: tmRNA; 10Sa; SsrA; SmpB; *trans*-Translation; Translation

1. Introduction

10Sa RNA, or tmRNA, encoded by the *ssr*A gene is one of the smallest stable RNAs first found in *Escherichia coli* [1] and commonly exists in the bacterial kingdom. A unique feature of tmRNA is that it acts both as a tRNA and mRNA. An alanine-tRNA-like domain, corresponding to acceptor stem and T-arm, endows tmRNA the capability of charging with alanine by alanyl-tRNA synthetase [2], while tmRNA encodes a template sequence corresponding to AANDENYALAA affording tag for proteolysis [3,4]. In cells, tmRNAs participate in peculiar translation reactions called *trans*-translation. Alanine-charged tmRNA enters the A-site of the ribosome stalled by truncated mRNA that lacks a stop codon (probably due to an attack of ribonuclease or by shortage of particular tRNA [5]), and the peptidyl-tmRNA is translocated to the P-site. Subsequently, tmRNA is substituted for the damaged mRNA as a template encoding the tag-peptide, adding the tag-peptide to the abortive polypeptide chain. Proteins labeled with tag-peptide are then degraded by the C-terminus-specific proteases recognizing the tag-peptide sequence [4,6]. It is proposed that through the consecutive reactions, tmRNA detaches stalled ribosomes and defective proteins are scavenged by specific proteases [7,8].

In 1999, Karzai and co-workers found an additional protein factor, SmpB, a unique RNA binding protein, required for the peptide-tagging system mediated by tmRNA [9]. They indicated that the deletion of the *smp*B gene in *E. coli* results in the similar phenotypes observed in the cell lacking *ssr*A gene. Either SmpB-defective or SsrA-defective strains are unable to add the tag-peptide to proteins derived from damaged mRNAs, and fail in growth of some phages. It was also shown that the purified SmpB protein binds to tmRNA with high affinity. Thus, it appears that SmpB protein participates in the peptide-tagging system of tmRNA, although how SmpB protein functions in the *trans*-translation process remains unclear.

Recently, we have succeeded in construction of the cell-free translation system reconstituted with purified components, named PURE system [10]. The PURE system contains only minimally required factors for the detailed translation of regular mRNAs and, therefore, allows the evaluation of molecular function of auxiliary factors in translation. Here, we describe the role of SmpB in the tmRNA-dependent peptide-tagging system by using pure translation components.

2. Materials and methods

2.1. Preparation of factors utilized in the in vitro translation experiments

The components indispensable to the cell-free protein synthesis, including ribosomes, IF1, IF2, IF3, EF-G, EF-Tu, EF-Ts, RF1, RF3, RRF, 20 aminoacyl-tRNA synthetase, methionyl-tRNA transformylase, T7 RNA polymerase and nucleoside-diphosphate kinase (NDK), are purified according to the previous report [10]. The gene encoding tmRNA was amplified by PCR from the *E. coli* genome and cloned into vector pET17b (Novagen) using a restriction site-free method described by Chen et al. [11] with a slight modification. The obtained plasmid, containing a T7 promoter directly upstream of the *ssr*A gene, was transformed into *E. coli* JM109/DE3 strain. tmRNA induced by the addition of 0.1 mM IPTG was purified as described [12] with a slight modification. The gene encoding SmpB protein was amplified by PCR from *E. coli* genomic DNA using primers with an *Nde*I site at the 5′ end and a *Bam*HI site at the 3′ end, and cloned into vector pET15b (Novagen). The resultant plasmid was transformed into *E. coli* BL21/DE3 strain. The BL21 cells were grown to an OD_{600} = 0.5–0.9 in 2 l LB broth. IPTG was added to a final concentration of 0.1 mM and the cells were grown for an additional 4 h at 37°C. Harvested cells were resuspended in a buffer (50 mM HEPES-KOH (pH 7.6), 1 M NH_4Cl, 10 mM $MgCl_2$, 0.3 mg/ml lysozyme, 0.1% Triton X-100, 0.2 mM PMSF, 7 mM 2-mercaptoethanol) and lysed by sonication. Cell debris was removed by centrifugation at 100 000×*g* for 1 h at 4°C and the supernatant was applied to a Ni^{2+} precharged 10 ml Hi-Trap chelating column (Amersham Pharmacia Biotech) and washed with 100 ml A buffer (50 mM HEPES-KOH (pH 7.6), 1 M NH_4Cl, 10 mM $MgCl_2$, 7 mM 2-mercaptoethanol) containing 10 mM imidazole. The protein was eluted with a linear gradient from 10 to 400 mM imidazole in A buffer, followed by a dialysis against B buffer (50 mM HEPES-KOH (pH 7.6), 100 mM KCl, 10 mM $MgCl_2$, 7 mM 2-mercaptoethanol). Purified SmpB protein was then digested by 7 U/ml thrombin (Sigma) to remove his-

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tag and purified by Mono-S column chromatography (Amersham Pharmacia Biotech) using a linear gradient from 100 to 500 mM KCl in B buffer. Fractions containing SmpB protein were collected, followed by the addition of glycerol (final concentration 30%). The concentration of the purified protein was determined by a Bio-Rad protein assay kit using bovine serum albumin as a standard.

2.2. In vitro translation experiment

Template plasmid for the truncated dihydrofolic acid reductase (DHFR) was prepared by digesting the plasmid encoding *E. coli* DHFR under the T7 promoter using *Eco*RI. Obtained plasmid, as well as the one encoding DHFR, was translated in the PURE system containing [^{35}S]methionine and visualized by SDS–PAGE as described previously [10]. The reaction mixtures (50 μl) for the poly(U)-programmed tag-peptide synthesis contained 9 mM magnesium acetate, 5 mM potassium phosphate (pH 7.3), 95 mM potassium glutamate, 5 mM ammonium chloride, 0.5 mM calcium chloride, 1 mM spermidine, 8 mM putrescine, 1 mM dithiothreitol (DTT), 1 mM each ATP and GTP, 10 mM creatine phosphate, 2.8 A_{260} units tRNA mixtures (Roche, Mannheim, Germany), 0.1 mM of each amino acid necessary for the production of tag-peptide-attached poly phenylalanine except for alanine, 9.25 kBq [^{14}C]alanine, and factor mix. The factor mix contained 12 pmol ribosome, 1 μg EF-G, 2 μg EF-Tu, 1 μg EF-Ts, 0.5 μg RF1, 0.5 μg RF3, 0.5 μg RRF, 30–300 U of phenylalanyl-tRNA synthetase and other aminoacyl-tRNA synthetase necessary for the translation of the tag-peptide sequence, 0.2 μg creatine kinase (Roche), 0.15 μg myokinase (Sigma), and 0.054 μg NDK. After the reaction mixture was incubated at 37°C for 5 min, 50 μg of poly(U) was added and the reaction was carried out at 37°C. At the times specified, an 8 μl aliquot was withdrawn and spotted on Whatman 3MM filter paper, and radioactivity in the 10% trichloroacetic acid-insoluble fraction was measured by a liquid scintillation counter.

2.3. In vitro aminoacylation experiment

The reaction mixtures (50 μl) contained 5 mM magnesium acetate, 5 mM potassium phosphate (pH 7.3), 95 mM potassium chloride, 5 mM ammonium chloride, 0.5 mM calcium chloride, 1 mM spermidine, 8 mM putrescine, 1 mM DTT, 1 mM ATP, 9.25 kBq [^{14}C]alanine and 30 pmol of purified tmRNA. After the preincubation at 37°C for 5 min, the reaction was started by the addition of 350 U of alanyl-tRNA synthetase. At the times specified, an 8 μl aliquot was withdrawn and spotted on Whatman 3MM filter paper, and radioactivity in the 5% trichloroacetic acid-insoluble fraction was measured by a liquid scintillation counter. Alanine-precharged tmRNA and alanine-precharged tRNA mixtures were prepared as follows. After 20 min incubation at 37°C, the aminoacylation reaction was quenched by 10% volume of 3 M potassium acetate (pH 4.8), followed by water-saturated phenol extraction. The aqueous layer containing aminoacylated RNA was dialyzed against 0.3 M potassium acetate (pH 4.8) by gel chromatography using an NAP-5 column (Amersham Pharmacia Biotech). Aminoacylated RNA is precipitated by isopropanol and used for the in vitro translation experiments.

3. Results

3.1. Construction of SmpB assay system using the PURE system

First, we addressed the construction of an in vitro system to examine the function of SmpB protein in the *trans*-translation system. According to Himeno et al. [13], using poly(U) as a truncated mRNA, tmRNA-dependent tag-peptide synthesis is easily detected by the incorporation of alanine encoded in the tag-peptide sequence. To clarify the roles of SmpB or tmRNA, the system was constructed with highly purified components, ribosomes, tRNAs, EF-G, EF-Tu, EF-Ts, RF1, RF3, RRF, six aminoacyl-tRNA synthetases for the tag-peptide sequence, phenylalanyl-tRNA synthetase, and poly(U).

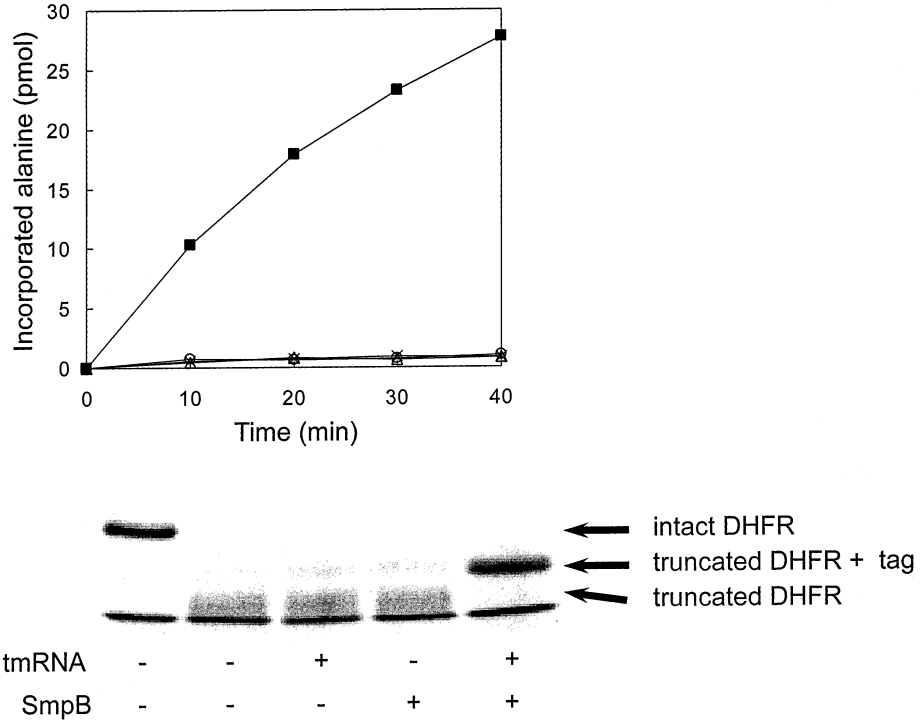

Fig. 1. Top: Time course of alanine incorporation in the product of poly(U)-directed *trans*-translation system in the presence and absence of tmRNA or SmpB protein. The reaction mixtures (50 μl) contained no tmRNA and no SmpB (○), 30 pmol tmRNA and no SmpB (×), no tmRNA and 1 μM SmpB (△), and 30 pmol tmRNA and 1 μM SmpB (■). Bottom: SDS–PAGE gel patterns of the product in the PURE system. The DHFR template (lane 1) and the truncated DHFR template digested by the restriction enzyme *Eco*RI (lane 2–5) were translated in the presence and absence of 30 pmol tmRNA or 1 μM SmpB protein for 1 h. Expected molecular weights of intact DHFR, truncated DHFR+tag, and truncated DHFR are 18.0 kDa, 16.7 kDa, and 15.6 kDa, respectively. Autoradiographed SDS–PAGE patterns of the ^{35}S-labeled products are shown. The row of bands at the bottom indicates the dye front.

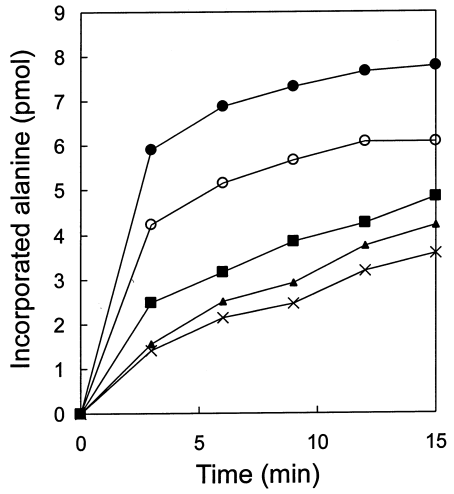

Fig. 2. Time course of aminoacylation of tmRNA (30 pmol) in the presence of a specified concentration of SmpB protein. The concentrations of SmpB protein were 0 μM (×), 0.14 μM (▲), 0.27 μM (■), 0.55 μM (○), and 1.1 μM (●), respectively.

As indicated in Fig. 1, top, omission of tmRNA or SmpB protein completely vanished alanine incorporation into peptides, indicating that tmRNA and SmpB are essential for *trans*-translation. We also employed the *E. coli* DHFR gene, which was digested by *Eco*RI in the open reading frame, as the truncated mRNA. The digested plasmid was translated in the transcription/translation-coupled PURE system, which is constructed from the purified components essential to the translation system [10], in the presence of tmRNA or SmpB protein. As shown in Fig. 1, bottom, only the reaction mixture containing both tmRNA and SmpB protein (Fig. 1, bottom, lane 5) produced a longer polypeptide chain, which is estimated to be the size of peptide with tag-sequence. The intensity of the protein band in lane 5 of Fig. 1, bottom, also indicates that the addition of tmRNA and SmpB protein is sufficient for the *trans*-translation to proceed in a multi-turnover reaction.

3.2. SmpB protein on the alanylation of tmRNA

tmRNA has a G–U base pair (G3–U357) in its tRNA-like structure corresponding to the determinant G–U pair (G3–U70) in the acceptor stem of $tRNA^{Ala}$ [14,15]. Because the minihelix containing G–U pair at the third position of the acceptor stem can be aminoacylated by alanyl-tRNA synthetase [16], tmRNA is likely to be aminoacylated with the help of no other accessory factor. As shown in Fig. 2, it is evident that tmRNA can be aminoacylated by alanyl-tRNA synthetase without the aid of any other factors. However, the rate of tmRNA alanylation was increased in proportion to the amount of added SmpB protein. These data are inconsistent with the result described by Karzai et al. [9], which reported that SmpB protein does not affect charging of tmRNA. Although we have no good explanation for this discrepancy, our observation suggests that SmpB protein enhances the rate of tmRNA aminoacylation.

3.3. SmpB protein on the translation of the tag-peptide

To examine the role of SmpB protein on ribosomes, we designed the following experimental system. After the preparation of cold alanine-precharged tmRNA and [^{14}C]alanine-precharged tRNA mixtures, these RNAs were subjected to the poly(U)-dependent *trans*-translation system constructed from purified components but containing no alanyl-tRNA synthetase, and the reaction was carried out in the presence or absence of SmpB protein. The incorporation of [^{14}C]alanine into peptide was shown to be completely dependent on the presence of SmpB (Fig. 3, left), indicating that SmpB is also essential for the addition of the tag-sequence on ribosome as well as the alanylation of tmRNA.

To study the function of SmpB protein on the ribosome more in detail, we prepared [^{14}C]alanine-precharged tmRNA and carried out the similar poly(U)-dependent *trans*-translation experiment. Because of omission of alanyl-tRNA synthetase and alanyl-$tRNA^{Ala}$ in the reaction mixture, the reaction directly reflects binding of tmRNA to the A-site of the ribosome and peptidyltransfer to tmRNA. As shown in Fig. 3, right, the [^{14}C]alanine was incorporated to the product only in

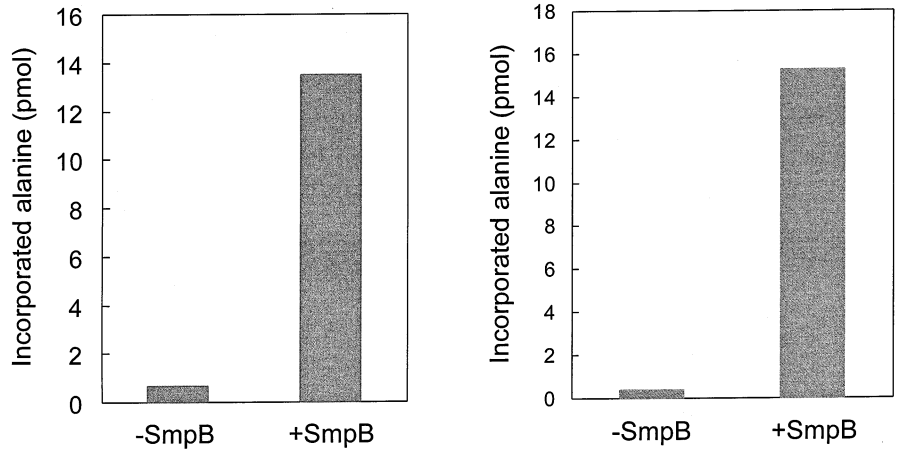

Fig. 3. Alanine incorporation in the product of poly(U)-directed *trans*-translation system containing no alanyl-tRNA synthetase. The translation was carried out for 1 h in the presence and absence of SmpB protein. The reaction mixtures contained 135 pmol of cold alanine-precharged tmRNA and 2.8 A_{260} units of [^{14}C]alanine-precharged tRNA mixtures (left) or 300 pmol of [^{14}C]alanine-precharged tmRNA and 2.8 A_{260} units of no charged tRNA mixtures (right) as a substitute for the removal of alanyl-tRNA synthetase. The concentrations of ribosomes, EF-G, EF-Tu, and EF-Ts were intensified five-fold, three-fold, three-fold, and three-fold, respectively, to detect the SmpB effect more clearly (right).

the presence of SmpB, demonstrating that SmpB protein is crucial for the tmRNA binding to the A-site of the ribosome.

4. Discussion

Here we demonstrate the SmpB protein is involved in alanylation and A-site binding of tmRNA in *trans*-translation. The construction of the in vitro translation system using purified components enabled us to easily examine the roles of SmpB protein and tmRNA in the *trans*-translation process, whereas it would be difficult when the conventional cell-free translation system using crude cell extract was employed. Thus, as a result of the in vitro translation experiment using both poly(U) and natural mRNA without in-frame stop codon as a truncated mRNA, the tag-peptide synthesis was not observed in the absence of SmpB (Fig. 1). This result is consistent with the in vivo results reported by Karzai et al. [9], in which the SmpB-defective *E. coli* strain is unable to add the tag-peptide to proteins synthesized from damaged mRNA as well as an SsrA-defective cell. Thus, it can be concluded that SmpB protein is an essential protein for the tmRNA-dependent peptide-tagging system. In addition, since the system is constructed from components purified to homogeneity, we can conclude that no other factor, except SmpB protein and tmRNA, is required in *trans*-translation.

The physiological roles of the tmRNA-dependent peptide-tagging system are proposed to be the clearance and recycling of the stalled ribosomes caused by truncated mRNA, followed by digestion of abortive protein by cellular protease targeting tag-sequence. The clearance and recycling of the stalled ribosomes are suggested to be more important, considering that some phenotypes of SsrA-defective *E. coli* can be complemented by SsrA mutants with mutant peptide tags that are poorly recognized by proteases [17]. In our experiment using truncated DHFR template, we observed a faint band corresponding to the truncated DHFR without tag-peptide in the absence of SmpB protein and tmRNA, whereas the tag-peptide-attached protein was produced as much as the DHFR of full length, suggesting that SmpB protein and tmRNA are able to recycle ribosome (Fig. 1, bottom). This finding fits the physiological role of tmRNA in the proposed model.

Previously it was shown that EF-Tu is capable of binding with alanyl-tmRNA, despite a low affinity [18,19]. Here we demonstrate that SmpB protein enhances the aminoacylation and is indispensable for the entry of tmRNA onto the A-site of the ribosome as shown in Figs. 2 and 3, indicating that SmpB protein synergistically participates in the *trans*-translation process with alanyl-tRNA synthetase and EF-Tu. Particularly, SmpB protein is significantly involved in binding of tmRNA to the ribosome, compared to the alanylation process. It is still unclear whether SmpB protein is required for EF-Tu binding or A-site binding. However, considering that EF-Tu is able to bind to tmRNA in the absence of SmpB protein, it is likely that SmpB protein plays roles in both steps.

EF-Tu binds the acceptor and TΨC stems [20], while alanyl-tRNA synthetase recognizes the acceptor stem including the G–U pair at the third position [14,15]. By binding to another region of tmRNA, SmpB protein might stabilize the tRNA-like structure important for the high affinity binding of alanyl-tRNA synthetase, EF-Tu and ribosome. Considering the fact that the regions corresponding to the anticodon helix and loop in the tmRNA are absent, SmpB may play a role on the tmRNA conformation equivalent to the effect of the codon–anticodon binding on the ribosome. In order to understand the detailed mechanism and function of this small protein in the *trans*-translation process, further structural study will be necessary.

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