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A novel class of bacterial translation factor RF3 mutations suggests specific structural domains for premature peptidyl-tRNA drop-off

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ABSTRACT

The bacterial translation factor RF3 promotes translation termination by recycling the tRNA-mimicking release factors, RF1 and RF2, after mature polypeptide release. RF3 also enhances the premature peptidyl-tRNA drop-off reaction in the presence of RRF and EF-G. Despite the recently resolved X-ray crystal structure of RF3, the molecular details of the bimodal functionality of RF3 remain obscure. In this report, we demonstrate a novel class of RF3 mutations specifically defective in the tRNA drop-off reaction. These mutations suggest differential molecular pathways closely related to the guanine nucleotide modes of RF3.

1. Introduction

The protein-translation termination step is catalyzed by class I and II release factors (RFs). There are two codon-recognizing class I RFs in prokaryotes (RF1 and RF2) and one in eukaryotes (eRF1) that directly recognize stop codons and induce hydrolysis of peptidyl-tRNA at the ribosomal peptidyl transferase center (PTC) [1]. Class II RFs belong to the translational GTPase family of proteins that include initiation factor 2 (IF2), elongation factor Tu (EF-Tu), and elongation factor G (EF-G). While the eukaryotic class II factor eRF3 binds eRF1 and works concertedly [2], the prokaryotic class II factor RF3 accesses the ribosome separately after mature peptide release and removes RF1 or RF2 from the ribosomal A site [3–5], indicating that definitive functional differences exist between prokaryotes and eukaryotes.

Interestingly, besides release-factor recycling, RF3 promotes the peptidyl-tRNA drop-off reaction during translation elongation [6]. The peptidyl-tRNA drop-off is found in the translation of certain very short open reading frames (minigenes) in the lambda-phage genome, in which translating ribosomes stall abnormally and release premature peptidyl-tRNAs [7]. Accumulation of the prematurely released peptidyl-tRNA results in depletion of free tRNA, which thus inhibits cell growth. To avoid such situations the essential peptidyl-tRNA hydrolase (PTH) releases tRNA from peptidyl-tRNAs. The relationship between the drop-off efficiency and the length of the open reading frame, as well as codon usages, has been investigated in detail [8] but the responsible factors are not well identified. Translation factors such as RRF, EF-G, and IF3, which are generally known to participate in ribosomal recycling after mature polypeptide release [7], promote the premature tRNA drop-off concertedly in vivo and in vitro. However, the molecular bases of these reactions by RF3 remain unresolved.

The cryo-EM structures of RF3·GDPNP stalled on the ribosome suggest putative bimodal conformational changes [9]. X-ray crystallography of RF3, in combination with the cryo-EM images, suggests that the conformation of the G-II domain is quite similar among the three translational GTPases (RF3·GDP, EF-G·GDP, and EF-Tu·GDP). This suggests a working model in which RF3 induces ribosomal conformation changes upon GTP hydrolysis in the RF-recycling reaction [10]. Alanine scanning mutational analyses of the putative interaction surface of the RF3-ribosome residues support this model.

In this study, we report novel RF3 mutations that are specifically involved in the tRNA drop-off reaction. The RF3 mutations inhibit dominantly the tRNA drop-off induced by minigene overproduction under the PTH-defective strain background; hence,

Abbreviation: RF, polypeptide-chain release factor

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they were named RF3^{DDI} mutations (drop-off dominant-inhibitory mutants). These mutations are clustered on the structural regions of RF3, some of which are highly conserved among translation factors such as EF-Tu and EF-G. Analyses of those RF3 mutations strongly suggest two differential ribosome-binding pathways for mature termination and premature tRNA drop-off reactions, which are closely related to the guanine nucleotide modes of RF3.

2. Materials and methods

2.1. Bacterial strains and plasmid vectors

The *Escherichia coli* K-12 strains used for initial mutation screenings were YW1 (CSH50 *pth*(ts)) transformants. The DNA fragment (5′-GAATTCCATGGAACGAGAAACGTAAA ATG AAA TGA ATATCTAGAGGATCC-3′), which included the synthetic *bar*A702 minigene (SD-fMet-Lys-Stop), was inserted into the pFLAG-CTC vector (Sigma), giving rise to the isopropyl-β-D-(−)-thiogalactopyranoside (IPTG)-inducible bar expression vector, pBar1 (Fig. 1A). The tRNA(Lys) constitutive expression plasmid pLysT carried the 409-base lysT operon DNA fragment attached to the crp terminator at the 3′ end. Information for the other strains and plasmids is listed in Table S1.

2.2. Mutagenesis and genetic selection of DDI mutants of RF3

The RF3 expression vector pRF3^{+} [11] (previously named as pSU-IQ-RF3^{+}) was mutagenized by using hydroxylamine treatment or error-prone PCR mutagenesis, as described previously [12]. The pBar1 transformant of YW1, referred to as YW1(pBar1), was transformed by using mutagenized pRF3^{+} and was selected on LB-agar plates [13] supplemented with selection antibiotics and a lethal concentration of IPTG (0.2 mM) at 32 °C. After 1–2 days, plasmids from the survivor colonies were prepared and introduced again to the YW1(pBar1) for phenotypic reconfirmation. The genuine RF3-mutant plasmids were analyzed by sequencing. Each single locus of the multiple-mutated genes was reconstituted separately by using site-directed mutagenesis with a QuickChange® Mutagenesis Kit (Stratagene) and the loci were reconfirmed.

2.3. Nonsense-suppression assay

The efficiency of nonsense suppression was determined by monitoring β-galactosidase activity in the *lacZ* UGA (RM698) strain transformed with each plasmid [13].

3. Results and discussion

3.1. E. coli strain with conditional lethal tRNA drop-off activity

To isolate the RF3 mutations that inhibit tRNA drop-off, we constructed a *pth*(ts) [14] strain that carries an IPTG-inducible synthetic lambda *bar*A702 (fMet-Lys-Stop) minigene [15] plasmid pBar1, referred to as YW1(pBar1) (Fig. 1A). The induction of the minigene was expected to induce severe growth inhibition of the *pth*(ts) strain at a permissive temperature (32 °C).

Colony growth of YW1(pBar1) was monitored at 32 °C on plates supplemented with various concentrations of IPTG. Cell growth was inhibited by minigene induction in the *pth*(ts) background at 32 °C (Fig. 1B, vector vs. pBar1) while no effect was observed in the *pth*$^{+}$ background (data not shown). The minigene-dependent growth defect was reversed by the over-expression of tRNA^{Lys} (Fig. 1B, bottom), suggesting that the accumulation of prematurely dropped-off Lysyl-tRNA at the second codon of the minigene (i.e., depletion of free tRNA^{Lys}) caused the inhibition in cell growth.

Over-expression of RF3 in the YW1(pBar1) strain enhanced minigene-dependent growth inhibition (Fig. 1C). Then, conversely, deletion of endogenous RF3 conferred apparent resistance to minigene expression (Fig. 1D). These observations clearly demonstrate that cell-growth inhibition of YW1(pBar1) is dependent on the amount of cellular RF3, thus YW1(pBar1) would be a suitable strain for the genetic selection of novel RF3 mutations that inhibit tRNA drop-off.

3.2. Genetic isolation of drop-off dominant-inhibitory RF3 (RF3^{DDI}) mutations

The YW1(pBar1) strain was transformed by using the mutagenized pRF3^{+} as described above and then was incubated on LB

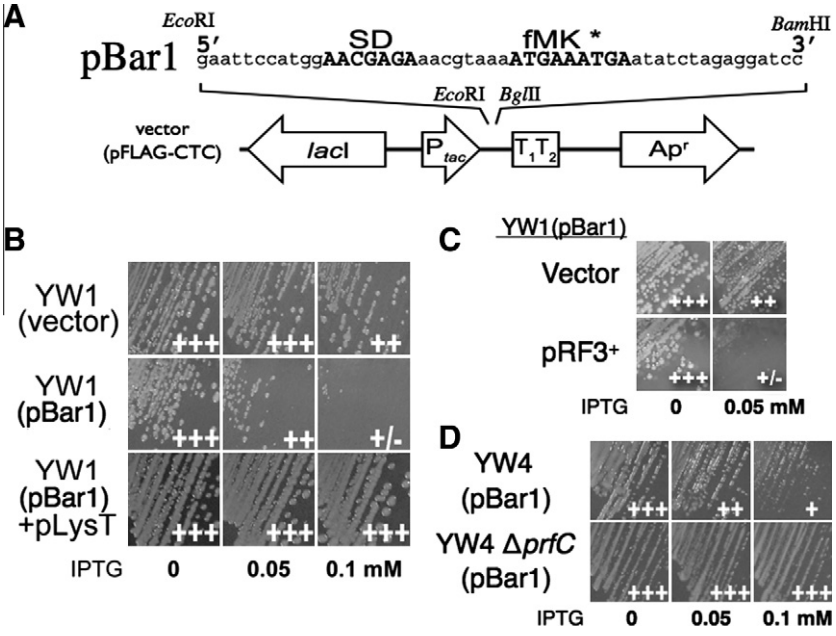

Fig. 1. Construction of the assay strains. (A) Scheme of pBar1 plasmid expressing *bar*A702 minigene. (B) IPTG conditional lethality of YW1 transformant: vector control (top), pBar1 plasmid (middle), pBar1 and pLysT (bottom). (C) Enhancement of tRNA drop-off dependent growth inhibition by RF3 over-expression. (D) Reduction of tRNA drop-off dependent growth inhibition by RF3 knockout. Strains used are YW4 [W3110 *pth*(ts)] (upper) and YW5 [YW4 *prfC*::Tn*10*] (lower).