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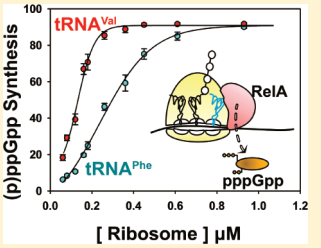
Dependence of RelA-Mediated (p)ppGpp Formation on tRNA Identity

Roshani Payoe and Richard P. Fahlman*

Department of Biochemistry, School of Molecular and Systems Medicine, University of Alberta, Edmonton, Alberta T6G 2H7, Canada

ABSTRACT: The bacterial stringent response is a cellular response to amino acid limitations and is characterized by the accumulation of the alarmone polyphosphate guanosine ((p)ppGpp). A key molecular event leading to (p)ppGpp synthesis is the binding of a deacylated tRNA to the vacant A-Site of a ribosome. The resulting ribosomal complex is recognized by and activates RelA, the (p)ppGpp synthetase. Activated RelA catalyzes (p)ppGpp formation until the deacylated tRNA passively dissociates from the ribosomal A-Site. In this report, we have investigated a novel role for the identity of A-Site bound tRNA in RelA-mediated (p)ppGpp synthesis. A comparison in the stimulation of RelA activity was made using ribosome complexes with either a tightly or weakly binding deacylated tRNA occupying the A-Site. *In vitro* analysis reveals that ribosome complexes formed with tight binding $tRNA^{Val}$ stimulate RelA activity at lower concentrations than that required for ribosome complexes formed with the weaker binding $tRNA^{Phe}$. The data suggest that the recovery from the stringent response may be dependent on the identity of the amino acid that was initially limiting for the bacteria.



The stringent response is a bacterial adaptive response historically linked to amino acid limitations but is also involved in other nutrient limitations and cellular stresses (for recent reviews see refs 1−3). The onset of the stringent response is marked by a rapid accumulation of the effector molecules, pentaphosphate guanosine (5′-triphosphate-3′-diphosphate guanosine or pppGpp) and tetraphosphate guanosine (5′,3′-dibisphosphate guanosine or ppGpp), collectively referred to as (p)ppGpp.[4,5] Despite the historically defined role as a negative regulator of ribosome biosynthesis,[6] transcriptome analysis has revealed that (p)ppGpp affects the expression of a wide range of genes such as the amino acid biosynthetic genes.[7] Global changes in gene expression appears to include mechanisms involving alternative sigma factor stabilization.[8] As a result of modulating gene expression, (p)ppGpp facilitates cell survival by regulating a range of processes within the cell, such as protein degradation,[9] DNA replication,[10] cell division,[11] fatty acid biosynthesis,[12] and biofilm formation.[13] It has also been reported that (p)ppGpp signaling is key to the pathogenicity of some infectious bacteria.[14−16] The regulatory effects of (p)ppGpp can be summarized as down-regulation of proliferation and growth phase processes and up-regulation survival and stationary phase processes.[13,17]

Two proteins are central to the stringent response, RelA and SpoT, which catalyze the synthesis and hydrolysis of (p)ppGpp. In *Escherichia coli*, RelA catalyzes the formation of the vast majority of (p)ppGpp synthesis during amino acid starvation,[18] while SpoT is responsible for basal level (p)ppGpp formation and (p)ppGpp degradation upon cessation of the stringent response.[19] The RelA/SpoT genes have been identified in most eubacterial[20] and some archaea[21] species. Homologues of RelA/SpoT have also been identified in plants.[22] Within the eubacterial kingdom there is an evolutionary dichotomy of RelA and SpoT genes. In some organisms, like *E. coli*, RelA and SpoT are expressed as two individual proteins,[18,23] and in others, like *M. tuberculosis*, there is a single RelA/SpoT fusion protein.[24]

Transfer RNAs (tRNAs) are essential to the bacterial stringent response. The synthesis of (p)ppGpp by RelA is stimulated by ribosomes with an uncharged or deacylated tRNA (deacyl-tRNA) bound in the A-Site. Under growth promoting conditions tRNAs are predominantly aminoacylated, are involved in active protein synthesis, and are rapidly re-aminoacylated by their respective aminoacyl-tRNA synthetases upon release from the ribosome. Under amino acids limiting conditions the deacylated forms of the respective tRNAs rapidly accumulate.[25−27] The resulting ratio of deacyl-tRNA to aminoacyl-tRNA is considered to be an important parameter to the binding of deacyl-tRNAs to the ribosomal A-Site.[26,27] When the concentration of a particular aminoacyl-tRNA:EF-Tu ternary complex is sufficiently depleted, translating ribosomes begin to pause at codons corresponding to the limiting aminoacyl-tRNA. These translanslational pauses present the opportunity for a deacyl-tRNA to bind the vacant ribosomal A-Site. Rapid delivery of aminoacyl-tRNAs to the ribosomsal A-Site by aminoacyl-tRNA:EF-Tu ternary complexes otherwise exclude deacyl-tRNA binding. The binding of the deacyl-tRNA traps the ribosome in complex referred to as the RelA Activating Complex (RAC). RelA is activated upon binding to the RAC and catalyzes the transfer of the γ,β-pyrophosphate from ATP to the 3′-hydroxyl of either GTP or GDP to form pppGpp and ppGpp, respectively.[26] It has been suggested that the formation of (p)ppGpp weakens the affinity of RelA for the RAC and RelA dissociates from the complex.[28] Additionally, it was reported that upon RelA dissociation from the ribosome the deacyl-tRNA remains bound to the ribosomal A-Site.[28] Dissociation of the deacyl-tRNA is a passive and RelA-independent process. The current tRNA-dependent model for RelA-mediated (p)ppGpp synthesis is illustrated in Figure 1. As long as the deacyl-tRNA remains bound to the ribosomal A-Site the cycle of

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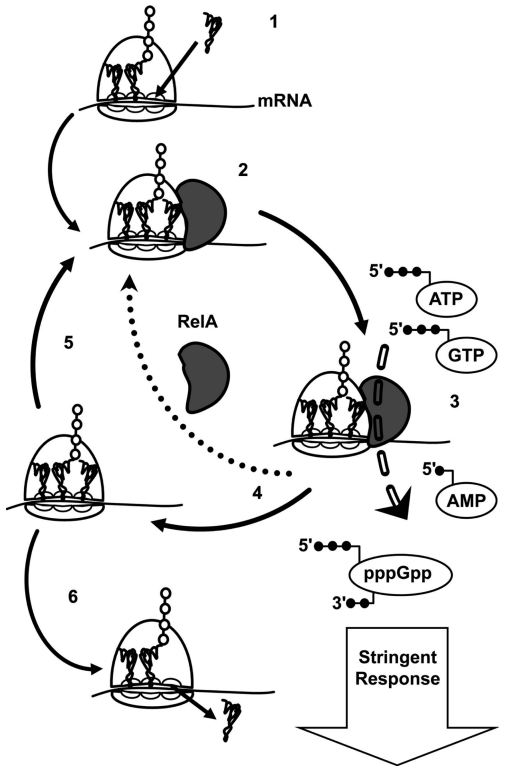

Figure 1. Model for (p)ppGpp synthesis and the stringent response. During amino acid starvation there is an accumulation of deacylated tRNAs in the cytosol (1). A deacylated tRNA binds to the cognate codon in the vacant A-Site and stalls the ribosome (2). RelA binds a stalled ribosome and catalyzes the formation of guanosine pentaphosphate (pppGpp) (3). After pppGpp synthesis RelA dissociates from the ribosome (4). The RelA can rebind or proceed to the next stalled ribosome (5). The cycle repeats until the deacylated tRNA passively dissociates from the ribosomal A-Site, preventing further pppGpp synthesis (6).

RelA binding can continue, and protein synthesis remains stalled as a result of the A-Site bound deacyl-tRNA blocking EF-Tu-dependent delivery of an aminoacylated tRNA.[29]

The passive dissociation of deacyl-tRNAs from the ribosomal A-Site implies that the inherent stability of the RAC plays an important role, not only on the regulation RelA but also on the duration of its activity. The cessation of RelA stimulation results from the passive dissociation of the A-Site bound deacyl-tRNA, a process that also releases the ribosome from the RAC to resume protein synthesis. We have previously demonstrated a significant variation in the rates of A-Site dissociation by deacyl-tRNAs.[30] In light of the variations in dissociation rates, we hypothesized that differences in tRNA binding would be reflected in RelA stimulation.

In this report we compare the *in vitro* stimulation of RelA by RACs formed with tRNAs that either rapidly ($tRNA^{Phe}$) or slowly ($tRNA^{Val}_{2A}$) dissociate from the ribosomal A-Site. These two tRNAs have exhibit A-Site dissociation rate constants that differ by approximately an order of magnitude.[30] The differences in binding leads to a prediction that RelA-mediated synthesis of (p)ppGpp would persist at lower RAC concentrations for complexes containing the tightly binding $tRNA^{Val}_{2A}$, in contrast to RACs containing $tRNA^{Phe}$.

MATERIALS AND METHODS

Ribosome Purification. 70S ribosomes from MRE600 cells were prepared according to previously described procedures.[31] Final ribosome pellets were suspended in ribosomal storage buffer (50 mM Tris-HCl pH 7.5, 100 mM NH_4Cl, 10 mM $MgCl_2$, 6 mM β-mercaptoethanol (β-ME)) and aliquots stored at −80 °C.

Protein Purification. A hexahistidine-tagged clone (ORF: JW2755) of RelA was obtained in a pCA24N plasmid from the National BioResource Project (NIG, Japan):E.coli ASKA collection.[32] RelA was expressed and purified from BL21 cells cultured in Luria−Bertani broth medium containing 25 μg/mL of chloramphenicol. Protein expression was induced with 1 mM isopropyl thio-β-D-galactosidase (IPTG) when the culture reached an OD_{600} = 0.5. The culture was then grown for an additional 4 h at 37 °C. Cells were harvested by centrifugation (5000*g* for 10 min at 4 °C). The cell pellet was washed and suspended in Lysis buffer (25 mM $NaHPO_4$ (pH 7.5), 50 mM NaCl, 5 mM imidazole, 1 mM β-ME, and 1 mM PMSF) and then lysed by sonication. Cell debris was removed by centrifugation (15 000 rpm for 15 min at 4 °C); the cleared lysate was then loaded onto a 1 mL HiTrap FF column (GE Healthcare); the proteins were eluted with a stepwise gradient of binding buffer (lysis buffer without PMSF) to elution buffer (25 mM $NaHPO_4$ (pH 7.5), 50 mM NaCl, 1.5 M imidazole, and 1 mM β-ME). The elution process was as follows: after a 10 mL wash with binding buffer, the elution buffer concentration was increased to 50% for 10 mL; this was followed by 100% elution buffer for the final 10 mL. The elution fractions were collected as 1 mL fractions, and those containing the protein were treated as previously described[33] with the following modifications. Fractions containing RelA were pooled and diluted to twice their original volume with imidazole-free binding buffer. This was done to suspend any RelA proteins that had already precipitated in the fraction tubes. The diluted sample was then dialyzed overnight against dialysis buffer A (10 mM Tris-HCl (pH 8.0), 14 mM $MgCl_2$, 60 mM KCl, 0.5 mM EDTA, 10% (v/v) glycerol, and 10 mM β-ME). Under these conditions RelA precipitates. The precipitate was then dissolved in suspension buffer (10 mM Tris-HCl (pH 8.0), 1 M KCl, 1 mM EDTA, 10% (v/v) glycerol, and 10 mM β-ME) and dialyzed exhaustively against RelA storage buffer (30 mM HEPES-KOH (pH 8.0), 150 mM KCl, 20% (v/v) glycerol, and 10 mM β-ME). Aliquots of the protein were flash-frozen and stored at −80 °C.

Unmodified tRNAs and mRNA Preparation. Purified $tRNA^{Phe}$ from *E. coli* was purchased from Chemical Block (Russia). Unmodified tRNAs and mRNAs were prepared by *in vitro* transcription. The DNA templates for $tRNA^{Val}_{2A}$ and $tRNA^{Phe}$ transcripts were generated by primer extension of overlapping DNA oligonucleotides (IDT) and have been used in previous *in vitro* transcription reactions.[30] The $mRNA^{Val}_{2A}$ (CAAGGAGGUAAAA**AUG**GUCGCACGU) and $mRNA^{Phe}$ (CAAGGAGGUAAAA**AUG**UUCGCACGU) sequences were generated from Milligan's transcription reactions[34] using synthesized DNA oligonucleotide templates. For each of the sequences the AUG start codon for P-Site binding is shown in bold while the A-Site codons are underlined. After transcription, all RNAs were purified by denaturing polyacrylamide gel electrophoresis.

End-Point *in Vitro* pppGpp Synthesis Assay. In a standard (p)ppGpp synthesis assay RelA activating complexes were assembled in the following method. An aliquot containing 10 μM 70S ribosomes was heat activated by incubating at 42 °C for 2 min and then slowly cooled to 22 °C. The 10 μM 70S ribosome stock was prepared in polyamine containing buffer (30 mM HEPES-KOH (pH 8.0), 15 mM $MgCl_2$, 15 mM KCl, 1 mM

β-ME, 50 μM spermine, and 2 mM spermidine). A 9.6 μL sample of heat activated ribosomes were programmed with mRNA ($mRNA^{Val}$ or $mRNA^{Phe}$) by the addition 5.8 μL of 25 μM mRNA and incubated for 2 min at 22 °C. To fully occupy the ribosomal P-Site, the programmed ribosomes were incubated with 5.8 μL of 25 μM $tRNA^{Met}$ for 10 min at 22 °C. Lastly, the appropriate A-Site tRNA (5.8 μL of 25 μM $tRNA^{Val}_{2A}$ or $tRNA^{Phe}$) was added, and the sample was incubated for 1 h at 22 °C. For all the described steps, the added solutions all contained 1× reaction buffer (20 mM HEPES-KOH (pH 8), 15 mM $MgCl_2$, 15 mM KCl, and 1 mM β-ME). All added RNAs were in a 1.5-fold excess with respect to the 70S ribosomes.

Reactions were initiated by the addition of the RAC to mixtures containing 0.18 μM RelA, 1 mM ATP, 0.1 mM GTP, and α[^{32}P]-GTP in 1× reaction buffer. The substrates were added from a 10× substrate mix containing 10 mM ATP, 1 mM GTP, and α[^{32}P]-GTP (1 μL of labeled GTP to 20 μL of unlabeled GTP). The reaction tube was incubated for 1 h (unless otherwise indicated) at 22 °C. Reactions were quenched by the addition of one volume of formic acid (88%) and then stored on dry ice until analysis to minimize hydrolysis of (p)ppGpp. From the quenched samples, 2 μL was removed and used for thin layer chromatography (TLC) analysis as previously described.[28] After the chromatography run, the TLC plates were exposed to a phosphorimager screen which was then visualized and quantified using a phosphorimager (GE Healthcare). ImageQuant and SigmaPlot were used for data analysis and curve fitting, respectively.

Time Course Analysis of (p)ppGpp Synthesis. The (p)ppGpp synthesis assay was carried out and visualized as described for the end-point (p)ppGpp synthesis assay with the exception that the total sample volume was increased to 50 μL to enable 3 μL aliquots to be taken over the course of the reaction.

3′-[^{32}P] Labeling of tRNAs. $tRNA^{Phe}$, $tRNA^{Met}$, and $tRNA^{Val}_{2A}$ were 3′-labeled with [α-^{32}P]-ATP (PerkinElmer) as previously described.[35]

Two-Layer Filter Binding. tRNA dissociation rate from ribosomes in the presence of active RelA was determined using a 96-well filtration protocol to quantify the amounts of ribosome-bound tRNA and unbound tRNA.[36,37] For these experiments the ribosome complexes were assembled in a similar manner to the ribosome complexes described in the (p)ppGpp synthesis assays, with the exception of using 3′-[α-^{32}P]-labeled tRNA for A-Site binding at a 10-fold lower concentration than the ribosomes.

Dissociation experiments were initiated by diluting the samples 40-fold with 1× reaction buffer containing 200 nM unlabeled tRNA as a cold chase. At given time points, 30 μL of the diluted sample were filtered through the nitrocellulose-nylon double layer of membranes and washed with an equal volume of 1× reaction buffer. The membranes were then removed from the filter apparatus and then separated and exposed to a phosphorimager screen for quantification.

RESULTS

The influence of tRNA identity on RelA activity was investigated using an *in vitro* enzymatic assay previously described by Wendrich et al.[28] To perform a comparative study between two tRNA species, the protocol was adjusted appropriately. The major alteration was the use of two 25 nucleotide "mini" mRNA sequences that only vary in sequence at the A-Site codon. The A-Site codons were either **G**UC for $tRNA_{2A}^{Val}$ binding or **U**UC for $tRNA^{Phe}$ binding. Each mRNA, being identically matched to the respective anticodons of each tRNA, facilitates the codon-specific occupation of the respective tRNA into the ribosomal A-Site. Since the mRNAs only differed by a single nucleotide at the first position of the valine and phenylalanine codons, their interaction with the ribosomes is unlikely to differ significantly. The mRNAs contain a Shine−Dalgarno (SD) sequence to facilitate tight 70S ribosome binding and A-Site selectivity.[38] The mRNAs also contain a single AUG codon for positioning the mRNA into the correct reading frame when $tRNA^{Met}$ is bound in the ribosomal P-Site. We have extensively used these mRNA designs for several investigations of tRNA binding to the ribosomal P- and A-Sites.[30,36,39−41]

The two tRNAs chosen to compare RelA stimulation are $tRNA^{Phe}$ and $tRNA^{Val}_{2A}$. Because of extensive use in other investigations of RelA activity,[28] $tRNA^{Phe}$ was chosen as an appropriate control for our comparative study. The second tRNA, $tRNA^{Val}_{2A}$, was chosen for several reasons. $tRNA^{Val}_{2A}$ is known to bind the ribosomal A-Site equally well whether it is deacylated or aminoacylated, in contrast to $tRNA^{Phe}$.[30] $tRNA^{Val}_{2A}$ is a low abundance isoacceptor and is present in *E. coli* at amounts similar to that of $tRNA^{Phe}$.[42] Additionally, $tRNA^{Val}_{2A}$ lacks post-transcriptional modifications in its anticodon stem loop.[43] While modifications in the anticodon stem loops of tRNAs often play significant roles in decoding and ribosome binding,[44,45] modifications within the body of the tRNA are generally for stabilizing tRNA folding.[46] Our previous work demonstrates that $tRNA^{Val}_{2A}$ binds the ribosomal A-Site equivalently regardless of the presence or absence of post-transcriptional modifications when appropriate buffer conditions are used.[30] Others have clearly demonstrated that the post-transcriptional modifications of the $tRNA^{Val}_{1}$ isoacceptor are important for tRNA folding and stability at low magnesium concentrations. The absence of post-transcriptional modifications can be accommodated during *in vitro* studies by having sufficient millimolar concentrations of magnesium ions present in the sample.[47,48] $tRNA^{Phe}$ only exhibits a marginal 2-fold reduction in A-Site binding in the absence of post-transcriptional modifications.[30] Unless noted otherwise, experiments were all performed with *in vitro* transcribed tRNAs and thus lacked all post-transcriptional modifications.

$tRNA^{Val}_{2A}$ Stimulation of RelA Activity. We verified the stimulation of RelA with transcribed $tRNA^{Val}_{2A}$ using the modified *in vitro* (p)ppGpp synthesis assay. The reaction consisted of tight-coupled 70S ribosomes, $mRNA^{Val}$, $tRNA^{Met}$, $tRNA^{Val}_{2A}$, RelA, ATP, and GTP (with [α-^{32}P]-GTP), which were added in a sequential order as described in the Materials and Methods section. The reaction was incubated in reaction buffer for 1 h and then quenched prior to resolving the nucleotides by thin layer chromatography to observe the conversion of [α-^{32}P]-GTP to [α-^{32}P]-(p)ppGpp (Figure 2). In accordance with previous results with $tRNA^{Phe}$,[28] nearly complete conversion of [α-^{32}P]-GTP to [α-^{32}P]-(p)ppGpp can be observed when all of the components are present in the reaction mixture (lane 7). Similar results were obtained when $mRNA^{Phe}$ and $tRNA^{Phe}$ are used in lieu of $mRNA^{Val}$ and $tRNA^{Val}_{2A}$ (data not shown). Our data mimics previous investigations using polyU[28] as an mRNA template, which suggests that the codon context and SD sequence do not significantly alter the RelA reaction. Additionally, basal (p)ppGpp synthesis is observed when individual RNAs or ribosomes are omitted from the reactions (lanes 1−4). This basal activity is a result of the unstimulated activity of *in vitro* purified

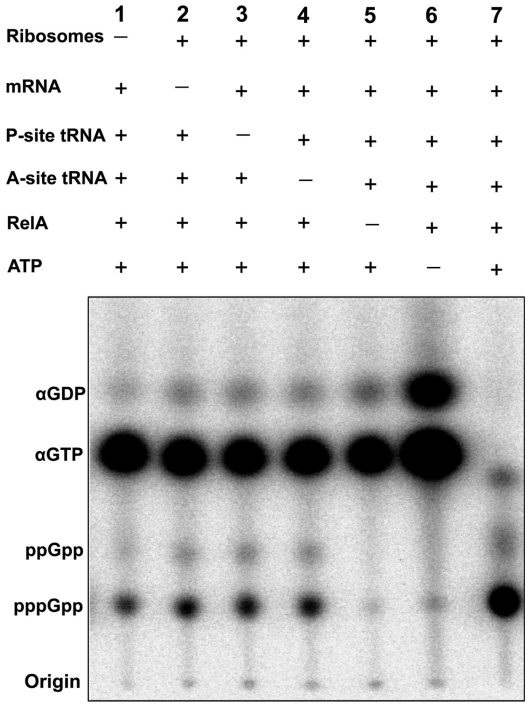

Figure 2. RelA-dependent (p)ppGpp synthesis. (A) RelA-mediated (p)ppGpp synthesis requires the presence of an uncharged $tRNA^{Val}$ in the A-Site of a mRNA programmed ribosome. Thin layer chromatography is used to monitor (p)ppGpp synthesis after incubating the reaction mixtures for 1 h. Samples contain either all reaction components (lane 7) or are missing individual components (lanes 1−6). Maximal (p)ppGpp synthesis was observed only when all components were present (lane 7). Lanes 1−4 reveal basal levels RelA-mediated (p)ppGpp synthesis.

RelA, as activity is not observed in the absence of ATP (lane 6) or RelA (lane 5). This ribosome free stimulation of RelA has also been reported by other investigators[33] and is assumed to be due to the poorly structured C-terminal domain.

Stimulation of RelA Activity by Unmodified tRNAs. A potential effect with lack of tRNA post-transcriptional modifications on the RelA activity was investigated. We compared (p)ppGpp synthesis between reaction mixtures containing RAC formed with either fully modified $tRNA^{Phe}$, purified from *E. coli*, or unmodified $tRNA^{Phe}$, generated by *in vitro* transcription, bound to the ribosomal A-Site. The data in Figure 3 show (p)ppGpp formation for fully modified and unmodified $tRNA^{Phe}$, when used in independent RelA synthesis assays. The data indicate no significant difference in RelA stimulation by the RACs containing either between the modified or unmodified tRNA. Small observed differences in the absolute amounts of (p)ppGpp formation are a result of variations in TLC spotting. All quantification is by measuring the relative ratios of all the observed species in a sample so measurements are independent of spotting volumes.

Passive Dissociation of tRNAs. The mechanism of passive dissociation of the A-Site bound deacylated tRNA as proposed by Wendrich et al.[28] is the basis for our rationale for the investigation on the role of tRNA identity on the duration of RelA stimulation. This current model contradicts the earlier model where tRNA dissociation was either concurrent with or successive to RelA catalysis and dissociation from the RAC.[49] As filter binding

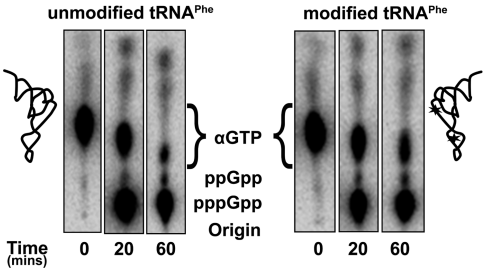

Figure 3. Dependence of tRNA post-transcriptional modifications on RelA activity. RelA-mediated (p)pppGpp synthesis was examined using ribosome complexes form using either purified and post-transcriptionally modified $tRNA^{Phe}$ (right) or unmodified $tRNA^{Phe}$ generated by *in vitro* transcription (left). The reaction was allowed to proceed for 20 or 60 min prior to TLC analysis.

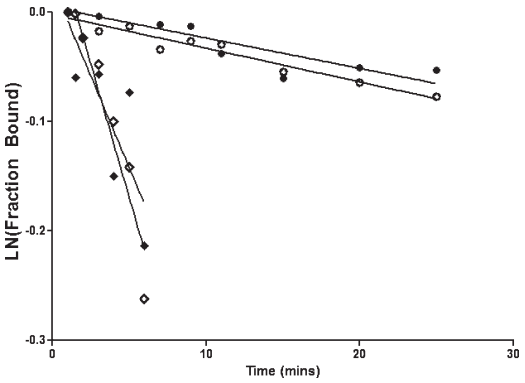

Figure 4. A-Site dissociation of tRNAs in the presence of RelA activity. The dissociation of $tRNA^{Val}$ from the ribosomal A-Site was measured using a double-filter binding assay in the presence (●) and absence (○) of RelA and its nucleotide substrates. The data reveal no measurable change in the dissociation rates. The data shown are from an individual experiment. For comparison, the dissociation of the weakly binding $tRNA^{Phe}$ is also shown in the presence (◆) and absence (◇) of RelA nucleotide substrates.

has been routinely used to quantify tRNA binding to ribosomes,[30,36,40,41] we used a two-layer filter binding assay[50] to validate whether the tRNA release from the A-Site was by passive dissociation or RelA-mediated active ejection after (p)ppGpp formation.

Reaction mixtures containing 70S ribosomes programmed with $mRNA^{Val}$ and $tRNA^{Met}$ in the P-site and 3′-[^{32}P]-labeled tRNA ($tRNA^{Val}_{2A}$ or $tRNA^{Phe}$) in the A-site were prepared as described in the Materials and Methods section. Dissociation was initiated by diluting the sample with an excess of unlabeled tRNA as a cold chase.[36] Aliquots of the diluted sample were collected at different time points and filtered through a nitrocellulose membrane layered on top of a nylon membrane. Ribosomes and bound RNAs were trapped on a nitrocellulose filter, and unbound tRNAs were retained by the second positively charged nylon membrane. Potential RelA-dependent alterations to tRNA dissociation rates were investigated by the inclusion or exclusion of the ATP and GTP substrates. The data in Figure 4 demonstrate that $tRNA^{Val}_{2A}$ dissociation rate from the ribosomal A-Site is unaffected by the presence or absence of the RelA and the triphosphate substrates. Data analysis reveals a dissociation rate constant of $(2.8-3.1) \times 10^{-3}$ min^{-1} from the ribosomal A-Site.

Table 1. Ribosomal A-Site Dissociation Rates in the Presence of RelA[a]

tRNA	+ ATP and GTP	− ATP and GTP
$tRNA^{Val}$	$(2.8 \pm 0.5) \times 10^{-3}$ min^{-1}	$(3.1 \pm 0.2) \times 10^{-3}$ min^{-1}
$tRNA^{Phe}$	$(48 \pm 6) \times 10^{-3}$ min^{-1}	$(21 \pm 12) \times 10^{-3}$ min^{-1}

[a] Dissociation rates of tRNAs from the ribosomal A-Site in the presence of RelA in the presence or absence of the RelA nucleotide substrates. Errors reflect the standard deviation from three independent experiments performed in triplicate.

Identical results were observed in similar experiments comparing the presence and absence of RelA from the reaction mixture. Additionally, the dissociation of $tRNA^{Phe}$ from the ribosomal A-Site is similarly unaffected by RelA and is significantly faster than the dissociation of $tRNA^{Val}_{2A}$. The summarized data from repeated analysis are reported in Table 1. As the dissociation of tRNAs from the ribosomal A-Site is unaltered by the presence or absence of RelA activity, our results concur with the current model that tRNA release from the ribosomal A-Site is independent of RelA dissociation.

Comparing tRNA-Dependent (p)ppGpp Synthesis. At the onset of amino acid starvation, we assume that deacyl-tRNAs are present at saturating concentrations. For this reason, stalled ribosome complexes will equivalently stimulate RelA activity regardless of the identity of the A-site occupying tRNA. We hypothesized that the tRNA identity may be influential in the recovery from the stringent state, i.e., during active amino acid biosynthesis and tRNA aminoacylation. As concentrations of aminoacyl-tRNA rise to levels that can support translation, protein synthesis will not resume until the A-Site occupying deacyl-tRNA dissociates. Since different decay-tRNAs dissociate from the ribosomal A-Site with rates that differ by over an order of magnitude,[30] we propose that slowly dissociating or tight binding tRNAs will form stable RACs that may result in a slower attenuation of RelA-dependent (p)ppGpp synthesis. These tight binding tRNAs are also predicted to result in a greater accumulation of stalled ribosome complexes at lower concentrations of deacylated tRNA.

The stimulation of RelA activity by stalled ribosome complexes formed with either the weakly binding $tRNA^{Phe}$ or tightly binding $tRNA^{Val}_{2A}$ at the A-Site were compared. For experimental comparison ribosomes were the limiting factor during RAC formation as opposed to the tRNAs. If tRNAs were the limiting factor during the association of ribosome complexes, differences in the folding efficiencies of the different tRNAs would be manifested as differences in RelA stimulation. Like many transcribed RNAs it is unlikely that the tRNAs will adopt the correct structure a 100% of the time, it would be expected to observe some quantity of misfolded RNAs in the sample. In addition, the extent of misfolding of the two tRNA sequences cannot be assumed to be equal, and any differences could impact the data. To avoid this challenge, the experiments were designed to be limiting in ribosomes such that the limiting factor is common between both samples.

Ribosome complexes formed with $tRNA^{Val}_{2A}$ stimulate RelA-dependent formation of (p)ppGpp at significantly lower concentrations than ribosome complexes formed with $tRNA^{Phe}$ (Figure 5). A fixed amount of RelA (0.18 μM) was titrated against a range of RAC concentrations. Ribosome complexes were initially formed at high ribosome concentrations (2.6 μM) and then subsequently diluted with reaction mixtures containing

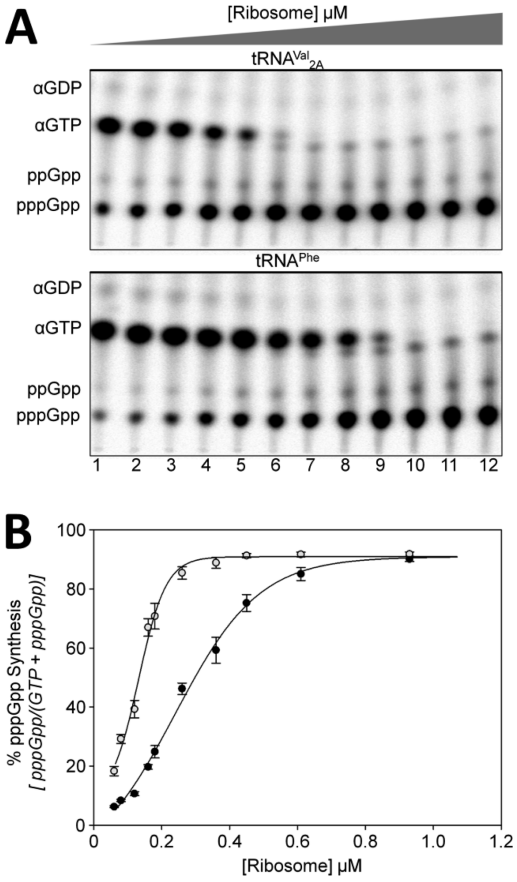

Figure 5. Differential (p)ppGpp synthesis. (A) The formation of (p)ppGpp was determined by thin layer chromatography using a range of concentrations (1 μM to 60 nM) of stalled ribosome complexes containing either $tRNA^{Val}$ (upper panel) or $tRNA^{Phe}$ (lower panel) in the ribosomal A-Site. (B) Summarized data from five independent replicate experiments using ribosomal complexes with either (●) $tRNA^{Phe}$ or (○) $tRNA^{Val}$ in the ribosomal A-Site.

RelA and nucleotide substrates (1 mM ATP, 0.1 mM GTP, and [α-^{32}P]-GTP). Formation of RAC at high concentrations ensures efficient ribosome complex formation. The subsequent dilution of the complex is analogous to when cells are recovering from stringent response, during which the amount of RelA activating complexes gradually decreases as the A-Site tRNA dissociates. The sigmoidal nature of the resulting data is a result of both the background activity of RelA at low ribosome concentrations and the complete substrate depletion in samples containing high ribosome concentrations. Repeat analysis of five independent experiments consistently reveals a more than 2-fold difference in RAC concentrations that effectively stimulate RelA-dependent (p)ppGpp formation (Figure 5B). The difference is highlighted by comparing the ribosome concentrations that result in 50% conversion of [α-^{32}P]-GTP into (p)ppGpp. The RACs formed with $tRNA^{Val}_{2A}$ stimulated RelA activity at concentrations lower than that obtained with similar ribosome complexes formed with $tRNA^{Phe}$. These results are in agreement with our hypothesis that the tighter binding $tRNA^{Val}_{2A}$ forms a stable ribosome complex and can effectively stimulate RelA activity at significantly lower stalled ribosome concentrations.

Time course analysis of (p)ppGpp formation was also performed with samples containing different RAC concentrations

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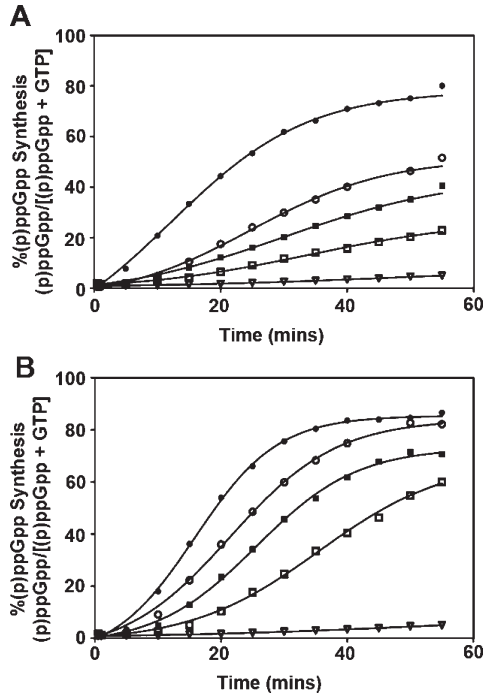

Figure 6. Rates of (p)ppGpp synthesis. RelA-dependent (p)ppGpp formation was measured in the presence of stalled ribosome complexes with either $tRNA^{Phe}$ (A) or $tRNA^{Val}$ (B) in the ribosomal A-Site. Synthesis over time was measured using stalled ribosome complexes that have been diluted to 1.8 (●), 0.63 (○), 0. 45 (■), or 0.25 μM (□). Synthesis in the absence of ribosomes was also quantified (▽).

(Figure 6). As with the experiment in Figure 5, the assembled RAC complexes were diluted prior to addition of RelA. Aliquots for analysis were removed from the samples at increasing time points after the addition of RelA. For RAC formed with either $tRNA^{Phe}$ or $tRNA^{Val}_{2A}$ there was a large enhancement of (p)ppGpp formation in comparison to the no ribosome controls. All samples contained 0.18 μM RelA, and the maximal rates of (p)ppGpp formation were 0.12 μM/min in the absence of RACs and 2.1 and 3.3 μM/min for the samples containing 1.8 μM RACs formed with $tRNA^{Phe}$ or $tRNA^{Val}_{2A}$, respectively. Comparison of the data from diluted RACs assembled with $tRNA^{Phe}$ (Figure 6A) and $tRNA^{Val}_{2A}$ (Figure 6B) reveals significant difference in (p)ppGpp formation. In all cases there appears to be a measurable delay in (p)ppGpp formation upon the addition of RelA; this may be a result of additional steps in RAC recognition by RelA prior to (p)ppGpp formation. As with the data from samples in Figure 5, the more tightly binding $tRNA^{Val}_{2A}$ exhibits enhanced RelA-dependent (p)ppGpp formation.

■ DISCUSSION

Our investigation into RelA stimulation by RACS with a tightly or weakly binding deacyl-tRNA in the ribosomal A-Site was rationalized by two previous observations that pertain to the termination of RelA-mediated (p)ppGpp synthesis. The first was the current model for passive dissociation of deacyl-tRNAs from the ribosomal A-Site for the attenuation of RelA activity (Figure 1). The second was the data demonstrating differences between the dissociation rates of different tRNA species from the ribosomal A-Site. Our hypothesis was that ribosome complexes with slowly dissociating A-site bound deacyl-tRNAs would stimulate RelA activity at lower concentrations than similar ribosomal complexes formed with rapidly dissociating tRNAs. Presented data reveal that the inherent thermodynamic differences to ribosomal A-Site binding by different deacyl-tRNAs are in fact reflected in RelA stimulation.

Stimulation of RelA Activity. Current models proposed that the binding of any deacyl-RNA to the ribosomal A-Site was sufficient to stimulate RelA activity. Therefore, in our initial investigation we examined the ability of $tRNA^{Val}_{2A}$ to stimulate RelA activity, as earlier studies of RelA activity *in vitro* were done using $tRNA^{Phe}$ and polyU mRNA. As clearly demonstrated in Figure 2, all the components including ribosomes, mRNA, P-Site tRNA, and A-Site $tRNA^{Val}_{2A}$, are required to maximally stimulate RelA-dependent (p)ppGpp formation, as was observed when polyU mRNA and $tRNA^{Phe}$ were used instead.[28] These data indicate that $tRNA^{Val}_{2A}$ bound to the A-Site of a stalled ribosome complex is functionally equivalent to $tRNA^{Phe}$ with respect to RelA-dependent (p)ppGpp formation.

Unmodified versus Modified tRNA Stimulation of RelA Activity. When either fully modified or unmodified $tRNA^{Phe}$ occupied the ribosomal A-Site, there was no significant difference in RelA activity, as shown in Figure 3. This outcome was predicted as previous results had reported only a marginal difference in A-Site binding for modified and unmodified $tRNA^{Phe}$.[30] Although, the result suggests that the post-transcriptional modifications do not play a role in RelA activation, it does not rule out the possibility for the tRNAs where post-transcriptional modifications are critical for A-Site binding.[51] If the modifications had a function beyond tRNA binding, we would expect a significantly reduced stimulation of RelA activity for the ribosome complexes formed with unmodified $tRNA^{Phe}$ than for similar complexes formed with fully modified $tRNA^{Phe}$. As previously mentioned, a possible role for the post-transcriptional modifications of $tRNA^{Val}_{2A}$ is unlikely as this tRNA isoacceptor is minimally modified and has no known modifications in the anticodon stem loop[52] nor do the post-transcriptional modifications in the body of the tRNA affect ribosome binding.[30]

tRNA Dissociation. Wendrich et al. had indirectly demonstrated, with $tRNA^{Phe}$, that the tRNA passively dissociates from the ribosomal A-Site and RelA activity does not enhance or catalyze its dissociation. As deacyl-$tRNA^{Phe}$ weakly binds the ribosomal A-Site, we investigated the dissociation of a tight binding tRNA in the presence and absence of active RelA. It was postulated that RelA could enhance the dissociation of tight binding tRNAs to match that of a rapidly dissociating tRNAs. The data presented in Figure 4 and summarized in Table 1 reveal no dependence of A-Site dissociation by $tRNA^{Val}$ on RelA activity. These data demonstrate that RelA does not participate in the dissociation or destabilization of tightly binding tRNAs as has been previously demonstrated for a weakly binding tRNA.[28]

Attenuation of RelA Activity. Time scales for tRNA dissociation are on the order of magnitude pertinent to bacterial responses and growth. If the typical half-lives for tRNAs bound to the ribosomal A-Site were on the order of seconds or milliseconds, differences between tRNAs would be insignificant with respect to cellular responses and growth. The reported half-lives for different tRNAs at the ribosomal A-Site range from 13 to 210 min,[30] which is a time scale relevant to bacterial growth. The longer half-lives surpass that of the doubling time of bacteria like *E. coli* during log phase growth in rich media and could be extremely detrimental if a significant number of ribosomes are trapped as stalled complexes. The toxicity of stalling a significant

portion of ribosomes by deacyl-tRNA binding would be analogous to treatment with a ribosome targeting antibiotic such as tetracycline. Deacyl-tRNA dissociation rates *in vivo* may be significantly faster than the *in vitro* measured rates as a result of differences in ion concentrations. Nonetheless, even rates 2 orders of magnitude faster, for the slowly dissociating tRNAs, would result in significant stalling times of translating ribosomes. These slow tRNA dissociation rates were a major reason for the evaluation of whether RelA activity enhances tRNA dissociation rates.

The proposed model for RelA[28] suggests that during the recovery from the stringent response the tRNA passively dissociates from the ribosomal A-Site to be aminoacylated by the respective aminoacyl-tRNA synthetase returning it into a substrate for active translation. During recovery from the stringent response there is a reintroduction of the limiting amino acids through the changes to the cellular metabolism and gene expression to support amino acid biosynthesis. This results in a dwindling amount of stalled ribosome complexes (or RACs) as the complexes passively dissociate and the deacyl-tRNAs are returned to active translation by aminoacylation. To mimic this decreasing abundance of RACs in the recovery phase in cells *in vitro*, a dilution procedure was used to emulate the dissociation model. Ribosome complexes, or RelA activating complexes (RACs), were initially formed at high concentrations to support maximal association. At high concentrations, the RACs stimulate RelA activity regardless of the identity of the tRNA bound to the ribosomal A-Site (Figure 5). The RACs are then diluted so the A-Site bound deacyl-tRNA can dissociate as the A-Site tRNA is generally the weakest binding component.[30,53] Comparative analysis of the stimulation of RelA activity by ribosome complexes formed with either $tRNA^{Phe}$ or $tRNA^{Val}_{2A}$ in the A-Site was performed using this dilution method.

Comparison of RelA-mediated (p)ppGpp formation (Figures 5 and 6) reveals that RAC formed with $tRNA^{Val}_{2A}$ more robustly stimulated RelA activity after dilution than for RACs formed with $tRNA^{Phe}$. There is complete conversion of [^{32}P]-GTP to (p)ppGpp at lower ribosome concentrations when $tRNA^{Val}_{2A}$ occupies the ribosomal A-Site (Figure 5). Moreover, $tRNA^{Val}_{2A}$ containing ribosome complexes resulted in higher levels of (p)ppGpp formation at all concentrations below 930 nM. Even at larger dilutions of ribosome complexes (to 60 nM), significant (~20%) conversion of GTP to (p)ppGpp is observed using ribosome complexes containing $tRNA^{Val}_{2A}$ in contrast to $tRNA^{Phe}$ where (p)ppGpp formation is essentially at background levels. Time course analysis (Figure 6) reveals reduced rates of (p)ppGpp synthesis after dilution of RACs formed with $tRNA^{Phe}$ in comparison to RACs forms with $tRNA^{Val}_{2A}$ in the ribosomal A-Site. All comparisons clearly demonstrate that at reduced RAC concentrations the $tRNA^{Val}_{2A}$ containing ribosome complexes are more potent stimulators of RelA activity. This difference at the lower concentrations of RACS would be the most significant *in vivo*. During the onset of the conditions leading to the stringent response there would be a gradual accumulation of the RACs until the initiation of the stringent response.

The absolute differences between the RelA stimulation by RACs assembled with $tRNA^{Phe}$ or $tRNA^{Val}_{2A}$ cannot be interpreted as a direct measurement reflecting the thermodynamic parameters of binding. The RelA-mediated (p)ppGpp synthesis assay is a composite of molecular processes, such as tRNA dissociation, RelA binding and dissociation, and RelA-catalyzed (p)ppGpp formation. The observed difference between the (p)ppGpp synthesis assays with the different tRNAs can therefore only be compared relatively. As consequence of being a multicomponent system, the absolute differences observed can be magnified or minimized by altering experimental parameters, such as the incubation time after ribosomal dilution and the addition of RelA. In light of these contributory factors, it is not unexpected that differences in binding affinity of the two tRNAs do not numerically match the differences between the RelA activity for reactions consisting of the different tRNAs.

Biological Implications. Differences in tRNA dissociation and subsequent stimulation of RelA activity have implications to the regulation of the metabolic pathways of *E. coli*. Our data predict that the absence or withdrawal of certain amino acid(s) will result in a more severe or long-lasting activation of the stringent response as the respective deacyl-tRNAs will dissociate with significantly slower rates from the ribosomal A-Site. Our observations are adding to the developing complexity emerging with respect to the stringent response and individual tRNA sequences.[25,54,55]

AUTHOR INFORMATION

Corresponding Author

*E-mail: rfahlman@ualberta.ca. Phone: (780)492-9566. Fax: (780)492-0886.

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ABREVIATIONS

Aminoacyl-tRNA,aminoacylated tRNA; deacyl-tRNA,deacylated tRNA; ppGpp,5′,3′-dibisphosphate guanosine; pppGpp,5′-triphosphate-3′-diphosphate guanosine; RAC,RelA activating complex.

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