Distinct Roles for Release Factor 1 and Release Factor 2 in Translational Quality Control*

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Background: Bacteria have two similar release factors: release factor 1 and release factor 2 for terminating protein synthesis. Translation terminates prematurely following a misincorporation event.

Results: Release factor 2 preferentially recognizes mismatched ribosomal complexes.

Conclusion: Release factor 1 and release factor 2 have evolved to carry out distinct functions beyond canonical termination.

Significance: Release factor 2 is a key translational quality control factor in bacteria.

In bacteria, stop codons are recognized by two similar class 1 release factors, release factor 1 (RF1) and release factor 2 (RF2). Normally, during termination, the class 2 release factor 3 (RF3), a GTPase, functions downstream of peptide release where it accelerates the dissociation of RF1/RF2 prior to ribosome recycling. In addition to their canonical function in termination, both classes of release factor are also involved in a post peptidyl transfer quality control (post PT QC) mechanism where the termination factors recognize mismatched (i.e. error-containing) ribosome complexes and promote premature termination. Here, using a well defined in vitro system, we explored the role of release factors in canonical termination and post PT QC. As reported previously, during canonical termination, RF1 and RF2 recognize stop codons in a similar manner, and RF3 accelerates their rate of dissociation. During post PT QC, only RF2 (and not RF1) effectively binds to mismatched ribosome complexes; and whereas the addition of RF3 to RF2 increased its rate of release on mismatched complexes, the addition of RF3 to RF1 inhibited its rate of release but increased the rate of peptidyl-tRNA dissociation. Our data strongly suggest that RF2, in addition to its primary role in peptide release, functions as the principle factor for post PT QC.

In vivo experiments nicely support the biochemical data suggesting that both factors promote termination through induced-fit mechanisms (6, 13–15).

RF3 is the bacterial class 2 RF. Like its eukaryotic and archaeal counterparts, RF3 is a GTPase, but appears to fulfill a distinct function in bacteria relative to the other two kingdoms (16). Whereas early studies hinted at an integral role for RF3 during the catalysis of peptide release (17–19), later studies suggest a role for this factor downstream of termination (20). RF3 uses the energy from GTP hydrolysis to accelerate the dissociation of the class I RFs following peptide release but has no effect on the maximal rate ($k_{cat}$) of release (4, 20).

In addition to their function in canonical release, both classes of RF are involved in a post peptidyl transfer quality control (post PT QC) mechanism (21). During post PT QC, a misincorporation event that results in a mismatched tRNA/mRNA interaction in the P site of the ribosome leads to substantial losses in fidelity for subsequent decoding steps. These mismatched complexes are ultimately recognized by release factors and protein synthesis terminates prematurely. Interestingly, whereas RF3 does not affect the rate of release by class I RFs on stop codons (21), it significantly accelerates the rate of premature release by RF2 on mismatched complexes (21). We note that RF1 was not tested extensively for a role in post PT QC in these early studies. In vivo experiments nicely support the biochemistry and establish a role for RF3 in fidelity during translation (22); deletion of RF3 has profound effects on premature termination and gene expression especially in error-prone backgrounds. Interestingly, RF3 is nonessential and is only

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‡ The abbreviations used are: RF, release factor; GDP-PNP, guanosine 5’-[(β,γ-imino)diphosphate; post PT QC, post peptidyl transfer quality control; RNC, ribosomal nascent chain; EFG, elongation factor G; IF, initiation factor; f-Met, N-formylmethionine.
found in a subset of bacteria (16), consistent with the idea that its primary function is in quality control rather than normal termination.

Here we sought to clarify the involvement of the RFs in these various processes using a well defined in vitro system. We show that whereas RF1 and RF2 display similar behaviors during canonical stop codon recognition, RF2 appears to be more efficient and broadly competent at promoting termination on mismatched complexes (post PT QC). These data suggest that RF2, in addition to its participation in canonical release, functions as a specialized quality control factor with RF3.

**EXPERIMENTAL PROCEDURES**

**Buffers and Reagents**—All assays were performed in buffer A (50 mM HEPES, pH 7.6, 70 mM NH₄Cl, 30 mM KCl, 10 mM MgCl₂, 5 mM β-mercaptoethanol). *Escherichia coli* MRE600 (ATCC29417) was overexpressed in BL21(DE3) cells, and the protein was purified on a 5-ml His-Trap FF FPLC column (GE Healthcare) as described previously (24). IF1 and IF2 (3 nM each), GTP (2 mM), and mRNA (60 μM) were transcribed by T7 RNA polymerase from double-stranded DNA templates (27). tRNAs were charged as described previously (21).

**Formation of Ribosomal Complexes**—The initiator fMet-tRNAfMet (3 μM) was enzymatically loaded into the P site by incubating with 70S ribosomes (2 μM), IF1, IF2, IF3 (3 μM each), GTP (2 mM), and mRNA (6 μM) in buffer A at 37 °C for 45 min. The initiation complex was mixed with preincubated ternary complex containing EFTu (15 μM), EFG (6 μM), GTP (2 mM), and Lys-tRNAlys (6 μM). The mixture was incubated for 5 min at 37 °C and purified from unincorporated tRNAs and factors over a sucrose cushion (1.1 M sucrose, 20 mM Tris-HCl, pH 7.5, 500 mM NH₄Cl, 10 mM MgCl₂, 0.5 mM EDTA) and spun at 70,000 rpm in a TLA 100.3 rotor for 2 h.

**Release Assays**—The experiments were performed by mixing the pre-formed ribosomal complexes (0.4 μM) and the release factors (10 μM) RF1 or RF2 with or without excess of RF3 (15 μM). Reactions were quenched with 6% formic acid. The hydrolized products F-[³⁵S]Met (N-formylmethionine) were separated from ribosome complex containing F-[³⁵S]Met-tRNA by electrophoresis on TLC-cellulose plates at 1200 V for 20 min in pyridine acetate buffer, pH 2.8. The fraction from the release product f-[³⁵S]Met versus the initial ribosomal complex were plotted, and the curves were fit to single exponential equations to obtain the rate constant for the reaction.

**Binding Assays**—Reactions were incubated in buffer A containing 0.4 μM ribosom complexes (MKX or MKY, pre-formed as in Ref. 21), and 1 μM His-tagged RF1 or RF2 for 10 min at 37 °C. Reactions were pelleted through 600 μl of buffer D (1 M sucrose, 20 mM Tris-HCl, pH 7.5, 500 mM NH₄Cl, 10 mM MgCl₂, 0.5 mM EDTA) for 2 h at 70,000 rpm in a TLA 100.3 rotor. Resulting pellets were resuspended in buffer A and analyzed via Western blotting with His5-HRP conjugate antibody.

**RESULTS**

**RF1 and RF2 Act Differently on Mismatched Complexes**—We first established that RF1 and RF2 behave similarly during canonical termination in our in vitro system as reported previously (13). Ribosomal nascent chain complexes (RNCs) programmed with an mRNA encoding Met-Lys-Stop (MKX) and carrying fMet-tRNAfMet in the P site (Fig. 1A) were generated and reacted with excess RF1/RF2 in the absence or presence of RF3 and GTP. As anticipated (13, 21), we observed essentially no change in the kcat of release when RF3 was added to the reaction (Fig. 1B).

We next evaluated the effects of RF3 on a multiple turnover release reaction where RF3 is thought to accelerate the rate of dissociation of RF1/RF2 (4, 20). RF1 and RF2 (3 nM) were provided in substoichiometric amounts relative to RNC (25 nM). In the absence of RF3, apparent rates of release of 0.0011 s⁻¹ and 0.0041 s⁻¹ were measured for RF1 and RF2, respectively. The addition of RF3-GTP accelerated the apparent rates of release for both factors (~10-fold for RF1 and ~5-fold for RF2) (Fig. 1C and Table 1), as observed previously (4, 20). We note that the rates of release under these multiple-turnover conditions (in the presence of RF3) are much slower than the single-turnover rates, suggesting that under these conditions, dissociation of RF1 and RF2 is rate-limiting.

Having established that both factors behave equivalently in our reconstituted systems during canonical termination, we next determined the release activity of RF1 and RF2 on mismatched complexes. We prepared matched and mismatched dipeptidyl-tRNA complexes as described earlier (21); for example, a mismatched complex was generated by forcing Lys-tRNAlys to decode the near cognate asparagine AAU codon (Fig. 2A) and a phenylalanine codon (UUU) was positioned in the A site. These complexes were reacted with 10 μM RF1 and RF2 in the absence or the presence of 15 μM RF3 and 2 mM GTP. As reported previously (13, 21, 28), RF1 did not promote release (hydrolysis) of the dipeptidyl product from the dipeptidyl-tRNA on the matched MKF complex, even in the presence of RF3 (Fig. 2B, left). However, whereas RF2 on its own also failed to promote hydrolysis on the mismatched complex, the addition of RF3 resulted in observable hydrolysis (Fig. 2B, right, filled symbols). These data hint at a difference in the biochemical behaviors of RF1 and RF2.

We next evaluated release activity on the mismatched RNCs. As reported previously (21), RF2 promotes release on the mismatched MNF complex, and RF3 increases the rate of this reaction by almost an order of magnitude (Fig. 2C, right). As reported previously (13), RF2, RF1 (and not RF2) may be codon-dependent, perhaps as a consequence of distinct motifs in the stop codon-recognizing domain 2 of the two factors (5).
Reactions were carried out in buffer A at 37 °C. Apparent rates of release for various complexes of RF1 and RF2 are shown in Table 1.

**TABLE 1**

| Complex | A site | $k_{app}$ (s$^{-1}$) | End point | $k_{app}$ (s$^{-1}$) | End point |
|---------|--------|---------------------|-----------|---------------------|-----------|
| MKX (excess) | UAA | 0.085 ± 0.0080 | 0.76 ± 0.022 | 0.14 ± 0.030 | 0.80 ± 0.044 |
| MKX (substoich.) | UAA | 0.0011 ± 0.00024 | 0.81 ± 0.091 | 0.0091 ± 0.0017 | 0.70 ± 0.035 |
| MKF | UUU | ND$^a$ | ND | ND | ND |
| MNF | UUU | ND | ND | ND | ND |
| MKI | AUC | ND | ND | ND | ND |
| MNI | AUC | 0.0038 ± 0.00036 | 0.9668 ± 0.031 | 0.015 ± 0.0020 | 0.42 ± 0.018 |
| MKD | GAU | ND | ND | ND | ND |
| MND | GAU | 0.0004 ± 0.00027 | 0.92 ± 0.38 | 0.0030 ± 0.00034 | 0.18 ± 0.0073 |
| MKG | GCC | ND | ND | ND | ND |
| MNG | GCC | ND | ND | ND | ND |
| MKP | CGG | ND | ND | ND | ND |
| MNP | CCG | ND | ND | ND | ND |
| MKV | GUG | ND | ND | ND | ND |
| MNY | GUG | ND | ND | ND | ND |
| MKA | GCC | ND | ND | ND | ND |
| MNA | GCC | ND | ND | ND | ND |
| MKL | UUG$^b$ | 0.0030 ± 0.00052 | 0.97 ± 0.16 | 0.012 ± 0.0044 | 0.89 ± 0.056 |
| MNL | UUG$^b$ | 0.0029 ± 0.0002 | 0.74 ± 0.24 | 0.010 ± 0.0038 | 0.49 ± 0.002 |
| MKY | UAC$^c$ | 0.0043 ± 0.00026 | 0.71 ± 0.011 | 0.017 ± 0.0077 | 0.69 ± 0.0073 |

$^a$ ND indicates the rates were immeasurably slow.
$^b$ UUG is a near-stop codon for RF1.
$^c$ UAC is a near-stop codon for RF1 and RF2.

**FIGURE 1.**

**A** Canonical termination by RF1 and RF2. A, schematic of the peptide release reaction depicting the dipeptidyl ribosomal nascent chains and release factors used in our assay. B, time courses of peptide release in the presence of substoichiometric amounts of RF1 and RF2. The addition of RF3 increases the apparent rate of release for both RF1 and RF2.

B, time courses of peptide release in the presence of saturating amounts of RF1 and RF2. The addition of RF3 has no effects on the release activity. C, time courses of peptide release in the presence of substoichiometric amounts of RF1 and RF2. The addition of RF3 increases the apparent rate of release for both RF1 and RF2.
A Central Role for RF2 in Quality Control

To further explore the involvement of RF1 in post PT QC, we prepared a series of RNCs with different sense codons positioned in the A site. RNCs with an isoleucine codon (AUC) in

the A site were reacted with RF1 and RF2 in the absence and the presence of RF3 (Fig. 2C). As expected, neither RF1 nor RF2 promoted substantial release on the matched MKI complex, although RF3 did modestly stimulate release by RF2 on this complex (Fig. 2D, right, filled symbols). On the mismatched MNI complex, RF1 only appeared to promote release of the dipeptide with a rate similar to that observed for RF2 (Fig. 2D, open circles, and Table 1). Interestingly, however, whereas the addition of RF3 resulted in an increase in RF2-mediated release, the addition of RF3 to the RF1 reaction resulted in a reproducible drop in its end point (Fig. 2D). We next prepared another pair of matched and mismatched complexes with a glutamate codon (GAU) positioned in the A site (MKD and MND, respectively) (Fig. 2E). Again, neither RF1 nor RF2 promoted release on the matched MKD complex, and the addition of RF3 had modest effects only on the RF2-mediated reaction (Fig. 2F, right, filled symbols). For the mismatched MND complex, RF1 stimulated the rate of release only modestly relative to the matched complex, and as observed with the MNI complex, the addition of RF3 diminished the end point of this reaction (Fig. 2F, left, open symbols). By contrast, RF2-mediated release on this mismatched complex (MND) was substantial and the addition of RF3 further increased this rate (Fig. 2F, right, open symbols). These data, together with those presented in Fig. 2B, suggest that the interaction of RF1 with mismatched complexes has a strong dependence on the A site codon.

To provide further support for this hypothesis, we prepared five new pairs of matched and mismatched complexes displaying various A site codons that included a glycine GGC codon, a proline CCG codon, a valine GUG codon, an alanine GCC codon, and a leucine UUG codon (Table 1). Except for the UUG complex, which displays a near-stop codon for RF1, all matched complexes failed to react with either factor. Moreover, as seen earlier, the addition of RF3 had only modest effects on premature termination on these complexes. As seen in the MNF and MND complexes (above), RF1 alone did not promote release on mismatched complexes, and the addition of RF3 only slightly stimulated the rate of release as compared with those measured on matched complexes; additionally, these reactions exhibited end point defects (Table 1). RF2, however, displayed robust release activity on all mismatched complexes, and the addition of RF3 increased the rate of premature termination to levels comparable with those observed on authentic termination complexes (Table 1). These observations suggest that RF2 is principally involved in the recognition of mismatched complexes, highlighting a distinct role for this factor during post PT QC.

RF1 and RF3 Promote Peptidyl-tRNA Dissociation—The observed decreases in end point triggered by RF3 on the RF1-mediated termination reactions (for the MNI and MND mismatched complexes) suggest the possibility of an RF3-dependent induction of a competing process. RF3 has previously been shown to stimulate the dissociation of peptidyl-tRNA from ribosomal complexes (29), a phenomenon that could readily explain the observed end point defects. We asked whether RF3 promotes peptidyl-tRNA dissociation from mismatched complexes by utilizing peptidyl-tRNA hydrolase to determine the

FIGURE 2. RF1 promotes peptide release on certain mismatched complexes, but the addition of RF3 has a deleterious effect on its activity. A, schematic of the matched MKF and mismatched MNF ribosomal nascent chains. B, time courses of peptide release for the denoted complexes in the presence of the indicated RFs. C, schematic representation of the matched MKI and mismatched MNI ribosomal nascent chains. D, time courses of peptide release for the MKI and MNI complexes in the presence of the indicated RFs. E, schematic representation of the matched MKD and mismatched MND ribosomal nascent chains. F, time courses of peptide release for the MKD and MND complexes in the presence of the indicated RFs.
amount of nonribosome bound peptidyl-tRNA in the various reactions (26).

Briefly, the mismatched MND complex was incubated in the presence of RF1 in the absence or presence of peptidyl-hydrolyase. The fraction of released product observed in the absence of peptidyl-hydrolyase was subtracted from that observed in the presence of peptidyl-hydrolyase to correct for the release activity by RFs. Indeed, we see that RF1 on its own promotes the dissociation of peptidyl-tRNA from the mismatched MND complex (Fig. 3). Moreover, when the release activity of RF1 is eliminated (through the use of a catalytically dead GAQ variant), the peptidyl-tRNA dissociation reaction proceeds even more efficiently (Fig. 3). The addition of RF3 was found to accelerate the rate of peptidyl-tRNA dissociation (by ~4-fold) such that the reaction proceeded nearly to completion (Fig. 3, left). As seen earlier (21), we observed little to no peptidyl-tRNA dissociation in the presence of RF2 (Fig. 4A, right).

RF2 Has Higher Affinity for Nonstop Complexes Than RF1—In light of differences that we observed between RF1 and RF2 during post PT QC, we next characterized the binding interactions between the RFs and various ribosomal complexes. For this experiment, matched stop codon (UAA) complexes were prepared as earlier (MKX, Fig. 1A) as well as matched near-stop codon complexes with UAC (tyrosine) positioned in the A site. The reactivity of the complexes with the RFs was first tested. As seen previously (13), RF1 and RF2 catalyzed the release reaction 10-fold more rapidly on the stop than on the near-stop complex, although both reactions proceeded to completion (Fig. 4A and Table 1). Furthermore, as reported earlier (13), the addition of RF3 increased the rate of release by ~4-fold on the near-stop complex.

To assess the binding interaction between the RFs and the RNCs qualitatively, we utilized a pelleting assay (4, 30). Briefly, RNCs were incubated with His-tagged RF, and the complexes were pelleted over a sucrose cushion. The fraction of ribosome-bound RF was determined using Western analysis (with anti-His antibody) where input was compared with the pelleted sample. As anticipated, both factors were found to associate quantitatively with the authentic stop codon complex. However, whereas RF1 failed to associate with the near-stop codon complex, RF2 was observed to associate strongly with this complex (Fig. 4B).
A Central Role for RF2 in Quality Control

**FIGURE 5. Dependence of premature termination on GTP hydrolysis by RF3.**

A, time courses of peptide release on the MKX complex in the presence of substoichiometric amounts of RF2 in the absence and presence of RF3 and GDP-PNP. Inhibition of GTP hydrolysis has no effect on RF3-mediated dissociation of RF1, RF2, or RF3 in the presence of GTP but not its hydrolysis (Figs. 3 and 5). These observations reinforce recent reports demonstrating that only GTP-bound RF3 binds the ribosome (4). Interestingly, our data suggest that after this initial binding event, downstream events diverge as function of the status of the ribosomal complex and the identity of the release factor. In particular, when there is a deacylated P-site tRNA, i.e. following canonical release, RF3 facilitates dissociation of the class 1 RF, independent of its identity. In the presence of a peptidyl-tRNA bound in the P site in the context of a matched complex with a near-stop codon in the A site, RF3 again accelerates the rate of premature termination independent of its identity of the class 1 RF. However, in the presence of a mismatched complex, RF3 appears to interact differently with RF1 and RF2; the downstream outcome is premature termination with RF2 and peptidyl-tRNA dissociation with RF1. Attempts to understand the structural/sequence elements that distinguish the two factors during post PT QC proved to be difficult. Specifically, our efforts to introduce RF2 domains into RF1 did not change the behavior of this factor in premature termination (data not shown). Further structural and biochemical studies are likely to illuminate these differences and how they facilitate the dominant role of RF2 in quality control.

**DISSCUSSION**

Biochemical characterization of the bacterial release factors RF1 and RF2 during canonical termination and quality control (post PT QC) has revealed unexpected differences between the two factors. During canonical stop codon recognition, the factors appear to behave very similarly. Both factors promote release with a maximal rate that does not change with the addition of RF3, consistent with earlier observations (4, 13, 21). Furthermore, the addition of RF3 was found to promote the dissociation of both factors following peptide release on stop codons with nearly equivalent rates in our recycling assays (Fig. 1C). Consistent with these similarities and earlier reports (13), both RF1 and RF2 appear to achieve their accuracy primarily by utilizing differences in rate constants for release on stop codon and near-stop codon complexes. The addition of RF3 to termination reactions appears to have a slightly adverse effect on accuracy on so-called matched complexes (Figs. 1 and 4), for which the rate of premature termination was measured to be 2–4-fold higher in the presence of RF3.

In contrast to these observations, during mismatched complex recognition the factors appear to behave distinctly. RF2 effectively acted on all tested mismatched complexes, and the addition of RF3 increased the rate of premature termination by nearly an order of magnitude (Fig. 2). However, RF1 effectively recognized only a subset of mismatched complexes, and the addition of RF3 tended to decrease the rate of premature termination (and increase the rate of peptidyl-tRNA dissociation) (Figs. 2 and 3). These observations highlight a profound distinction between RF1 and RF2 during noncanonical termination, for which our data suggest that in addition to its primary role in canonical termination, RF2 plays a key role in post PT QC.

Similar to RF3-dependent dissociation of RFs during canonical release (4, 20), RF3-dependent acceleration of premature termination by RF2 or peptidyl-tRNA dissociation is dependent on the presence of GTP but not its hydrolysis (Figs. 3 and 5). These observations reinforce recent reports demonstrating that only GTP-bound RF3 binds the ribosome (4). Interestingly, our data suggest that after this initial binding event, downstream events diverge as function of the status of the ribosomal complex and the identity of the release factor. In particular, when there is a deacylated P-site tRNA, i.e. following canonical release, RF3 facilitates dissociation of the class 1 RF, independent of its identity. In the presence of a peptidyl-tRNA bound in the P site in the context of a matched complex with a near-stop codon in the A site, RF3 again accelerates the rate of premature termination independent of the identity of the class 1 RF. However, in the presence of a mismatched complex, RF3 appears to interact differently with RF1 and RF2; the downstream outcome is premature termination with RF2 and peptidyl-tRNA dissociation with RF1. Attempts to understand the structural/sequence elements that distinguish the two factors during post PT QC proved to be difficult. Specifically, our efforts to introduce RF2 domains into RF1 did not change the behavior of this factor in premature termination (data not shown). Further structural and biochemical studies are likely to illuminate these differences and how they facilitate the dominant role of RF2 in quality control.

The identification of specificity differences between RF1 and RF2 are not without precedent. Previous reports indicated that certain ribosomal mutations have differential effects on termination by RF1 and RF2 (32); mutations in helix 69 of the large subunit inhibited RF2-mediated release on UGA codons but
not RF1-mediated release on UAA and UAG. Further support for these ideas comes from an earlier report showing that RF1 and RF2 utilize different strategies to achieve accuracy during termination (13); RF2 utilizes kinetic discrimination whereas RF1 utilizes thermodynamic discrimination. More specifically, the $K_{\text{cat}}$ differences between stop codon and near-stop codons are smaller for RF2 relative to RF1; in contrast, the $k_{\text{cat}}$ differences are higher for RF2 relative to RF1. These observations suggest that RF2 is more likely to interact with noncanonical termination complexes, but under normal conditions, this interaction is unproductive. We reason that the addition of RF3 induces a conformational change on RF2 that increases the likelihood of a productive interaction forming between RF2 and the ribosome. Consistent with these ideas, we found that RF2 has higher affinity for near-stop complexes than RF1 (Fig. 4).

Our data are also consistent with studies indicating that RF2 functions in a different quality control process, during which it works cooperatively with the bacterial factor ArfA to rescue stalled ribosomes (33, 34). We note that RF1 is nonessential for E. coli, admittedly after some engineering, even in the presence of the ∼200 amber (UAG) codons in the genome of the bacterium (35). Further studies are likely to illuminate the complexities of the roles of these various factors in ribosome function and rescue. It is interesting to note that despite fundamental differences between bacterial and eukaryotic termination, quality control processes in both domains of life have evolved to utilize release factors themselves, or RF-like proteins, for quality control. In euukaryotes, for example, multiple mRNA surveillance pathways depend on the eRF1- and eRF3-related factors Dom34 and Hbs1 for ribosome rescue (36). Rationally, for all of these processes, the signals for crisis and rescue must originate in the decoding center of the ribosome where decisions of this nature are made.

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