Functional Interactions between the G′ Subdomain of Bacterial Translation Factor EF-G and Ribosomal Protein L7/L12* [S]

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Protein L7/L12 of the bacterial ribosome plays an important role in activating the GTP hydrolytic activity of elongation factor G (EF-G), which promotes ribosomal translocation during protein synthesis. Previously, we cross-linked L7/L12 from two residues (209 and 231) flanking α-helix A\(_{G}\) in the G′ subdomain of *Escherichia coli* EF-G. Here we report kinetic studies on the functional effects of mutating three neighboring glutamic acid residues (224, 228, and 231) to lysine, either singly or in combination. Two single mutations (E224K and E228K), both within helix A\(_{G}\), caused large defects in GTP hydrolysis and smaller defects in ribosomal translocation. Removal of L7/L12 from the ribosome strongly reduced the activities of wild-type EF-G but had no effect on the activities of the E224K and E228K mutants. Together, these results provide evidence for functionally important interactions between helix A\(_{G}\) of EF-G and L7/L12 of the ribosome.

Elongation factors (EF)\(^3\) Tu and G are the bacterial counterparts of universal translation factors, members of the G protein superfamily (1), which hydrolyze GTP to GDP and inorganic phosphate (P\(_i\)). The ribosome activates the latent GTPase activities of EF-Tu and EF-G. These factors, in turn, regulate protein synthesis by promoting specific molecular movements in the ribosome during protein synthesis. EF-Tu delivers aminoacyl-tRNA substrates to the ribosome, dependent on codon-anticodon pairing. EF-G promotes ribosomal translocation, involving movement of two tRNAs and mRNA in the ribosomal cavity, following the formation of each peptide bond.

GTP hydrolysis by EF-Tu and EF-G occurs on their G domains, which are similar to one another in their amino acid sequences and their core tertiary structures. The hydrolysis reaction involves in-line nucleophilic attack of a water molecule on the γ-phosphorus of GTP (2). On EF-Tu (and possibly also EF-G), this reaction is catalyzed by a conserved histidine residue (3), whose side chain is believed to rotate to a position next to the water molecule (4). In contrast to EF-Tu, EF-G contains a G′ subdomain, which is invariably inserted between α-helices D\(_G\) and E\(_G\) (5). The G′ subdomain is also present in the same location in EF-2, the eukaryotic cytoplasmic homolog of EF-G (6). However, the G′ subdomains of EF-G and EF-2 are unrelated in their amino acid sequences and tertiary structures. The functional significance of the G′ subdomain in either factor has not been determined.

How the bacterial ribosome activates GTP hydrolysis by EF-Tu and EF-G remains obscure. Early research identified L7/L12, a protein component of one of the peripheral stalks of the ribosome, as an important contributor to GTPase activation of both factors (7, 8). More recent studies identified residues of the C-terminal domain (CTD) of L7/L12, important for GTPase activation and rapid association of both factors with the ribosome (9, 10). Nucleotides of 23S RNA (11) and unidentified ribosomal components (12) have also been implicated in GTPase activation of these factors.

The sites on EF-Tu and EF-G that interact with L7/L12 are only beginning to be elucidated. For EF-Tu, mutational studies identified negatively charged residues in its helix D\(_G\) that may interact with positively charged residues of L7/L12 (9). For EF-G, no functional studies of this type have been reported so far. However, two topographic studies indicated that the G′ subdomain of EF-G is proximal to L7/L12. First, residues 209 and 231, flanking helix A\(_{G}\), of *Escherichia coli* EF-G were cross-linked to L7/L12 (13). In the same study, no cross-links were detected from two other EF-G residues (156 and 160) of helix D\(_G\), whose sequence is completely different from the corresponding helix of EF-Tu. Second, a cryo-EM study localized EF-G residue 209 near the base of the ribosomal stalk, which was interpreted as an interaction with the CTD of L7/L12 (14).

This study was undertaken to investigate the potential functional roles of the G′ subdomain of EF-G. We present results from kinetic experiments that provide evidence for interactions between residues of helix A\(_{G}\) of EF-G and L7/L12, which are important for activating GTP hydrolysis and ribosomal translocation.

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\(^{[3]}\) The abbreviations used are: EF, elongation factor; CTD, C-terminal domain; mant-GTP, 2′,3′-O-(N'-methylanthraniloyl)-GTP; TK, triple lysine mutant; P\(_i\), inorganic phosphate.

\(^{[4]}\) L7 and L12 refer to the N-acetylated and unacetylated forms of the same protein.
EXPERIMENTAL PROCEDURES

Materials—E. coli 70 S ribosome and phage T4 gene 32 mRNA were prepared as described (15). L7/L12 was specifically stripped from the ribosome by an ethanol-NH₄Cl washing procedure (12). The extent of L7/L12 stripping was assessed by immunoblotting using a L7/L12 polyclonal antibody (13). E. coli tRNA^fMet and tRNA^the were purchased from Sigma. The mRNA, 5′-AAGGAGGUAAGGUUGUCG(N₃)-3′, was synthesized by Dramar and conjugated at its 3′ end with pyrene as described (16). Mant-labeled nucleotides were purchased from Invitrogen.

Buffers—Buffer 1 consists of 50 mM Tris-HCl (pH 8.0), 100 mM KCl, 10 mM MgCl₂, 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 mM β-mercaptoethanol in water. Buffer 2 consists of 80 mM HEPES-KOH (pH 7.7), 50 mM NH₄Cl, 10 mM MgCl₂, 1 mM dithiothreitol in water. Buffer 3 consists of 50 mM HEPES-KOH (pH 7.7), 100 mM NH₄Cl, 20 mM MgCl₂, 1 mM dithiothreitol in water. The AIF₃ complex consists of 100 μM AlCl₃, 10 mM NaF in buffer 2.

GTP Binding and Hydrolysis Assays—All fluorescence measurements described below were made by a QM-6 fluorimeter (Photon Technology International). Binding of mant-GTP to EF-G was measured by titrating mant-GTP (120 μM) and EF-G (25 μM) in 1.2 ml of buffer 3. Reactions were started by rapidly mixing 200 μl samples from each syringe. Syringe B contained the various EF-G proteins (1.25–12 μM) and GTP (2 mM) in 1.2 ml of buffer 3. Reactions were started by rapidly mixing 200 μl samples from each syringe. Fluorescence (excitation 362 ± 4 nm; emission 438 ± 4 nm) was recorded.

Multiple-turnover GTP hydrolysis was assayed as described (13), with the following modifications. EF-G (0.04 μM) and ribosomes (variable concentration) were preincubated (37 °C, 10 min) in 9 μl of buffer 2. Reactions (10 μl) were started by adding 1 μl of 500 μM GTP (containing 0.05 μCi of [γ-³²P]GTP). Samples (2 μl) were withdrawn after appropriate time intervals, during the linear kinetics of the reaction. Samples were quenched and analyzed by TLC as described (13). Data of GTP molecules hydrolyzed per EF-G molecule/s (ν) as a function of ribosome concentration [R] were calculated and fitted (via SigmaPlot2000 software) to: ν = kcat × [EF-G] × [R]/(Kₘ + [R]).

Multiple-turnover mant-GTP hydrolysis was detected by the fluorescence change between mant-GTP and mant-GDP bound to EF-G. The fluorescence of mant-GTP (120 μM) in 250 μl of buffer 2 was monitored by exciting the fluorophore at 362 nm and detecting its emission at 438 nm. EF-G (25 μM) and vacant ribosomes (0.7 μM) were added in successive steps and mixed by pipetting. Samples (20 μl) were removed from the cuvette and precipitated with 0.2 M HCl. The supernatants of the samples were concentrated in a vacuum centrifuge to ~8 μl, and 2 μl of each sample (~500 pmol) was analyzed by TLC (17).

Single-turnover mant-GTP hydrolysis was monitored in a stopped-flow device (MiniMixer, KinTek), which contained two reactant syringes. Syringe A contained 1 ml of 24 μM vacant ribosomes in buffer 2 at ~22 °C. Syringe B contained 1 ml of 20 μM EF-G and 10 μM mant-GTP (same buffer and temperature). In some experiments, AIF₃ complex (100 μM) was included in syringe B. Samples (~200 μl) from each syringe were rapidly mixed together (dead time of ~3.5 ms) and injected into a cuvette in our fluorometer. Fluorescence (excitation 362 ± 2 nm; emission 438 nm ± 4) was monitored over 40–400 s, with measurements taken every 50–1000 ms, depending on the rate constant of the EF-G protein being tested. Data for each reaction were fitted to a single exponential equation, F(t) = Fₙ₋₋ × ΔFₙ₋₋ × exp(−kobs × t), where F₀ is the initial fluorescence at time = 0; Fₙ₋₋ is the fluorescence at time t; ΔFₙ₋₋ is the maximum fluorescence change (F₀ − F₋₋), and kobs is the observed reaction rate constant.

Ribosomal Translocation Assays—Multiple-turnover ribosomal translocation was monitored by the toeprinting method (18). A pretranslocation complex (1 μM) was formed in 90 μl of buffer 3 with E. coli ribosomes (1 μM) containing phage T4 gene 32 mRNA (0.8 μM) and uncharged tRNA^fMet (1.2 μM) and tRNA^the (1.2 μM) bound in the P and A sites, respectively, of the ribosome (15). EF-G (0.1 μM) and GTP (500 μM) were added, and reactions were incubated at 37 °C. Samples (10 μl) were removed after various time intervals (as indicated) and analyzed by toeprinting (15).

Single-turnover ribosomal translocation was monitored by the change in fluorescence of mRNA labeled with pyrene at its 3′ end (16). A pretranslocation complex (0.5 μM) was formed in 5 ml of buffer 3, similar to the above complex except substituting pyrene-mRNA (0.4 μM). This complex was divided into five equal aliquots, which were loaded successively into syringe A of the stopped-flow device. Syringe B contained the various EF-G proteins (1.25–12 μM) and GTP (2 mM) in 1.2 ml of buffer 3. Reactions were started by rapidly mixing 200 μl samples from each syringe. Fluorescence (excitation 332 ± 4 nm; emission 377 ± 8 nm) was monitored over 200–300 s, with averaged measurements taken every 60–200 ms, depending on the rate constant of the EF-G protein being tested. Data were fitted to a double exponential equation: F(t) = F₋₋ × ΔF₋₋ × exp(−k₋₋ × t) + ΔF₂ × exp(−k₂ × t). Observed translocation rates as a function of EF-G concentration were fitted to Reaction 1.

\[
\begin{align*}
A₁ + B₁ & \rightarrow A₂ · B₁ \rightarrow A₂ · B₂ \\
A₁ & \rightarrow A₂ · B₂
\end{align*}
\]

RESULTS

To investigate the function of the G′ subdomain, we first genetically replaced the entire subdomain of E. coli EF-G (residues 166–261) with a single Gly residue. The N- and C-terminal ends of the G′ subdomain are closely juxtaposed in the x-ray crystal structures of Thermus thermophilus EF-G proteins (5, 19), suggesting that this deletion would not perturb the folding of the rest of EF-G. We introduced this genetic deletion into a plasmid encoding E. coli EF-G with a C-terminal His₆ tag (15). Although the mutant protein could be produced at high levels in E. coli, it aggregated in cellular inclusion bodies, and we were unable to refold it after purification under denaturing conditions.
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We then replaced single amino acid residues of the G’ subdomain. In selecting these mutations, we considered three sources of information as follows: (i) our previous cross-linking results indicating the proximity of L7/L12 to EF-G residues 209 and 231, which flank helix A_G’; (ii) an alignment of the amino acid sequences of the G’ subdomain from bacterial and mitochondrial phyla (supplemental Fig. 1); and (iii) a study identifying conserved, positively charged residues of L7/L12 important for EF-G GTPase activity (10), suggesting that mutations would repel interactions with L7/L12. The His6- derivative, mant-GTP, with affinities (K_d values: 2.2–3.7 μM) that were indistinguishable from the wild type (Fig. 2B).

FIGURE 1. Locations of mutated residues in the G’ subdomain of EF-G. Schematic is based the structure of T. thermophilus EF-G-2 bound to GTP; Protein Data Bank accession code 1WDT (19). Yellow, G’ subdomain; orange, residues equivalent to E. coli Glu-224, Glu-228, and Glu-231.

FIGURE 2. G’ mutations affect GTP hydrolysis but not binding. A, GTP hydrolysis catalyzed by EF-G proteins in the presence of the vacant ribosome, under multiple-turnover conditions (see “Experimental Procedures”). Reaction “none” contained only the ribosome and GTP. B, binding of mant-GTP to EF-G proteins in the absence of the ribosome. Data were normalized with respect to wild type EF-G. In both panels, error bars are centered on the averages ± S.D. of three independent reactions.

G’ Mutations Hinder Ribosome-activated GTP Hydrolysis—We first examined the GTP hydrolysis activities of the purified EF-G proteins, activated by vacant ribosomes (lacking tRNA and mRNA). Experiments were performed under multiple-turnover conditions of fixed EF-G concentration (0.04 μM), saturating GTP concentration (500 μM), and increasing ribosome concentration. As shown in Fig. 2A, the initial velocity (v_o) of GTP hydrolysis catalyzed by wild type EF-G increased with increasing ribosome concentrations up to ~2 μM, followed by a drop in v_o at higher ribosome concentrations, because of turnover inhibition effects as reported previously (20). Based on fitting the Michaelis-Menten equation to the data up to 2 μM ribosome, wild type EF-G and the E231K mutant displayed nearly the same turnover (k_cat = 9.9 and 7.7 s⁻¹, respectively) and apparent ribosome binding (K_m = 0.91 and 1.5 μM). In contrast, the other three G’ mutants, E224K, E228K, and the triple Lys mutant (TK), were severely defective, to the extent that their v_o values were not measurable at any ribosome concentration above a control reaction containing only the ribosome.

The latter three G’ mutants were specifically defective in ribosome-activated GTP hydrolysis. In the absence of the ribosome, the basal GTPase activities of all four G’ mutants varied between ~3 × 10⁻³ and 3 × 10⁻² s⁻¹, slightly faster than ~4 × 10⁻³ s⁻¹ for the wild type protein, at the same EF-G and GTP concentrations indicated above (data not shown). Under multiple-turnover conditions, the defects in ribosome-activated GTP hydrolysis could, in principle, be manifested at one or several kinetic steps in the uncoupled GTPase reaction cycle (20). We could exclude effects on the initial binding of GTP to EF-G, as all four G’ mutants bound a fluorescent substrate derivative, mant-GTP, with affinities (K_d values: 2.2–3.7 μM) that were indistinguishable from the wild type (Fig. 2B).
To try to localize the defects of the $G'$ mutants to specific steps in the reaction cycle, we sought a GTPase kinetic assay performed under single-turnover conditions. Using mant-GTP as a fluorescent reporter, we observed that its fluorescence increased substantially when wild type EF-G was added (Fig. 3A), as reported previously (21). When we subsequently added a limiting amount of the ribosome, the fluorescence gradually dropped to a lower plateau. This suggested that mant-GTP was slowly hydrolyzed to mant-GDP, although it was bound to EF-G, activated by a limiting amount of ribosome that was recycled.

To test this hypothesis, we removed three samples ($S_1$, $S_2$, and $S_3$) at different time points during the fluorescence decay. These samples were immediately quenched by acid precipitation and subsequently analyzed by TLC (Fig. 3B). According to
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the mobilities of known molecular markers resolved on the same TLC, S1 (taken after EF-G addition) contained only mant-GTP; S2 (taken after ribosome addition, near the end of the fluorescence decay) contained mostly mant-GDP and small amounts of mant-GTP and S3 (taken in the fluorescence plateau) contained only mant-GDP. Thus, these experiments clearly show that the fluorescence decrease was because of mant-GTP hydrolysis.

The fluorescence decrease of the mant group presumably reflects a conformational change in EF-G. It is unclear whether this occurs during cleavage of the phosphoanhydride bond or during the subsequent release of the P1 product from EF-G. To characterize the mechanism further, we monitored single-turnover mant-GTP hydrolysis by using a stopped flow device that rapidly mixed EF-G-mant-GTP with a stoichiometric amount of the ribosome. We compared reactions in the presence or absence of AlF4⁻, an analog of the planar transition state structure of the γ-phosphate leaving group, which binds tightly to many G protein-GDP complexes.

Under single-turnover conditions, the fluorescence signal decayed exponentially with much faster kinetics and similar in amplitude (Fig. 3C). When AlF4⁻ was included with EF-G-mant-GTP before mixing them with the ribosome, the rapid fluorescence decrease was followed by an increase, which was slower and smaller in amplitude. As a positive control, when EF-G-mant-GDP and AlF4⁻ were mixed with the ribosome, only a larger fluorescence increase was observed. As a negative control, when EF-G-mant-GDP was mixed with the ribosome, a smaller fluorescence increase was observed. Collectively, these results suggest that the initial fluorescence decrease encompasses both mant-GTP hydrolysis and P1 release from EF-G. The subsequent fluorescence increase is because of AlF4⁻ binding to EF-G-mant-GDP, which follows P1 release. The small fluorescence increase seen in the negative control most likely reflects binding of EF-G-mant-GDP to the ribosome.

We then applied the above assay to compare the G’ mutants and wild type EF-G proteins in catalyzing mant-GTP hydrolysis (Fig. 4). Under single-turnover conditions, wild type EF-G catalyzed this reaction with kobs of 1.8 s⁻¹ (Table 1). This value is ~10-fold slower than previously reported for P1 release (20 s⁻¹), and much slower than GTP hydrolysis (80 s⁻¹), as measured by other methods under comparable conditions (22). These comparisons further suggest that the fluorescence decrease is not caused by the hydrolysis reaction itself, but rather because of a slower EF-G conformational change that follows P1 release. Alternatively, the slower rate may arise from the mant group partially interfering with the hydrolysis reaction.

Regardless of the precise explanation, the G’ mutants showed substantially reduced rates of mant-GTP hydrolysis, in comparison to wild type EF-G (Table 1). The most defective single G’ mutants were E224K and E228K, whose kobs values were both 65-fold lower than wild type. The E231K mutant was the most active, with kobs only 2-fold lower than wild type, in accord with the multiple-turnover GTP hydrolysis assay (Fig. 2A). The TK mutant was the most defective of all, with kobs that was 200-fold lower than wild type. However, the TK mutant still displayed significant ribosome-activated GTPase activity, relative to controls of the ribosome alone (Fig. 4) and the basal GTPase activity of the TK mutant in the absence of the ribosome.

In summary, results from multiple- and single-turnover assays both identified mutants E224K and E228K as having large defects in GTP hydrolysis. The single-turnover assays provide the more direct and accurate measurements, but they are ambiguous with regard to effects on hydrolysis or P1 release. It is also possible that the apparent defects in mant-GTP hydrolysis are manifested because of an impaired association of G’ mutants with the ribosome. The latter possibility is addressed below.

G’ Mutations Reduce Ribosomal Translocation Kinetics—Previous studies have indicated that GTP hydrolysis by EF-G is strongly coupled to ribosomal translocation, the coordinated movement of tRNA and mRNA in the ribosomal cavity. Upon binding of EF-G-GTP to a pretranslational ribosome complex, GTP hydrolysis occurs rapidly (23), which is thought to induce conformational rearrangements in the complex that drive the subsequent steps of P1 release from EF-G-GDP and translocation (22, 24). Although translocation can occur in the presence of nonhydrolyzable GTP analogs, GTP hydrolysis strongly promotes translocation both kinetically and thermodynamically (24, 25).

Given the effects of the G’ mutations on GTP hydrolysis activated by the vacant ribosome, we reasoned that these mutations might also exert effects on translocation if, as in the wild type situation, GTP hydrolysis and translocation remain strongly coupled mechanisms. We assembled a pretransloca-

**TABLE 1**

**Functional effects of stripping L7/L12 from the ribosome**

Values represent the averages ± S.D. of three or four independent reactions (stopped flow injections).

| +EF-G | Mant-GTP hydrolysis, kobs (s⁻¹) | mRNA translocation, kobs (s⁻¹) |
|-------|-------------------------------|-------------------------------|
|       | +L7/L12 | − L7/L12 | +L7/L12 | − L7/L12 |
| Wild type | 1.8 ± 0.1 | 0.149 ± 0.007 | 0.58 ± 0.02 | 0.067 ± 0.004 |
| E224K | 0.028 ± 0.001 | 0.027 ± 0.001 | 0.055 ± 0.003 | 0.051 ± 0.006 |
| E228K | 0.027 ± 0.001 | 0.024 ± 0.001 | 0.059 ± 0.002 | 0.050 ± 0.005 |
| E231K | 0.8 ± 0.1 | 0.088 ± 0.003 | 0.27 ± 0.01 | 0.057 ± 0.006 |
| TK | 0.009 ± 0.001 | 0.022 ± 0.001 | 0.029 ± 0.001 | 0.029 ± 0.001 |

**FIGURE 5.** G’ mutations affect both the pattern and kinetics of EF-G-dependent ribosomal translocation. Ribosome movement on mRNA was monitored by the toeprinting method under multiple-turnover conditions (see “Experimental Procedures”).
RIBOSOMAL TRANSLOCATION

Ribosome movement on mRNA was monitored by pyrene-mRNA fluorescence under single-turnover conditions (see “Experimental Procedures”). Trace labeled GTP control contained only GTP and pretranslocation complex; other traces contained the indicated EF-G proteins, GTP, and pretranslocation complex. For clarity, traces are offset along the vertical axis.

To measure accurately the kinetics of translocation under single-turnover conditions, we took advantage of a fluorescence assay that monitors the movement of pyrene attached to the 3' end of a short mRNA that just spans the ribosome (16). A pretranslocation complex (0.5 μM) containing pyrene-labeled mRNA and the same two tRNAs was rapidly mixed with wild type EF-G (1.25 μM) and saturating GTP. The fluorescence decay associated with translocation followed distinctly biphasic kinetics (supplemental Fig. 3). In the first phase of the reaction, $k_{obs}$ was 0.58 s$^{-1}$, whereas $k_{obs}$ of the second phase was 0.056 s$^{-1}$. It should be noted that the rate constant of the faster phase is comparable with the original report (16) but slower than reported in other studies (22, 26). This discrepancy has been recently attributed to the C-terminal His$_6$ tag, which is present in the EF-G proteins of our study, and other smaller factors (26).

Nonetheless, the G’ mutants promoted single-turnover translocation at rates that were significantly slower than wild type (Fig. 6; Table 1). Their relative rates were in the same order as measured in single-turnover mant-GTP hydrolysis. However, the quantitative differences between wild type and G’ mutants were smaller in translocation (e.g. ~10-fold between wild type, E224K, and E228K). The biphasic kinetic behavior was retained in all reactions. The G’ mutants were largely affected in the first reaction phase, and only slight affected in the second phase.

To dissect these effects further, we monitored translocation rate as a function of EF-G concentration, which was always in
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excess over pretranslational complexes (i.e., pseudo-unimolecular conditions). The $k_{obs}$ of the first phase of translocation was strongly dependent on the concentration of wild type EF-G up to ~6 μM (Fig. 7), whereas the slower phase was only slightly dependent (data not shown).

These results indicated that the first phase of translocation was rate-limited by the bimolecular association of EF-G with the ribosome. Compared with the wild type behavior, translocation catalyzed by all four G’ mutants was almost unaffected by their concentrations. Thus, the differences between wild type and the G’ mutants became even greater at the highest EF-G concentrations we tested. These results argue against activity defects of the G’ mutants being due to their impaired, productive association with the ribosome. Rather, they suggest that the G’ mutants are rate-limited by the subsequent (unimolecular) step of GTP hydrolysis, which leads to reduced ribosomal translocation kinetics.

G’ Mutant Defects Result from Disrupted Interactions with L7/L12—To examine whether the defects of the G’ mutants result from their disrupted interactions with L7/L12, we specifically stripped L7/L12 from the ribosome by an ethanol-NH$_4$Cl washing procedure (12). This procedure removed ~95% of L7/L12 while leaving other proteins on the ribosome intact (Fig. 8). We then compared the GTPase and ribosomal translocation activities of the G’ mutants and wild type EF-G.

Removal of L7/L12 from the ribosome reduced the rate of GTP hydrolysis by wild type EF-G by a factor of ~140 (from 9.9 to 0.070 s$^{-1}$) under multiple-turnover conditions (data not shown). In mant-GTP hydrolysis by wild type EF-G under single-turnover conditions, the effect of removing L7/L12 from the ribosome was smaller, ~12-fold (Table 1), which again suggests that the mant group partially interferes with GTP hydrolysis. In contrast, the most defective G’ mutants (E224K, E228K, and TK) catalyzed mant-GTP hydrolysis at nearly the same rates either in the presence or the absence of L7/L12. In the absence of L7/L12, these mutants and wild type EF-G became closer in their relative activities, although the wild type protein remained the most active. E231K displayed a behavior between the most severe G’ mutants and wild type EF-G.

L7/L12 effects on ribosomal translocation mirrored those measured in GTP hydrolysis assays. Removal of L7/L12 from the ribosome had no effect on the abilities of the most defective G’ mutants to promote translocation. The largest effect of L7/L12 removal was again observed in the wild type EF-G reactions. Consequently, in the absence of L7/L12, all four EF-G proteins catalyzed translocation with similar kinetics (Table 1). In summary, the two sets of data in Table 1 provide strong evidence that the defects of the G’ mutants result largely from disrupted interactions with L7/L12.

DISCUSSION

L7/L12 is one of a few bacterial ribosomal proteins of which accumulated evidence points to its functional roles in protein synthesis, whereas other ribosomal proteins appear to play structural roles in folding and modulating the conformations of the ribosomal RNAs, which catalyze peptide bond formation and decoding. Studies during the 1970s first demonstrated the requirement of L7/L12 for the functions of a wide variety of essential translation factors, including G and non-G proteins, in protein synthesis in vitro (7, 8, 27, 28). Subsequent studies isolated mutants of L7/L12, which altered the fidelity and speed of protein synthesis in vivo (29). Recent in vitro studies have converged on a small cluster of conserved, positively charged or aliphatic residues of L7/L12, which may interact with the G protein factors EF-Tu, EF-G, RF3, and IF2 (9, 10, 30).

From the latter studies, one might have anticipated that L7/L12 would interact with the G domains that are shared among the G protein factors. Instead, other studies (summarized in the Introduction) suggested that L7/L12 interacts with helix D$_G$ of EF-Tu and helix A$_G$ of EF-G (9, 13, 14). These two helices have been proposed to contact a common (unspecified) component of the ribosome (31). In RF3 a portion of the G’ subdomain, including helix A$_G$’$, is present (32), but in IF2 the G’ subdomain is completely missing, which suggests that these factors also may interact distinctly with L7/L12. This idea has precedent in other G proteins, which interact with GTPase-activating proteins (GAPs) in structurally distinct ways (33).

Functional Interactions between the G’ Subdomain and L7/L12—Our present study provides evidence for functionally important interactions between helix A$_G$’ of EF-G and L7/L12. Of the three single mutants we characterized, E224K and E228K exhibited the largest defects in EF-G activities on the ribosome. These mutations involve conserved Glu residues located near the C-terminal end of helix A$_G$. These G’ mutants were most severely defective in ribosome-activated GTP hydrolysis, whereas their basal GTPase activities in the absence of the ribosome were not affected.

Consistent with the notion that these mutations disrupt electrostatic interactions with L7/L12, we found that removing L7/L12 from the ribosome had little or no effect on the GTPase activities of the E224K and E228K mutants. In contrast, removal of L7/L12 from the ribosome resulted in a substantial reduction in the GTPase activity of wild type EF-G, in accord with previous studies (7, 8, 10, 12). From these results, we anticipate that residues Glu-224 and Glu-228 in helix A$_G$’ are involved in ion pairs with positively charged residues of L7/L12, which have been identified previously (10). By analogy to EF-Tu (9), several conserved hydrophobic residues adjacent to Glu-224 and Glu-228 in helix A$_G$’ may also interact with L7/L12 and thereby stabilize the electrostatic interactions.

The same order of activity of EF-G proteins (TK < E224K < E228K < E231K < wild type) was found in GTP hydrolysis and ribosomal translocation assays. The quantitative effects were uniformly larger in GTP hydrolysis, relative to translocation. This suggests that the G’ mutations exert direct effects on GTP hydrolysis, which in turn leads to indirect effects on translocation (23–25). Alternatively, the differences in the observed effects on GTP hydrolysis and translocation may simply reflect a differential interference by the fluorescent probes or the His$_6$ tag on EF-G (see “Results”). Our mant-GTP hydrolysis experiments, which monitor a conformational change in EF-G, suggest that the G’ mutations perturb GTP hydrolysis and/or $P_i$ release from EF-G. These results are in partial accord with a study analyzing mutations in the CTD of L7/L12, which reported effects on $P_i$ release but surprisingly no significant effects on translocation (34).

Implications—Several questions remain unanswered. First, L7/L12 is the only protein in bacterial ribosomes that is present...
in multiple copies, which varies depending on species (10). Thus, it is unclear whether EF-G interacts with single or multiple L7/L12 proteins.

Second, it is unclear how interactions of L7/L12 with the G’ subdomain leads to activation of GTP hydrolysis on the G domain, some 35 Å away from helix A\textsubscript{G}. Possibly, these interactions may allosterically alter the configurations of catalytic residues of the G domain, or may affect the positioning of another ribosomal component that directly contacts the GTP substrate. According to cryo-EM models, nucleotides of helix 95 (or Sarcin-Ricin loop) of 23 S RNA may directly contact the G domain (19, 35).

Finally, in translation systems in vitro, EF-G and EF-Tu are not interchangeable with their eukaryotic cytosolic homologs, EF-2 and EF-1\textalpha, respectively (36). This is despite the high degree of sequence conservation in these factors and ribosomes. One major difference is that the eukaryotic factors interact with P proteins, unrelated to L7/L12, on the stalk of some ribosomes (37). Experiments in which L7/L12 was replaced with P proteins on the E. coli ribosome demonstrated functionality of the eukaryotic factors on these hybrid ribosomes (38). Considered in light of our present results, we speculate that the specificity determinants of EF-G and EF-2 may involve the G’ subdomains of these factors, which have unreported sequences and tertiary structures (6). The specificity determinants of EF-Tu and EF-1\textalpha remain unknown, but it is interesting to note that their G domains differ by the addition or deletion of several \alpha-helices (39), and their shared D\textsubscript{G} \alpha-helices have different sequences.

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