GTPase Activation of Elongation Factors Tu and G on the Ribosome†

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ABSTRACT: The GTPase activity of elongation factors Tu and G is stimulated by the ribosome. The factor binding site is located on the 50S ribosomal subunit and comprises proteins L7/12, L10, L11, the L11-binding region of 23S rRNA, and the sarcin-ricin loop of 23S rRNA. The role of these ribosomal elements in factor binding, GTPase activation, or functions in tRNA binding and translocation, and their relative contributions, is not known. By comparing ribosomes depleted of L7/12 and reconstituted ribosomes, we show that, for both factors, interactions with L7/12 and with other ribosomal residues contribute about equally and additively to GTPase activation, resulting in an overall 10^7-fold stimulation. Removal of L7/12 has little effect on factor binding to the ribosome. Effects on other factor-dependent functions, i.e., A-site binding of aminoacyl-tRNA and translocation, are fully explained by the inhibition of GTP hydrolysis. Based on these results, we propose that L7/12 stimulates the GTPase activity of both factors by inducing the catalytically active conformation of the G domain. This effect appears to be augmented by interactions of other structural elements of the large ribosomal subunit with the switch regions of the factors.

Ribosomes synthesize proteins with the help of translational factors for initiation, elongation, and termination. During the elongation cycle, two elongation factors (EF),[1] EF-Tu and EF-G, act on the ribosome. EF-Tu and EF-G are GTP-binding proteins (*1*) whose GTPase activity is induced by the ribosome. EF-Tu in the GTP-bound conformation forms a high-affinity complex with aminoacyl-tRNA (aa-tRNA) ("ternary complex") that delivers aa-tRNA to the ribosomal A site where it takes part in ribosome-catalyzed peptide bond formation. Subsequently, EF-G completes the cycle by translocating the peptidyl-tRNA from the A to the P site. The mechanism by which the ribosome stimulates the GTPase activity of the factors is not known. High-resolution structures of ribosome complexes with elongation factors are not available so far. Low-resolution cryo-EM structures of ribosomal complexes with EF-Tu and EF-G (*2*−*8*), as well as a variety of biochemical (*9*−*16*) and genetic (*17*−*26*) data, suggest that the factors interact with proteins L7/12, L10, L11, the L11-binding region of 23S rRNA, and the sarcin-ricin loop (SRL) of 23S rRNA. However, it is not known to which extent these contacts contribute to factor binding and/or GTPase activation.

The stalk of the 50S ribosomal subunit is comprised of two dimers of the 12 kDa protein L7/12 (L7 differs from L12 by an acetylated N terminus). The structure of the protein on the ribosome is not known, because it is not resolved in the crystal structure of either the 50S subunit (*27*) or the 70S ribosome (*28*). The isolated protein consists of two domains (*29*). The N-terminal domain is required for dimer formation and for anchoring the protein to the ribosome by binding to protein L10, while the C-terminal domain is involved in factor binding (*30*). Previously, we reported that isolated ribosomal protein L7/12 strongly stimulates GTP hydrolysis by EF-G, but not by EF-Tu, indicating a major contribution of L7/12 to GTPase activation of EF-G on the ribosome (*31*). The single, highly conserved arginine residue in the C-terminal domain of L7/12 is not essential for the activation, excluding an "arginine finger"-type mechanism reported for other GTP-binding proteins, such as Ras, Rho, and Rac (*32*). Rather, L7/12 seems to function by stabilizing the GTPase transition state of EF-G in a similar way as the G_α subunits of the heterotrimeric G proteins are activated by regulators of G protein signaling (RGS) (*33*). However, the rate of GTP hydrolysis by EF-G as stimulated by isolated L7/12 was about 500-fold lower than that on the ribosome, indicating that other elements of the ribosome might be involved in GTPase activation as well.

In addition to L7/12, the factor interaction site contains proteins L10 and L11 bound to the 1060-region of 23S rRNA, as well as the SRL around nucleotide 2660 of 23S rRNA (*27*, *34*). The two proteins, L10 and L11, have a number of conserved arginine residues which could act as "arginine fingers" provided in trans into the catalytic site of the factors. However, L10 does not contribute to the GTPase activation of EF-G, as the rate of GTP hydrolysis in the presence of the complex of L10 and four copies of L7/12 (L8 complex) is similar to that in the presence of L7/12 alone

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[1] Abbreviations: EF-Tu, elongation factor Tu; GEF, guanine nucleotide exchange factors; GAP, GTPase activating protein; RGS, regulator of G protein signaling; aa-tRNA, aminoacyl-tRNA; mant-GDP, 3′(2′)-*O*-(*N*-methylanthraniloyl)guanosine 5′-diphosphate; PBP, phosphate-binding protein; MDCC, 7-diethylamino-3-((((2-maleimidyl)ethyl)-amino)carbonyl)coumarin; Pm, puromycin; SRL, the sarcin-ricin loop of 23S rRNA.

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(*31*). Also L11 is unlikely to be important for GTPase stimulation, because bacteria lacking L11 are viable (*35*, *36*). SRL and the 1060-region of 23S rRNA are known to be important for the binding of the factors to the ribosome (*10*, *13*), but their role in GTPase activation or other functions is not understood.

L7/12 proteins can be selectively removed from and reconstituted back into the ribosome. Ribosomes depleted of L7/12 are impaired in factor functions, and the activity is restored by the addition of L7/12 (*37*−*39*). It is generally accepted that L7/12 is important for EF-G-dependent GTP hydrolysis on the ribosome, because the removal of L7/12 resulted in a 85−98% inhibition of the reaction measured under multiple turnover conditions (*38*−*42*). The effect of L7/12 on EF-G binding to the ribosome is less clear, as reported reductions of binding varied between 15 and 90% (*38*, *39*, *41*). EF-G-dependent translocation was impaired by the lack of L7/12, but to a considerably smaller extent than the GTPase (*38*). The GTPase activity of EF-Tu on L7/12-depleted ribosomes was reported to be very low (*38*, *39*, *42*); on the other hand, only a 10−20% reduction of the ribosome-induced GTPase activity of EF-Tu was found in the presence of kirromycin (*43*). Together, these data strongly suggest that L7/12 is important for EF-G and EF-Tu function on the ribosome, although neither the exact steps that are impaired in the absence of L7/12 nor the quantitative contribution of L7/12 to the catalysis of these reaction is known. Furthermore, the relative contribution of other ribosomal elements to the factors' function remains unclear.

This prompted us to analyze the kinetics of EF-G and EF-Tu reactions on the ribosome in the presence and absence of L7/12 with the aim to determine the contribution of L7/12 and other ribosomal residues to GTPase activation of both factors and to clarify the role of these ribosome elements in factor binding and other factor-dependent functions. Using rapid kinetic techniques, we measured rate constants of GTP hydrolysis by EF-G and EF-Tu, factor-dependent translocation, and A-site binding. Together with the previous report on the activity of isolated L7/12 in promoting GTP hydrolysis in EF-G (*31*), the present data reveal the relative contributions of L7/12 and other ribosomal elements to the stimulation of GTP hydrolysis by the two factors.

EXPERIMENTAL PROCEDURES

Buffers and Materials. Buffer A: 20 mM Tris-HCl, pH 7.5, 0.6 M NH_4Cl, 20 mM $MgCl_2$, 5 mM β-mercaptoethanol; buffer B: 50 mM Tris-HCl, pH 7.5, 30 mM KCl, 7 mM $MgCl_2$, 25% glycerol; buffer C: 50 mM Tris-HCl, pH 7.5, 70 mM NH_4Cl, 30 mM KCl, and 7 mM $MgCl_2$; buffer D: 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 10 mM $MgCl_2$. GTP, phosphoenolpyruvate, pyruvate kinase, and poly(U) were from Roche Diagnostics. 2′(3′)-*O*-(*N*-Methylanthraniloyl)-guanosine 5′-diphosphate (mant-GDP) was from JenaBioScence. Radioactive compounds were from ICN. All other chemicals were from Merck.

70S ribosomes from *E. coli* MRE 600 were prepared as described (*44*). f[^{3}H]Met-tRNA^{fMet}, [^{14}C]Phe-tRNA^{Phe}, MFTI-mRNA, EF-Tu, EF-G, and initiation factors were prepared and purified as described (*45*−*48*). Protein L7/12 was expressed in *E. coli* BL21 from plasmid pGEX-5x-3-L7/12 containing the gene for glutathione-*S*-transferase fused to the gene of L7/12 (*31*). L7/12 was purified by affinity chromatography on glutathione-Sepharose 4B (Pharmacia) as described (*31*), except that cleavage of the fusion protein by factor Xa (Novagen) was carried out directly on the affinity matrix. The purity of the resulting preparations of L7/12 protein was >90% according to SDS−PAGE.

Depletion and Reconstitution of Ribosomes. L7/12 were removed from the ribosomes by NH_4Cl/ethanol treatment (*49*) with modifications. A 450 pmol sample of purified 70S ribosomes was incubated in 450 μL of buffer A on ice for 10 min. The solution was mixed with 250 μL of cold ethanol and stirred on ice. After 10 min, another 250 μL of ethanol was added and the mixture stirred for another 5 min. The mixture was centrifuged at 150000*g* for 30 min, and the ribosomal pellet was dissolved in buffer B. According to the immunoblot analysis with anti-L7/12 antibodies (provided by R. Brimacombe, MPI Berlin), the NH_4Cl/ethanol-treated ribosomes contained about 15% L7/12 protein. The removal of ribosomal proteins other that L7/12 was controlled by precipitating proteins from the NH_4Cl/ethanol supernatant with acetone; according to SDS−PAGE, the supernatant contained L7/12, trace amounts of L10, and no other protein. For reconstitution, ribosomes lacking L7/12 were incubated with a 5-fold excess of purified L7/12 for 30 min at 37 °C.

GTP Hydrolysis. To measure ribosome-stimulated multiple-turnover GTP hydrolysis by EF-G, ribosomes (0.2 μM) were mixed with EF-G (0.04 μM) in buffer C, and the reaction was started at 37 °C by the addition of [γ-^{32}P]GTP (20 μM). Samples (30 μL) were quenched by adding 30 μL of 1 M $HClO_4$/3 mM potassium phosphate and analyzed by thin-layer chromatography on PEI-cellulose in 0.5 M potassium phosphate, pH 3.5. The extent of hydrolysis was quantified using a Bio-Rad phosphoimager. Blanks of [γ-^{32}P]GTP hydrolysis by EF-G or ribosomes alone were measured separately and subtracted. Measurements of ribosome-stimulated GTP hydrolysis by the ternary complex EF-Tu·[γ-^{32}P]GTP·[^{14}C]Phe-tRNA^{Phe} were performed in buffer D at 20 °C using a quench-flow apparatus (KinTek). Ribosomes at concentrations indicated in the figures were incubated with 1 mg/mL poly(U) and a 1.5-fold excess of AcPhe-tRNA^{Phe} in buffer D at 37 °C for 20 min, rapidly mixed with ternary complex, EF-Tu·[γ-^{32}P] GTP·[^{14}C]Phe-tRNA^{Phe}, purified by gel filtration (*46*), and concentrated by ultrafiltration on Centricon 3 filtration membranes (Amicon) (1−3 μM final concentration). Samples were quenched with 25% formic acid and analyzed by thin-layer chromatography as described above.

Dissociation of Inorganic Phosphate (Pi). Pi release from EF-G after GTP hydrolysis was measured as described (*50*). Ribosomes (0.2 μM, concentration after mixing) were mixed with EF-G at varying concentrations, GTP (25 μM), and 2.5 μM MDCC-labeled phosphate-binding protein (PBP) (*51*) in a stopped-flow apparatus (Applied Photophysics), and the fluorescence change of MDCC-PBP upon binding of Pi was monitored. Amplitudes of the burst phase of Pi release during the first 0.1 s of incubation were estimated after subtraction of the linear function, accounting for the multiple-turnover phase between 0.2 and 0.5 s. The maximum amplitude of the burst phase (see Figure 1C, curve 1) was set to 1. Rates of multiple-turnover Pi release were calculated from the linear parts following the burst phase of the time courses.

Translocation. To prepare 70S initiation complexes, ribosomes (0.5 μM) were incubated with a 3-fold excess of MFTI-mRNA (coding for fMetPheThrIle...), a 1.5-fold excess of each IF1, IF2, IF3, f[^{3}H]Met-tRNA^{fMet}, and 1 mM GTP in buffer C for 30 min at 37 °C. The ternary complex EF-Tu·GTP·[^{14}C]Phe-tRNA^{Phe} was prepared by incubating EF-Tu (2 μM) with 1 mM GTP, 3 mM phosphoenolpyruvate, and 0.5 mg/L pyruvate kinase for 30 min at 37 °C and with 1 μM [^{14}C]Phe-tRNA^{Phe} for an additional 5 min. Equal volumes of the ternary complex were added to the initiation complex and incubated at 20 °C for 5 min to form pretranslocation complex. Multiple-turnover translocation was induced by the addition of EF-G (40 or 0.5 nM final concentration). The extent of translocation was followed by the puromycin (Pm) assay, monitoring the formation of fMetPhe-Pm [1 mM Pm, incubation for 10 s at 37 °C; (*47*)]. In the experiment with 0.5 nM EF-G, which required rather long incubation, the Mg^{2+} concentration was raised to 14 mM in order to increase the stability of the pretranslocation complex.

Kinetics of single-round translocation were measured by fluorescence stopped-flow (*47*). Pretranslocation complex was prepared as above, except that fluorescence-labeled [^{14}C]-Phe-tRNA^{Phe}(Prf16/17) was used (Prf, proflavin) and the complex EF-Tu·GTP·[^{14}C]Phe-tRNA^{Phe}(Prf16/17) was purified by gel filtration (*52*). Pretranslocation complex (0.1 μM, final concentration after mixing) with f[^{3}H]Met[^{14}C]Phe-tRNA^{Phe}(Prf16/17) in the A site and tRNA^{fMet} in the P site was mixed with EF-G in the presence of GTP (1 mM) in buffer C at 37 °C. Prf fluorescence was excited at 460 nm and measured after passing a KV500 filter (Schott). The results of several (four to nine) experiments were averaged, and the data were evaluated by exponential fitting (*47*, *52*).

Steady-State Fluorescence Measurements. Mant-GDP (1 μM) was mixed with EF-G (2 μM), $AlCl_3$ (60 μM), and NaF (10 mM) in buffer C, ribosomes were added as indicated, and the fluorescence was measured on a Schoeffel RRS 1000 spectrofluorometer using excitation and emission wavelengths of 349 and 430 nm, respectively.

A-Site Binding. Purified ternary complex EF-Tu·GTP·[^{14}C]-Phe-tRNA^{Phe}(Prf16/17) (0.3 μM final concentration) was mixed in buffer D at 20 °C with poly(U)-programmed ribosomes containing AcPhe-tRNA^{Phe} in the P site prepared as described above (see GTPase activity) in a stopped-flow apparatus. Prf fluorescence was measured as described above (see Translocation). To measure dipeptide formation, poly-(U)-programmed ribosomes (0.2 μM) containing AcPhe-tRNA^{Phe} in the P site were mixed with ternary complex (0.4 μM) in buffer D at 20 °C in a quench-flow apparatus. The reaction was stopped by 0.8 M KOH, and the amount of AcPhePhe formed was analyzed by HPLC (*52*).

RESULTS

EF-G-Dependent GTP Hydrolysis and Pi Release. Ribosomes lacking L7/12 proteins (cores) were prepared by NH_4-Cl/ethanol treatment. According to immunoblot analysis (Experimental Procedures), about 85% of L7/12 were removed, whereas only trace amounts of L10 and no other ribosomal proteins were lost. Harsher conditions led to uncontrolled loss of proteins. The presence of about 15% of intact ribosomes in the preparation of core ribosomes was taken into account for quantitative assessments of the effects of L7/12 removal on the functions of EF-G and EF-Tu, as described in detail below.

Removal of L7/12 had no effect on the saturation level of tRNA binding to P and A sites or peptide bond formation, which remained close to 100% (data not shown). In agreement with earlier reports, EF-G-dependent GTP hydrolysis was strongly inhibited by removing L7/12. Reconstitution of intact ribosomes by adding excess L7/12 to cores completely restored the ability to promote GTP hydrolysis (Figure 1A), indicating that the inhibition of GTP hydrolysis was caused by the lack of L7/12, rather than of any other protein. In the following, the contribution of L7/12 to GTPase activation of EF-G was determined by comparing the properties of cores and L7/12-reconstituted ribosomes in kinetic experiments.

Time courses of EF-G-dependent GTP hydrolysis were followed by the release of Pi using an assay in which the fluorescence change of MDCC-labeled PBP phosphate-binding protein (PBP) that takes place upon Pi binding was monitored (*51*). This method is sensitive enough to monitor both single and multiple rounds of GTP hydrolysis in the same experiment. The release of Pi after the first round of GTP cleavage on intact ribosomes resulted in a rapid ($\sim$40 s^{-1}) burst phase of the time curve (Figure 1B). The burst was followed by a slower multiple-turnover reaction which was linear in the early phase and saturated after prolonged incubation due to saturation of PBP with Pi (Figure 1C). In line with the strong inhibition of GTP hydrolysis (Figure 1A), the release of Pi was inhibited when L7/12 was removed (Figure 1B,C). The burst amplitude of Pi release was strongly reduced, suggesting that rapid GTP hydrolysis in EF-G was not induced in the absence of L7/12. In fact, the quantitative analysis of the burst amplitudes at increasing concentration of EF-G (Figure 1D) revealed that approximately 20% of NH_4Cl/ethanol-treated ribosomes were capable of stimulating the first round of rapid GTP hydrolysis with a rate of about 40 s^{-1}, in agreement with the estimated 15% ribosomes still containing L7/12 present in the preparations of cores used for the experiments.

Multiple rounds of GTP hydrolysis, measured by Pi release, were very slow in the absence of L7/12. The turnover rate, k_{cat}, at saturation was 0.04 s^{-1} with the cores, compared to 1.5 s^{-1} with reconstituted ribosomes (Figure 1E). Taking into account that the 15% intact ribosomes also contribute to the turnover reaction, the true rate of the turnover reaction on ribosomes lacking L7/12 must be even lower than 0.04 s^{-1}. The turnover rate on intact ribosomes is determined by rate-limiting conformational changes of EF-G on the ribosome, rather than by GTP hydrolysis which is rapid, $k_{GTP} =$ 170 s^{-1} (*47*). In contrast, the turnover rate on cores is limited by the hydrolysis step, because the rapid burst of the reaction is not observed; thus, the value of $k_{cat} < 0.04$ s^{-1} for the turnover reaction probably represents the rate constant of GTP hydrolysis, k_{GTP}. Comparison of the k_{GTP} values of intact ribosomes, 170 s^{-1}, and of cores, $<$0.04 s^{-1}, suggests that L7/12 accelerates GTP hydrolysis by EF-G on the ribosome $>$4000-fold.

The concentration dependence of the turnover rate of GTP hydrolysis was similar with cores and reconstituted ribosomes (Figure 1E), suggesting that the interaction with L7/12 does not contribute significantly to the affinity of EF-G binding.

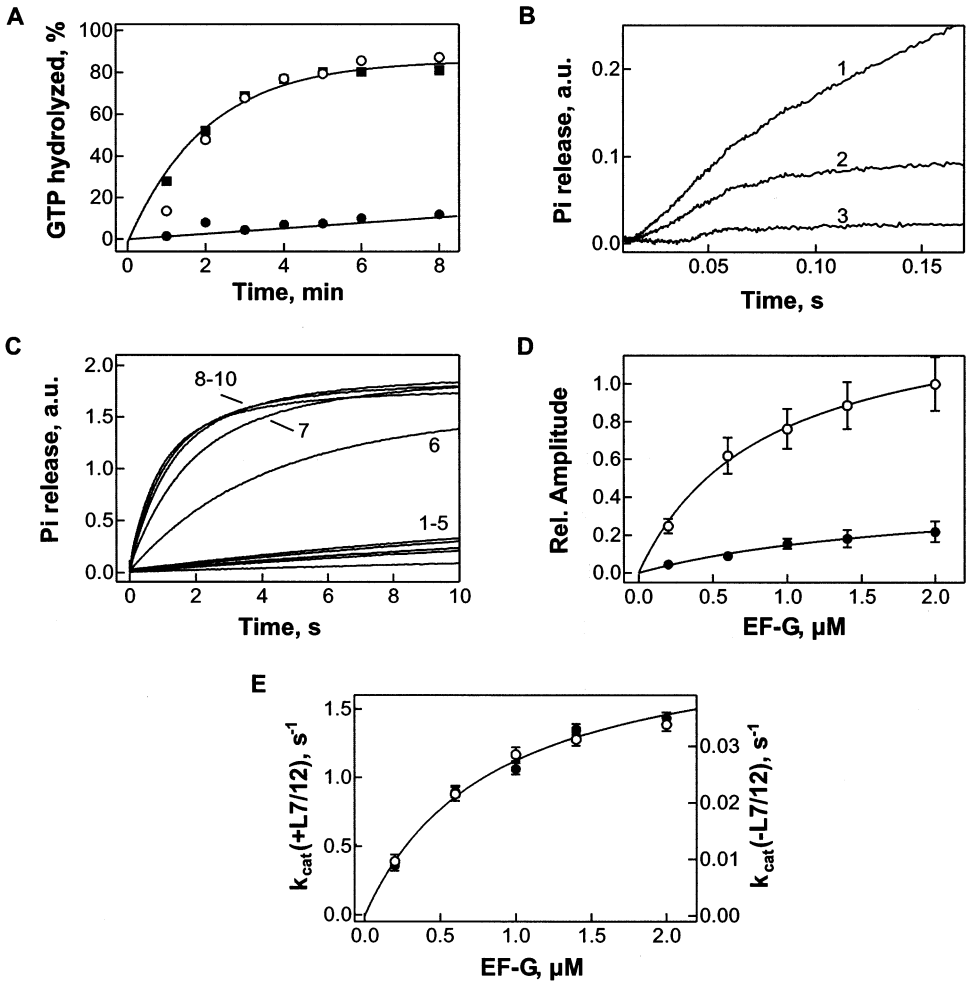

FIGURE 1: EF-G-dependent GTP hydrolysis. (A) Turnover reaction measured with [γ-^{32}P]GTP. For details, see Experimental Procedures. Native 70S ribosomes (■), reconstituted 70S (○), and 70S cores (●). (B) Initial burst of Pi release from EF-G following GTP hydrolysis on the ribosome. EF-G (1 μM final concentration) was rapidly mixed with ribosomes (0.2 μM), and liberated Pi was monitored by the fluorescence of MDCC-labeled PBP (Experimental Procedures), given in arbitrary units (a.u.). Transients are shown for reconstituted 70S as measured (1) and after subtraction of the linear multiple-turnover phase (2), and for 70S cores as measured (3). (C) Dependence of turnover Pi release upon EF-G concentration. Time courses of Pi release were measured as in (B). Transients 1−5, 70S cores with EF-G, 0.2, 0.6, 1, 1.4, and 2 μM, respectively; transients 6−10, reconstituted 70S with EF-G at the same concentrations as 1−5. (D) Relative amplitudes of the burst phase (first round) of Pi release of EF-G on reconstituted 70S (○) and 70S cores (●). Burst amplitudes were determined from the transients of Figure 1C (first 0.1 s) after subtraction of the linear function accounting for the multiple-turnover reaction between 0.2 and 0.5 s (as illustrated in Figure 1B) and were normalized by setting the maximum amplitude to 1. (E) Dependence of the multiple-turnover rate of Pi release on the concentration of EF-G. Values of k_{cat} were derived from the initial linear slopes (0.2−0.5 s) of the time courses depicted in (C). Left axis, reconstituted 70S (○); right axis, 70S cores (●).

However, the same type of concentration dependence would be observed if only the residual ribosomes carrying L7/12 induced GTP hydrolysis and the cores were completely inactive and therefore did not contribute to the reaction. The two possibilities can be distinguished by single-turnover experiments where 10−20% residual intact ribosomes contribute very little to the overall reaction. Because such experiments cannot be done using the GTPase assay (GTP turnover cannot be avoided), the binding of EF-G to cores and intact ribosomes was compared in assays of EF-G-dependent translocation.

Translocation. Multiple-turnover translocation assays were carried out with substoichiometric amounts of EF-G and monitored by the puromycin reaction. The extent of EF-G-promoted translocation on ribosomes lacking L7/12 and ribosomes reconstituted by the addition of L7/12 was the same, about 80%, whereas the rate of turnover translocation was strongly reduced by the removal L7/12 (Figure 2A,B). The time courses yielded turnover rates of 0.009 s^{-1} for cores and 0.4−0.5 s^{-1} for intact and reconstituted ribosomes, indicating an at least 50-fold rate reduction of the turnover reaction by removal of L7/12. It should be noted that, due to the presence of residual amounts of intact ribosomes in the preparation of cores, the precise values of k_{cat} and K_M for translocation on cores cannot be deduced from these turnover experiments.

In single-turnover experiments carried out in excess of EF-G over pretranslocation complexes carrying fMetPhe-tRNA^{Phe}(Prf16/17) in the A site, a fluorescence increase is observed that is due to the translocation of fMetPhe-tRNA^{Phe}-(Prf16/17) to the P site (*47*). The extent of translocation was the same on cores and reconstituted ribosomes (not shown), in agreement with the results of multiple-turnover experiments. The rate of translocation, as measured by fluorescence stopped-flow (*47*) at increasing concentration of EF-G, was reduced 30-fold by the removal of L7/12 (Figure 2C). Because EF-G-dependent GTP hydrolysis is very slow on cores (<0.04 s^{-1}), translocation on cores (0.4 s^{-1}) is due to

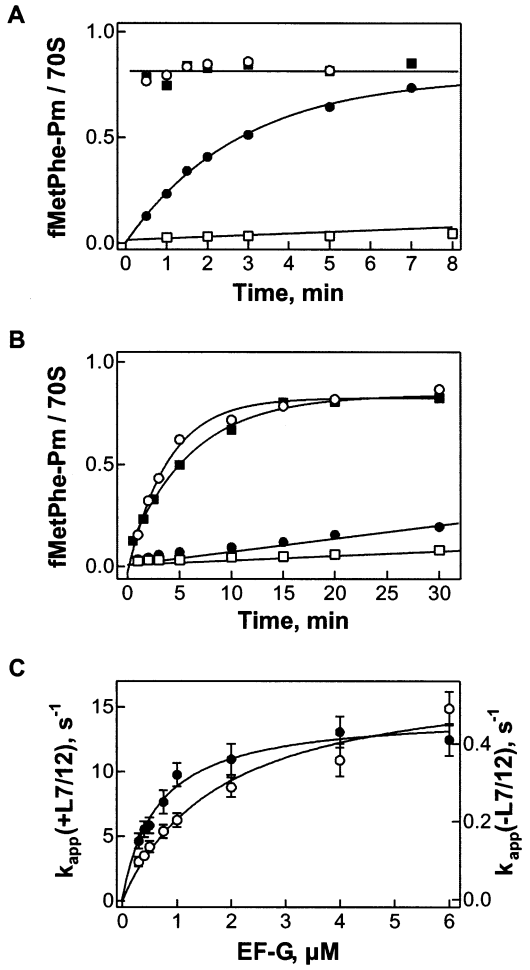

FIGURE 2: EF-G-dependent translocation. (A) Multiple-turnover translocation measured with 40 nM EF-G. 0.2 μM pretranslocation complexes prepared from 70S (■), 70S cores (●), and reconstituted 70S (○) were incubated with EF-G or without the factor (□) in the presence of GTP (1 mM). Translocation was measured by the formation of fMetPhe-puromycin. (B) Multiple-turnover translocation with 0.5 nM EF-G. Conditions as in Figure 3A, except for the lower concentration of EF-G. (C) Dependence of translocation rates, k_{app}, on EF-G concentration. Left axis, reconstituted 70S (○); right axis, 70S cores (●).

factor binding only, and thus one may expect the rate to be similar to that measured in the absence of GTP hydrolysis. In fact, the rates of EF-G-promoted translocation on cores were similar with GTP and a nonhydrolyzable GTP analogue, GDPNP, 0.4 and 0.1 s^{-1}, respectively (not shown). Importantly, both the extent of single-round translocation at different EF-G concentrations (not shown) and the concentration of EF-G at which half-saturation of the concentration dependence is achieved (Figure 2C) were the same for cores and reconstituted ribosomes, indicating that the affinity of the ribosome to EF-G is not affected by the presence or absence of L7/12.

GTPase Transition State. Several GTP-binding proteins, including EF-G, G_α, and Ras, form complexes with GDP and AlF_4, the structure of which is different from both GTP- and GDP-bound forms and believed to represent the GTPase transition state (*53*, *54*). When a fluorescent derivative of GDP is used, mant-GDP, a large fluorescence change is observed upon formation of the complex between the GTP-binding protein, mant-GDP, and AlF_4 (*55*). A similar effect is observed when the same experiment is performed with

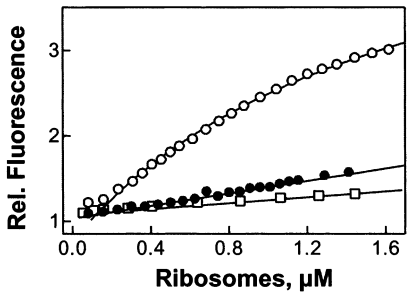

FIGURE 3: Interaction of EF-G·mant-GDP with ribosomes in the presence of AlF_4. EF-G (2 μM) was mixed with mant GDP (1 μM), $AlCl_3$ (60 μM), and NaF (10 mM) and increasing concentrations of native 70S (○) and 70S cores (●). As a control, the titration was performed with native 70S without addition of $AlCl_3$/NaF (□).

EF-G−ribosome complexes (A. Savelsbergh, unpublished data). This prompted us to test the effect of L7/12 on the formation of the AlF_4-induced GTPase transition state of EF-G on the ribosome (Figure 3). In the absence of L7/12, the fluorescence change of mant-GDP is not observed, except for a small effect which is attributed to the 15% remaining L7/12. It is important to note that, based on the translocation experiments, binding of EF-G to ribosomes with and without L7/12 is the same (see above). This suggests that the GTPase transition state of EF-G is not, or not properly, formed in the absence of L7/12. In either case, the effect is consistent with the strong inhibition of GTP hydrolysis observed in the absence of L7/12, indicating that the interaction with L7/12 is crucial for the formation of the fully activated GTPase state of EF-G on the ribosome.

EF-Tu-Dependent Reactions. EF-Tu in the GTP-bound conformation forms a high-affinity ternary complex with aa-tRNA that binds to the ribosomal A site. The interaction between the A-site codon and a cognate ternary complex results in conformational changes of the ribosome which stabilize tRNA binding and trigger GTP hydrolysis by EF-Tu (*56*). As a result of GTP hydrolysis and release of Pi, EF-Tu changes conformation from the GTP- to the GDP-form whereby it loses the affinity for aa-tRNA. aa-tRNA that is released from EF-Tu·GDP accommodates in the peptidyl transferase center and takes part in peptide bond formation, while EF-Tu·GDP dissociates from the ribosome. On native ribosomes, the slowest step in this sequence, which determines the rate of peptide bond formation, is the accommodation step.

The role of L7/12 in binding of the ternary complex to the A site was studied under single-round conditions in the presence of stoichiometric or substoichiometric amounts of ribosomes in order to minimize the contribution from intact ribosomes. Binding and codon−anticodon recognition were monitored by a fluorescence increase of proflavin-labeled $tRNA^{Phe}$ that takes place upon interaction of EF-Tu·GTP·Phe-$tRNA^{Phe}$(Prf16/17) with the ribosome that is followed by a decrease due to accommodation of aa-tRNA in the A site (*46*, *52*). A similar fluorescence increase was observed upon ternary complex binding to ribosomes lacking L7/12, whereas the fluorescence decrease due to accommodation was not observed within the time range of the stopped-flow experiments (Figure 4A). At the concentrations of ternary complex and ribosomes used, the bimolecular binding reaction, which is dominated by ribosome−EF-Tu interactions, was rate-limiting (*52*). Under these conditions, the rate

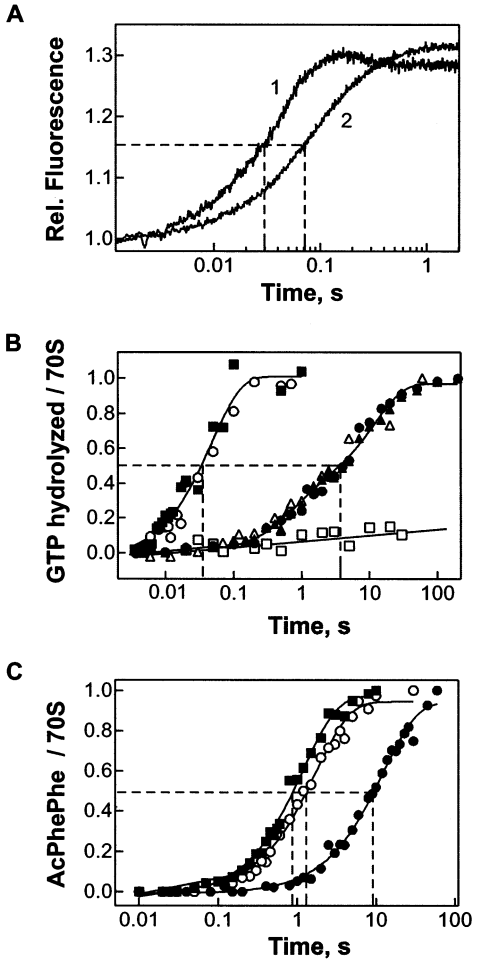

FIGURE 4: EF-Tu-dependent A-site binding of Phe-tRNA^{Phe}. (A) Binding of EF-Tu·GTP·Phe-tRNA^{Phe}(Prf16/17) to the A site. Poly(U)-programmed 70S (1) or 70S cores (2) with AcPhe-tRNA in the P site (concentration after mixing 0.3 μM) were rapidly mixed with purified ternary complex containing Phe-tRNA^{Phe}(Prf16/17) (0.1 μM) in a stopped-flow apparatus, and proflavin fluorescence was monitored. (B) GTP hydrolysis. Purified ternary complex containing [γ-^{32}P]GTP and Phe-tRNA^{Phe} (1−3 μM) was rapidly mixed with poly(U)-programmed 70S ribosomes with AcPhe-tRNA in the P site in the quench-flow apparatus as follows: native 70S (■) (final concentration 0.5 μM), reconstituted 70S (○) (0.5 μM), 70S cores (●, 3 μM; ▲, 2 μM; △, 1 μM). The control was performed with buffer B alone (□). (C) Dipeptide formation. Native 70S ribosomes (■), reconstituted 70S (○), or 70S cores (●) programmed with poly(U) and containing AcPhe-tRNA in the P site (final concentration 0.2 μM) were rapidly mixed with ternary complex EF-Tu·GTP·[^{14}C]Phe-tRNA^{Phe} (0.4 μM) in the quench-flow apparatus, and AcPhePhe formed was measured (Experimental Procedures).

of ternary complex binding to the ribosome was decreased 2-fold by the removal of L7/12, from about 20 s^{-1} to 10 s^{-1} (indicated as half-life times in Figure 4A). This suggests that the binding of the ternary complex to the ribosome is only slightly affected by the lack of L7/12.

The role of L7/12 in stimulating GTP hydrolysis by EF-Tu in the ternary complex on poly(U)-programmed ribosomes was studied by quench flow. Intact or reconstituted ribosomes induced rapid GTP hydrolysis; at the ribosome concentration used, the observed rate was about 20 s^{-1} (Figure 4B), in agreement with previous data (*52*). In the absence of L7/12, the rate of GTP hydrolysis was reduced to about 0.2 s^{-1} (calculated from the half-life time of about 4 s, Figure 4B). Because this rate did not increase at high ribosome concentrations, and was not limited by the preceding steps of ternary complex binding to the ribosome and codon recognition, 10 s^{-1} (Figure 4A), the value of 0.2 s^{-1} represents the rate constant of GTP hydrolysis. On intact ribosomes, k_{GTP} was 500 s^{-1}, as determined previously by complete kinetic analysis on the basis of the concentration dependence of all steps (*52*). Thus, L7/12 stimulates GTP hydrolysis by EF-Tu on the ribosome by a factor of about 2500.

Peptide bond formation on ribosomes lacking L7/12 (0.08 s^{-1}) was also slower than on intact or reconstituted ribosomes (about 0.7 s^{-1}) (Figure 4C). The inhibition was mainly due to slow GTP hydrolysis which limited the rates of all subsequent steps. In addition, one or several of the steps following GTP hydrolysis, i.e., the conformational change of EF-Tu from the GTP- to the GDP-bound conformation, the release of aa-tRNA from the factor, and aa-tRNA accommodation, were 5 times slower on ribosomes lacking L7/12, as calculated from the difference in the half-life times of peptide bond formation and GTP hydrolysis, which were 5 s in the absence and 1 s in the presence of L7/12. Thus, the predominant effect of L7/12 on EF-Tu function on the ribosome is on GTP hydrolysis; the rates of other steps are reduced by factors of 2−5 only.

DISCUSSION

The binding site for elongation factors on the ribosome comprises proteins L7/12, L10, L11, and two regions of 23S rRNA, i.e., the L11-binding region around nucleotide 1060 and the SRL around nucleotide 2660. The present data show that these elements have distinct and different roles for the functions of EF-G and EF-Tu on the ribosome. Both L7/12 and ribosomal elements present in cores contribute to the stimulation of GTP hydrolysis by EF-G and EF-Tu. Factor binding to the ribosome is dominated by core elements and is either not affected (EF-G) or the affinity is decreased about 2-fold only (EF-Tu) by the removal of L7/12. Other factor-dependent functions, i.e., A-site binding of aa-tRNA and translocation, are impaired to the extent which is expected for these reactions when there is no, or very slow, GTP hydrolysis. In the absence of L7/12, the rates of single-round and multiple-turnover translocation are reduced by factors of 30−50, which is also observed when the reactions are performed on intact ribosomes with either wild-type EF-G and nonhydrolyzable GTP analogues or mutant EF-G deficient in GTP hydrolysis and GTP (*47*, *50*; Katunin et al., submitted for publication). In EF-Tu-dependent A-site binding of aa-tRNA, aa-tRNA accommodation in the A site and peptide bond formation are slow on ribosomes lacking L7/12, mainly because GTP hydrolysis that precedes these steps is strongly impaired; additional effects which may not be directly related to the inhibition of GTP hydrolysis do not exceed 5-fold rate reductions. Thus, for both factors the inhibition of GTP hydrolysis is the main primary cause for impaired function resulting from the removal of L7/12 proteins.

GTP hydrolysis by elongation factors is accelerated on the ribosome by at least 7 orders of magnitude, from $<10^{-5}$ and 5×10^{-5} s^{-1} (*57*, *58*) to about 170 and 500 s^{-1} for EF-G and EF-Tu, respectively (*47*, *52*). Removal of L7/12 decreases the GTPase rate of EF-G more than 4000-fold. Thus,

L7/12 provides a major contribution to GTPase activation of EF-G. Core ribosome elements that accelerate the reaction by another factor of up to 4000 are likely to play a role in binding and correct positioning of EF-G. GTP hydrolysis in EF-Tu is stimulated 4000-fold by core elements and by another factor of 2500 by L7/12; thus, the contributions of L7/12 and cores to overall GTPase stimulation of EF-Tu are roughly comparable and additive.

The GDP·AlF_4-bound form of a number of GTP-binding proteins, including EF-G (but not EF-Tu) (*54*), mimics the GTPase transition state, and GTPase-activating proteins (GAPs) and RGSs induce and/or stabilize the structure (*32*, *33*, *59*). The removal of L7/12 from the ribosome prevents the formation of the GDP·AlF_4-dependent GTPase transition state of EF-G, whereas it is readily formed in the presence of native ribosomes. Notably, isolated L7/12, which stimulates GTP hydrolysis by EF-G about 500 times less efficiently than the intact ribosomes (*31*), does not induce the high-fluorescence mant-GDP·AlF_4 state of EF-G (A. Savelsbergh, unpublished data). Thus, both L7/12 and ribosomal core elements are required to efficiently induce or stabilize the GTPase transition state of EF-G and to promote rapid GTP hydrolysis. The ribosomal core elements may either provide additional interactions with EF-G or preferentially stabilize an arrangement of L7/12 that is required for the GTPase stimulation.

Generally, the GTPase activity of GTP-binding proteins is activated by proteins, GAPs or RGSs, that contact the switch 1 and 2 regions of the G domain (*32*). The events at the active site that result from activator binding are different in detail for different GTPases. GTPase activation in Ras (and Ras-like GTPases) or G_α subunits of heterotrimeric G proteins requires positioning of a catalytic arginine that either is donated by Ras-GAP or else resides in the effector region of G_α and is stabilized in the catalytically active position by RGS binding (*32*). For GTPase activation of EF-G and EF-Tu, either mechanism seems unlikely. The single, universally conserved arginine in L7/12, Arg74, does not function as an arginine finger (*31*). Furthermore, GTP hydrolysis by EF-G is stimulated about 100-fold by the addition of alcohol in the absence of ribosomes (*60*), and the inhibition of EF-G-dependent GTP hydrolysis by L7/12 depletion of ribosomes is partially reversed by the addition of alcohol (*40*), suggesting that the GTPase activation in EF-G requires structural rearrangements within the active site, rather than a catalytic residue donated *in trans*. On the other hand, those intrinsic arginine residues that are located in the effector loop of EF-G (Arg59) or EF-Tu (Arg58) in positions homologous to that of the catalytic arginine in G_α proteins are not essential for GTPase activity (*50*, *61*, *62*). Unexpectedly, an arginine residue in EF-G (Arg29) which is 11 Å away from the β-phosphate of GDP, and is not present in EF-Tu, turned out to be crucial for the GTPase activity of EF-G on the ribosome (*50*). However, direct participation of Arg29 in GTP hydrolysis would require an extensive conformational change of the G domain to bring Arg29 closer to GTP. Mutational analysis of EF-Tu failed to identify an arginine residue in the vicinity of the GTP-binding pocket that would specifically affect the GTPase activity of the factor. Thus, GTP hydrolysis in elongation factors may not require an arginine residue at the catalytic site.

The crystal structure of the small GTP-binding protein Ran in the complex with RanBP1 and RanGAP, which are both required to induce GTP hydrolysis in Ran, indicates that correct positioning of a glutamine residue in the catalytic site and its shielding from solvent are sufficient for GTP hydrolysis by Ran (*63*). EF-Tu and EF-G have histidines at positions homologous to that of glutamine in Ran, His84 and His91, respectively, that may serve a catalytic function. In fact, His84 in EF-Tu was shown to be essential for GTP hydrolysis (*62*). The orientation of His84 is such that an extensive conformational change within the G domain of EF-Tu is necessary in order to position the side chain of histidine into the catalytic center (*64*). The interaction with L7/12 may initiate the required conformational change of the G domain, thereby inducing the formation of the GTPase transition state. Interactions of the switch regions of the factors with structural elements of the ribosomal core may additionally stabilize the catalytically active conformation, augmenting the effect of L7/12.

Structural details of the interactions of elongation factors with the ribosome during GTPase activation and GTP hydrolysis are not known, as there are no crystal structures of ribosome−factor complexes. Furthermore, most of the available cryo-EM reconstructions of ribosome−EF-G complexes are of low resolution and show the factor bound to the ribosome in the post-translocation state. The complex that may be closest to the GTPase state is one in which EF-G was stalled in the pretranslocation state immediately after GTP hydrolysis by binding thiostrepton to the 50S subunit. In the cryo-EM reconstruction of this complex, the G domain of the factor is involved in extensive interactions with structural elements of the 50S stalk, possibly including interactions with L7/12; however, structural details of this interaction as well as of other EF-G−ribosome interactions were not resolved (*5*). The cryo-EM structure of EF-G with ribosomes with free A site (i.e., representing the post-translocation state) reveals that the switch regions of the G domain are in contact with the SRL of 23S rRNA (*65*), whereas an arc-like interaction of an element of the 50S subunit with the G′ subdomain of the factor was assigned as a connection to L11 (*6*); the recent reconstruction of EF-G in a post-termination complex has identified a contact of the G′ subdomain to L7/12 (M. Valle and J. Frank, personal communication). The SRL is known to bind EF-G both on the ribosome (*10*) and free in solution (*13*). However, in complexes stalled immediately after GTP hydrolysis, either by thiostrepton (*48*) or by introducing an intramolecular cross-link into EF-G (*66*), no contact of EF-G with the SRL was found by chemical footprinting. These results argue against, though do not exclude, an early contact between EF-G and the SRL which may be involved in GTPase activation. On the other hand, the interaction of EF-G with the L11-binding region seems to take place early (*66*) and persist throughout the functional cycle of EF-G on the ribosome (*10*). However, so far there is no evidence suggesting that the L11-binding region is involved in the GTPase activation of EF-G, since thiostrepton binding to this region does not impair single-round GTP hydrolysis (*48*), and mutations at the crucial position 1067 in the L11-RNA had essentially no effect on EF-G function (*23*). Furthermore, an involvement of proteins L10 and L11 seems unlikely, because L10 does not increase the stimulation of GTP

hydrolysis by EF-G brought about by isolated L7/12 (*31*), and L11 is a nonessential protein (*35*, *36*). Thus, the question of which elements of the 50S subunit other than L7/12, for instance the SRL and the L11-region, are involved in GTPase activation of EF-G cannot be answered until further structural information becomes available.

The cryo-EM reconstruction of the ternary complex stalled on the ribosome after GTP hydrolysis, but before the conformational rearrangement into the GDP form took place, shows extensive interactions of the switch regions of the G domain with the SRL, whereas L11 and the L11-binding region of 23S rRNA contact the elbow region of aa-tRNA in the ternary complex, rather than EF-Tu itself (*2*, *7*, *8*). An earlier reconstruction, obtained at lower resolution, suggested a contact of L7/12 to helix D of EF-Tu (*2*); in the 13 Å map, density corresponding to such an interaction was not observed, which may indicate a weak interaction and/or high flexibility of L7/12 (*8*). Thus, the available structural information suggests that the interaction of L7/12 with EF-Tu is transient compared to other contacts in the complex and may involve regions of the factors other than the switch regions. This suggests a mechanism in which interactions of both L7/12 and the SRL with different regions of the G domain stimulate the GTPase activity of EF-Tu in an additive fashion. Most likely, L7/12 induces the rearrangement of the G domain required to bring the catalytic residue(s), in particular the essential His84, into the active site, and the SRL stabilizes the transition state conformation of the switch regions of the factor.

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