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Control of phosphate release from elongation factor G by ribosomal protein L7/12

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Ribosomal protein L7/12 is crucial for the function of elongation factor G (EF-G) on the ribosome. Here, we report the localization of a site in the C-terminal domain (CTD) of L7/12 that is critical for the interaction with EF-G. Single conserved surface amino acids were replaced in the CTD of L7/12. Whereas mutations in helices 5 and 6 had no effect, replacements of V66, I69, K70, and R73 in helix 4 increased the Michaelis constant (K_M) of EF-G·GTP for the ribosome, suggesting an involvement of these residues in EF-G binding. The mutations did not appreciably affect rapid single-round GTP hydrolysis and had no effect on tRNA translocation on the ribosome. In contrast, the release of inorganic phosphate (Pi) from ribosome-bound EF-G·GDP·Pi was strongly inhibited and became rate-limiting for the turnover of EF-G. The control of Pi release by interactions between EF-G and L7/12 appears to be important for maintaining the conformational coupling between EF-G and the ribosome for translocation and for timing the dissociation of the factor from the ribosome.

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Introduction

In the ribosomal elongation cycle, elongation factor G (EF-G) promotes the translocation step. EF-G is a large GTPase that consists of five domains (Ævarsson *et al*, 1994; Czworkowski *et al*, 1994). The GTPase activity of EF-G, which is very low intrinsically ($<10^{-5}\,s^{-1}$; Parmeggiani and Sander, 1981), is stimulated by several orders of magnitude upon binding of EF-G·GTP to the ribosome (Rodnina *et al*, 1997). GTP hydrolysis by EF-G is stimulated by isolated L7/12 (Savelsbergh *et al*, 2000b) and the GTPase activity of EF-G on the ribosome is decreased about 1000-fold when ribosomes are depleted of L7/12 (Mohr *et al*, 2002; Diaconu *et al*, 2005), indicating that a major contribution to GTPase activation on the ribosome comes from protein L7/12. In comparison, the rate of translocation on ribosomes lacking L7/12 is reduced only 50-fold, that is, to the rate observed when there is no GTP hydrolysis (Mohr *et al*, 2000, 2002; Katunin *et al*, 2002). On native ribosomes containing L7/12, the steps following GTP hydrolysis, including tRNA–mRNA translocation and the release of inorganic phosphate (Pi) from EF-G·GDP·Pi, are much slower than GTP hydrolysis and delayed by about 30 ms. The rate-limiting step for both translocation and Pi release is a rearrangement of the ribosome ('unlocking') that is induced by the binding of EF-G·GTP to the ribosome and promoted by GTP hydrolysis (Savelsbergh *et al*, 2003). Translocation is much less efficient, and has different activation parameters, with non-hydrolyzable GTP analogs or GDP, compared to GTP, indicating different reaction pathways (Katunin *et al*, 2002). The active form of EF-G in promoting unlocking and, thereby, translocation is EF-G·GDP·Pi, indicating that the retention of Pi in the nucleotide-binding pocket following GTP hydrolysis is important for conformational coupling between EF-G and the ribosome (Savelsbergh *et al*, 2003). On the other hand, the turnover of EF-G is very slow with GTP analogs (Belitsina *et al*, 1976; Kaziro, 1978; Katunin *et al*, 2002), indicating that the release of Pi is required for EF-G to dissociate from the ribosome.

The L7/12 stalk of the large ribosomal subunit forms part of the binding site of EF-G on the ribosome. The stalk comprises protein L10 and, in *Escherichia coli*, four copies of protein L7/12. Protein L7/12 comprises two domains (Dey *et al*, 1995; Wahl *et al*, 2000). The N-terminal domain consists of two α helices (α1, 2) and is responsible for dimer formation and for anchoring the L7/L12 dimers to helix α8 of protein L10 on the 50S subunit (Diaconu *et al*, 2005). N- and C-terminal domains are connected by a long hinge assigned as helix α3 in the crystal structure (Wahl *et al*, 2000). The hinge is likely to be unstructured in solution (Bocharov *et al*, 2004; Mulder *et al*, 2004) and confers mobility to the C-terminal domain (CTD) (Dey *et al*, 1995; Wahl *et al*, 2000; Diaconu *et al*, 2005). The globular CTD, which is the site of factor binding, consists of three α helices (α4–6) and a double-stranded β sheet (Leijonmarck and Liljas, 1987). Mutagenesis and kinetic studies suggested that helices 4 and 5 in the CTD of L7/12 are involved in the initial contact with helix D of elongation factor Tu (EF-Tu), promoting ternary complex binding to the ribosome (Kothe *et al*, 2004). Except for the partial functional analysis of a single mutant, K70A, in helix 4 of the L7/12 CTD (Savelsbergh *et al*, 2003), the interactions between the CTD of L7/12 and EF-G that are involved in regulating EF-G functions on the ribosome have not been characterized in detail and the location of the binding site of EF-G in the CTD of L7/12 is not known. In the present work, the interaction between the CTD of protein L7/12 and EF-G was studied in the *E. coli* system by introducing amino-acid changes in the CTD of L7/12. Ribosomes reconstituted with

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mutant L7/12 were studied with respect to EF-G functions on the ribosome, including GTP hydrolysis, translocation, and Pi release.

Results

Effects of amino-acid replacements in ribosomal protein L7/12 on multiple-turnover reactions of EF-G

Single amino acids were exchanged at conserved surface positions in the CTD of *E. coli* L7/12 (Figure 1). Mutagenesis was performed and mutant L7/12 expressed and purified as described in Materials and methods. Ribosomes were depleted of L7/12 by NH_4Cl/ethanol treatment and reconstituted with mutant or wild-type (wt) L7/12 (Mohr *et al*, 2002). Under conditions where L7/12 depletion was complete, only trace amounts of L10 and no other ribosomal proteins were lost. In agreement with earlier reports (Kischa *et al*, 1971; Hamel *et al*, 1972; Sander *et al*, 1975), ribosome-dependent GTP hydrolysis by EF-G was strongly inhibited by the removal of L7/12, and full activity was restored by adding back excess wt L7/12 to depleted cores (Figure 2).

Ribosomes reconstituted with mutant L7/12 were first assayed for their ability to stimulate GTP hydrolysis by EF-G under conditions of multiple turnover, using catalytic amounts of EF-G. Amino-acid replacements at four positions in helix 4 of the CTD of L7/12 (V66A/D, I69A, K70A, and R73M) reduced the rates of EF-G-dependent turnover GTP hydrolysis (Figure 2A). Two other mutations in helix 4, R73K and K65A, had no effect (data not shown). Alanine substitutions in positions K84, E88, K107, K100, and K120 did not affect turnover GTP hydrolysis (data not shown). Helix 4 mutations that had an effect on the activity of EF-G on the ribosome were studied in more detail.

To quantify the effects of helix 4 mutations on k_{cat} and K_M of GTP hydrolysis, initial rates were measured at various concentrations of ribosomes added in excess over EF-G. The mutations lowered k_{cat} up to 15-fold and increased K_M up to about three-fold, resulting in an up to 50-fold decrease of the catalytic efficiency, k_{cat}/K_M (Table I). The strongest effect (50-fold decrease of k_{cat}/K_M) was observed when valine at position 66 was replaced with the charged amino acid aspartate (V66D). The more conservative V66A exchange decreased k_{cat}/K_M about five-fold. A 4- to 10-fold decrease of k_{cat}/K_M resulted from the replacements I69A, K70A, and R73M.

Independent evidence for the effect of mutations in L7/12 on EF-G function was obtained when the translocation of fMetPhe-tRNA^{Phe} was studied under multiple-turnover conditions, using catalytic amounts of EF-G. In this assay, the

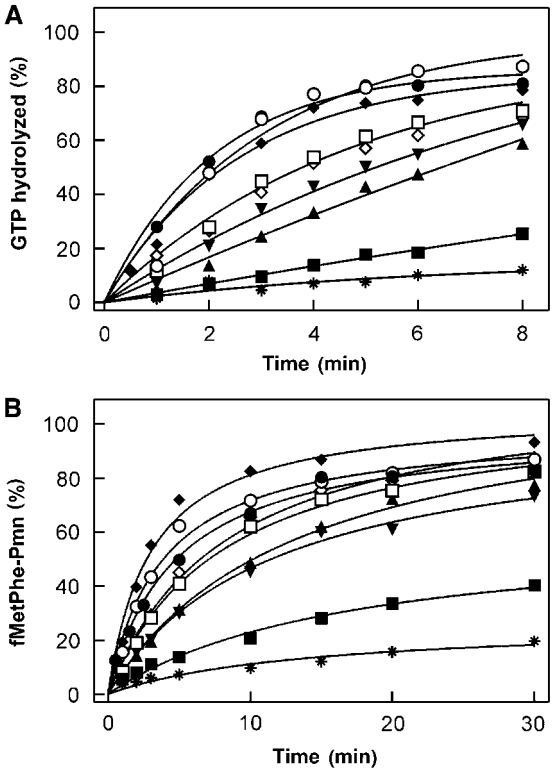

Figure 2 Effect of L7/12 mutations on turnover reactions of EF-G. (**A**) GTP hydrolysis. Time courses were measured with native ribosomes (●), ribosomes reconstituted with wt L7/12 (○), R73K (◆), V66A (□), R73 M (◇), I69A (▼), K70A (▲), or V66D (■), and ribosome cores lacking L7/12 (*). EF-G (0.04 μM) was incubated with [γ-^{32}P]GTP (20 μM) and ribosomes (0.2 μM) at 37°C. (**B**) Translocation. Pretranslocation complex carrying fMetPhe-tRNA^{Phe} in the A site (0.2 μM) was incubated with EF-G (0.5 nM) and GTP (1 mM) at 37°C. At the indicated times, puromycin (Pmn; 1 mM) was added and the reaction stopped after 10 s. The extent of fMetPhe-Pmn formation is given relative to the initial amount of pretranslocation complex. Symbols as in panel A.

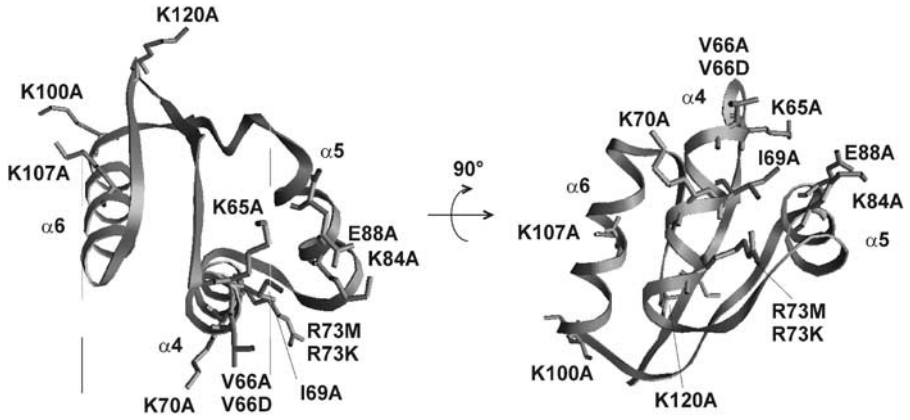

Figure 1 Positions of mutations in the CTD of protein L7/12 from *E. coli*. Amino acids are numbered according to the sequence of the protein as in the crystal structure (Leijonmarck and Liljas, 1987).

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Table I Effect of mutations in the CTD of protein L7/12 on EF-G turnover during GTP hydrolysis and translocation on the ribosome

L7/12	GTP hydrolysis			Translocation		
	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M ($\mu M^{-1} s^{-1}$)	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M ($\mu M^{-1} s^{-1}$)
wt	3.0±0.1	0.07±0.02	43±12	1.6±0.1	0.07±0.01	20±4
V66A	1.3±0.1	0.17±0.04	8±2	0.7±0.1	0.16±0.05	4±1
V66D	0.2±0.1	0.22±0.06	0.9±0.5	0.4±0.1	0.47±0.06	1.0±0.3
I69A	0.9±0.1	0.08±0.04	11±6	0.7±0.1	0.13±0.03	5±1
K70A	0.7±0.1	0.17±0.07	4±2	0.8±0.1	0.12±0.03	6±1
R73K	1.9±0.2	0.05±0.02	38±15	n.d.	n.d.	n.d.
R73M	1.6±0.3	0.20±0.04	8±2	0.7±0.1	0.17±0.02	8±1

wt, wild type; n.d., not determined.

replacement R73K had no effect, whereas the mutations R73M, V66A, I69A, K70A, and particularly V66D, substantially reduced the turnover rate of translocation (Figure 2B). To quantify the effects on the kinetic parameters of multiple-turnover translocation, Michaelis–Menten titrations were performed with pretranslocation complexes prepared from ribosomes carrying mutant L7/12 (Table I). The mutations decreased k_{cat} and increased K_M values for EF-G-dependent translocation in a similar fashion as observed for GTP hydrolysis (Table I). Again the strongest effect was observed for the V66D mutant (20-fold decrease of k_{cat}/K_M), whereas V66A, I69A, K70A, and R73M had moderate effects (two- to five-fold decrease of k_{cat}/K_M).

The two turnover assays yielded values for k_{cat} and K_M that were similar within a factor of two (Table I). Thus, on both vacant ribosomes (GTP hydrolysis assay) and pretranslocation complexes (translocation assay), the turnover of EF-G is controlled by an interaction between L7/12 and EF-G, which involves specific amino-acid residues that are all located on one side of helix 4 of the CTD of L7/12.

Single-round GTP hydrolysis and translocation

To study the functional importance of the interaction between L7/12 and EF-G for specific functions of EF-G, we measured the effects of helix 4 mutations on GTP hydrolysis, Pi release, and tRNA translocation under single-round conditions. Single-round GTP hydrolysis was measured at saturating EF-G concentration (Figure 3A). Although the presence of L7/12 is required to stimulate rapid GTP hydrolysis by EF-G on the ribosome (Mohr *et al*, 2002), there was no systematic effect of the mutations in helix 4 on the first round of rapid GTP hydrolysis, as GTP hydrolysis rates observed for ribosomes reconstituted with mutant L7/12 were higher or lower by at most a factor of two compared to ribosomes with wt L7/12 (Figure 3A and B). Notably, ribosomes containing mutant L7/12(V66D), which had the strongest inhibitory effect on EF-G turnover (Table I), stimulated GTP hydrolysis at the same rate as ribosomes containing wt L7/12. We concluded that none of the mutated amino-acid side chains in L7/12 is essential for ribosome-induced rapid GTP hydrolysis by EF-G and that GTP hydrolysis was invariably much faster than the following steps (see below).

The extent of translocation was not affected by any of the mutations in L7/12 (Figure 3C), consistent with the previous observation that even in the absence of L7/12 translocation was complete (Mohr *et al*, 2002). Also the rate of translocation, as measured by fluorescence stopped-flow, was not significantly altered by any of the helix 4 mutations (Figure 3C), as shown previously for the K70A mutation (Savelsbergh *et al*, 2003). This result indicates that the contact of L7/12 with EF-G is not important for translocation and that the inhibition observed under conditions of multiple turnover is not due to an inhibition of translocation.

Release of inorganic phosphate

The release of Pi from ribosome-bound EF-G·GDP·Pi was monitored by the fluorescence of MDCC-labeled phosphate-binding protein (PBP) that strongly increases upon binding of Pi (Brune *et al*, 1994; Savelsbergh *et al*, 2003) (Figure 4A). Pi release took place in two phases: The first round of Pi release gave rise to a rapid burst, which was followed by a slower phase due to turnover; the curves leveled off after a few seconds (not shown in Figure 4A) due to the saturation of PBP with Pi and the uptake of Pi by the 'Pi mop' (Materials and methods). Exponential fitting yielded values for both rates and amplitudes of the burst as well as of the turnover reaction. Burst rates were in the range of $20 \pm 10\ s^{-1}$ for wt and mutant L7/12, that is, there was no systematic influence of the mutations on the burst rate. However, the burst amplitudes were decreased with the L7/12 mutants, except R73K and R73M (Figure 4B). Compared to the amplitude of GTP hydrolysis, the amplitude of Pi release was lower by 30–40% (I69A, V66A) or 75–85% (K70A, V66D).

The observation of an up to 85% decrease of the burst amplitude with no change of the burst rate can only be explained by a branched model of translocation of the type we have derived from kinetic analyses (Savelsbergh *et al*, 2003) (Figure 4C). Alternative models, such as the early model suggested by Spirin (1985) according to which tRNA translocation preceded GTP hydrolysis or a recent model that postulates a rate-limiting nucleotide exchange on EF-G on the ribosome (Zavialov *et al*, 2005), do not contain a branch and no explicit Pi release step and are, therefore, unsuitable to interpret the present observations. Furthermore, these models are inconsistent with the kinetic data that define the timing of EF-G binding, GTP hydrolysis, and tRNA–mRNA movement during translocation (Rodnina *et al*, 1997; Savelsbergh *et al*, 2000a, b, 2003; Seo *et al*, 2004).

According to the branched model (Figure 4C), following ribosome unlocking translocation may occur first, followed by Pi release (upper branch), or, alternatively, Pi release may take place first, followed by translocation (lower branch). On native ribosomes, the latter two reactions are intrinsically rapid and their rates are determined by the unlocking step; therefore, rate constants of translocation and Pi release cannot be determined in the wt situation, and the observed

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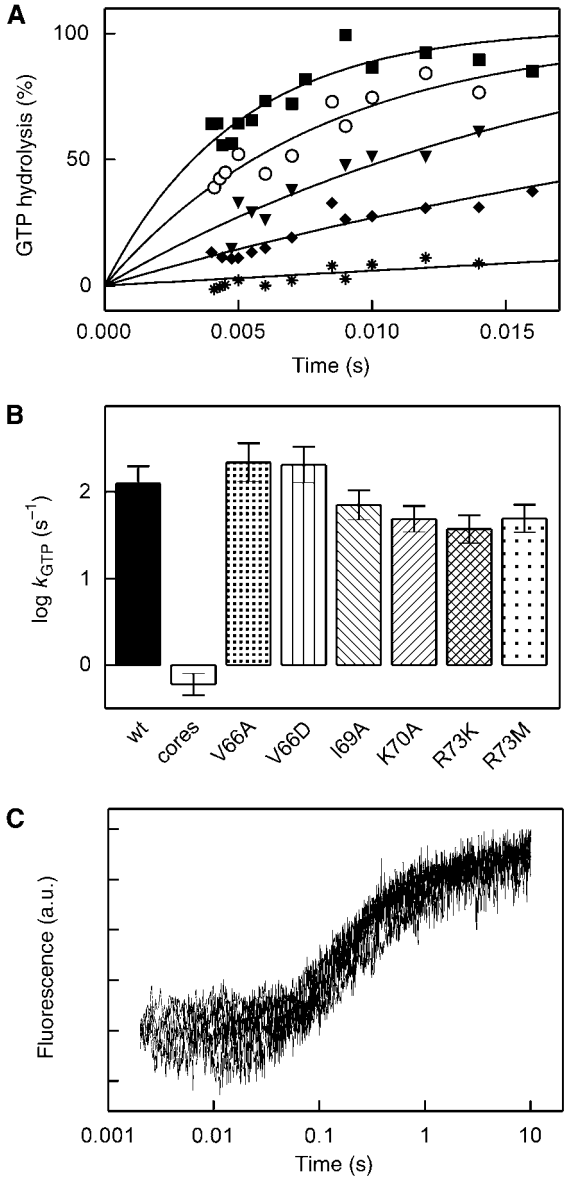

Figure 3 Effect of L7/12 mutations on single-round GTP hydrolysis and translocation catalyzed by EF-G. (**A**) Time courses of GTP hydrolysis. EF-G and [γ-^{32}P]GTP were rapidly mixed with ribosomes (Materials and methods). Unlabeled GTP (1 mM) was added together with the ribosomes in order to limit [γ-^{32}P]GTP hydrolysis to a single round (Rodnina *et al*, 1997). Symbols as in Figure 2. (**B**) Rate constants of GTP hydrolysis. Time courses shown in panel A were evaluated by exponential fitting to yield rate constants as indicated. (**C**) Time courses of translocation as monitored by the fluorescence of fMetPhe-tRNA^{Phe}(Prf16/17). Overlaid stopped-flow traces are shown for ribosomes reconstituted with wt L7/12, V66A, V66D, I69A, K70A, R73M, and R73K.

burst amplitude of Pi release reflects the sum of the upper and lower branches of the reaction. However, when Pi release becomes slower than unlocking, while translocation remains fast, the lower branch will be disfavored and the amplitude of the rapid burst of Pi release will decrease, due to partitioning between the lower and upper branches of the scheme (for mathematical treatment of partitioning, see Fersht, 1985). The fact that the burst amplitude of Pi release was decreased by mutations in L7/12, while the amplitude of rapid GTP hydrolysis was not (Figure 4), indicated that the mutations disfavored Pi release relative to the parallel reaction. On vacant ribosomes (used in the present experiments), where there is no translocation, the parallel reaction presumably consists in a conformational rearrangement of the ribosome that follows unlocking (and accompanies translocation on translating ribosomes). Vacant ribosomes can be used for the analysis of Pi release, because kinetic studies have revealed that the rates of first-round and multiple-turnover Pi release on translocating and vacant ribosomes are about the same (Savelsbergh *et al*, 2003; data not shown), indicating that Pi release is not affected by the functional state of the ribosome. Depending on the actual rates, the burst rate of Pi release is given either by the sum of the rates of the two competing reactions, Pi release and ribosome rearrangement (or translocation), or is determined by the rate of the unlocking step that precedes both reactions (Savelsbergh *et al*, 2003). The rate of the unlocking step was not affected by the mutations, as the rate of translocation was not changed significantly by any of the mutations (Figure 3C). Consequently, the apparent rate of the residual burst of Pi release also should not be affected by the mutations, which is exactly what was observed (Figure 4A).

After the burst phase, a slower phase of Pi release is observed (Figure 4A), which on wt ribosomes reflects the turnover of EF-G. However, on ribosomes carrying mutant L7/12, part of the amplitude of slow Pi release is due to first-round Pi release, which was slowed down by the mutations. For example, the V66D mutation, which had the strongest effect on turnover, reduced the burst amplitude of Pi release to 15%, compared to the native situation (100%), whereas the rate of translocation was not changed (Figure 3C). Thus, in this case, 85% of the reaction followed the upper branch of the kinetic scheme (Figure 4C), and 85% of Pi was released at the rate of the following multiple-turnover reaction, which was about $1\,s^{-1}$ for the V66D mutant, comparable to the numbers measured by turnover GTP hydrolysis and translocation (Table I). Compared to the burst rate of Pi release on native ribosomes, $30\,s^{-1}$, the V66D mutation lowered the apparent rate of Pi release about 30-fold. The other mutations (V66A, I69A, K70A, and R73M) inhibited Pi release about 15-fold, to around $2\,s^{-1}$, again comparable to the other two turnover assays (Table I). This suggests that the L7/12 mutations slowed down Pi release to such an extent that it became rate-limiting for the turnover of EF-G, that is, k_{cat} of the turnover reaction(s) represented the rate of Pi release. On core ribosomes lacking L7/12, there was no burst of Pi release, as GTP hydrolysis was very slow ($0.6\,s^{-1}$), and turnover Pi release was slow as well (Figure 4A). In this case, GTP hydrolysis is rate-limiting for Pi release and EF-G turnover (Mohr *et al*, 2002).

To examine whether the effect of L7/12 mutations on Pi release was specific for EF-G, we performed measurements with EF-Tu. Mutations in helices 4 and 5 of L7/12 (K65A, V66A, V66D, I69A, K70A, R73M, and K84A) reduced the rate of association of the ternary complex EF-Tu·GTP·aa-tRNA with the ribosome, but had no effect on the rate constant of GTP hydrolysis by EF-Tu (Kothe *et al*, 2004; Diaconu *et al*, 2005). Helix 4 mutations lowered the apparent rate of the first round of Pi release from EF-Tu about three- to four-fold (multiple rounds were not observed due to very slow nucleotide exchange on EF-Tu) (Figure 5). However, this inhibition is fully explained by the reduction of the association rate

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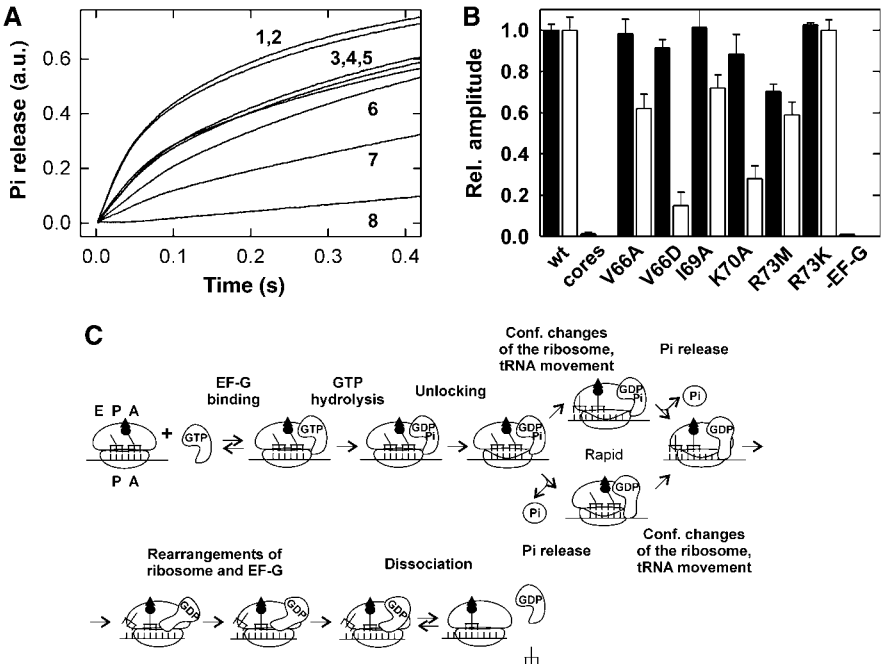

Figure 4 Pi release from EF-G after GTP hydrolysis. (**A**) Time courses of Pi release. EF-G (3 μM) and GTP (30 μM) were mixed with ribosomes (0.5 μM) in the stopped-flow apparatus and the liberation of Pi was monitored by the fluorescence change of MDCC-labeled PBP (Materials and methods). Traces are shown for ribosomes reconstituted with wt L7/12 (1), R73K (2), R73M (3), V66A (4), I69A (5), K70A (6), V66D (7) and for ribosome cores depleted of L7/12 (8). Exponential fitting yielded burst rates of $20 \pm 10\,s^{-1}$ in all cases, except for cores depleted of L7/12 where the burst phase is lacking. The rates of the slower turnover phase were 3–4 s^{-1} (wt, R73M, R73K), 2 s^{-1} (V66A, I69A), or 1 s^{-1} (V66D, K70A). The final level of all time courses was identical within 10% and was normalized to 1.0. (**B**) Relative burst amplitudes of single-round GTP hydrolysis (closed bars) and Pi release (open bars). The extent of [γ-^{32}P]GTP hydrolysis was determined either from quench-flow data (Figure 3) or from the amount of [γ-^{32}P]GTP hydrolyzed after 10 s at conditions where multiple turnover was suppressed by the addition of unlabeled GTP. Amplitudes of Pi release were estimated from the data in panel A. Burst amplitudes for GTP hydrolysis and Pi release determined for ribosomes reconstituted with wt L7/12 were set to 1.0 and relative numbers are given for cores and reconstituted mutant ribosomes. (**C**) Kinetic scheme of translocation used for the evaluation of rate constants of Pi release (Savelsbergh *et al*, 2003). Binding of EF-G to the ribosome is followed by rapid GTP hydrolysis (50–200 s^{-1}, cf. Figure 3B); a conformational change ('unlocking'; 10–35 s^{-1}; Savelsbergh *et al*, 2003) precedes and limits (fully or partially) the following tRNA–mRNA translocation. Translocation (or a structural rearrangement accompanying tRNA movement) and Pi release are parallel and independent of one another. The rate of Pi release is decreased to 1–2 s^{-1} by mutations at positions 66, 69, and 70 of the L7/12 CTD. Subsequent conformational changes of the ribosome and EF-G are important for EF-G dissociation from the ribosome.

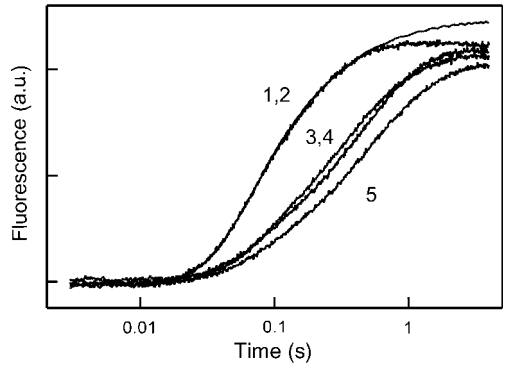

Figure 5 Time courses of Pi release from EF-Tu. Purified ternary complex EF-Tu·GTP·Phe-tRNA^{Phe} (1 μM) was rapidly mixed with poly(U)-programmed ribosomes (1 μM) containing AcPhe-tRNA^{Phe} in the P site. Traces are shown for native ribosomes (1) and ribosomes reconstituted with wt L7/12 (2), R73M (3), K70A (4), or K84A (5).

constant caused by the mutations, from $100\,\mu M^{-1}\,s^{-1}$ (wt L7/12) to about $20–30\,\mu M^{-1}\,s^{-1}$ (mutant L7/12) (Kothe *et al*, 2004; Diaconu *et al*, 2005). Thus, mutations in L7/12 do not directly affect Pi release from EF-Tu, suggesting that the control of Pi release from EF-G by contacts with helix 4 of L7/12 is specific for EF-G.

Discussion

The presence of protein L7/12 is important for the function of GTP-binding translation factors on the ribosome. The highly mobile CTDs of L7/12 reach out from the ribosome and promote the recruitment of the factors to the ribosome (Diaconu *et al*, 2005). Furthermore, L7/12 contributes to the stimulation of the GTPase activity of ribosome-bound factors by inducing or stabilizing their active conformation (Mohr *et al*, 2002; Diaconu *et al*, 2005). Sequence alignment of bacterial L7/12 proteins has revealed highly conserved patches in the CTD, in particular in helices 4 and 5, suggesting a site of interaction with translation factors (Leijonmarck and Liljas, 1987; Wahl *et al*, 2000; Wieden *et al*, 2001). Further evidence suggesting a role of this region of L7/12 in factor function came from the observations that the E82K mutation led to altered translational accuracy, decreased growth rates *in vivo* (Kirsebom and Isaksson, 1985; Kirsebom *et al*, 1986), and reduced translational efficiency *in vitro* (Bilgin *et al*, 1988). Chemical modification of R73 of L7/12 decreased the translational activity of the ribosomes

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(Koteliansky *et al*, 1977; Hernandez *et al*, 1984). Finally, amino-acid exchanges in helices 4 and 5 in the CTD of L7/12 decreased the rate of ternary complex binding to the ribosome, most likely by compromising interactions between L7/12 and helix D in the G domain of EF-Tu (Kothe *et al*, 2004).

The present data show that hydrophobic residues (V66, I69) as well as positively charged residues (K70, R73) in helix 4 of L7/12 are involved in the interaction with EF-G. All four mutations resulted in increased K_M values for the multiple-turnover reactions of EF-G on the ribosome, suggesting that helix 4 is (part of) the binding site and that the mutated residues may be directly involved in the interaction. The four residues are located on the surface of L7/12 and are either strictly conserved (K70, R73) or appear to allow only conservative replacements (66, I/V/L; 69, I/L) (Wieden *et al*, 2001). The presence of a basic residue at position 73 seems to be important, because the replacement R73K had essentially no effect on activity of EF-G on the ribosome, in contrast to the significant effect of the mutation R73M. In the crystal structure of L7/12, these residues form a ridge–groove–ridge surface, where the ridges are formed by charged amino acids and the bottom of the groove by hydrophobic amino acids. The dimensions of the surface would allow an α helix to bind along the groove, with charged residues positioned at opposite sides of the helix.

The region of EF-G that is involved in binding to L7/12 has not been characterized so far. Three-dimensional reconstructions of EF-G–ribosome complexes from cryo-electron microscopic images indicate that there is a contact between protein L7/12 and the G′ domain of EF-G (Valle *et al*, 2003; Diaconu *et al*, 2005), which is located at a considerable distance from the phosphate side of the nucleotide-binding pocket. Taking into account the nature of the amino-acid side chains in L7/12 that according to the present analysis appear to be involved in the interaction, the complementary surface in EF-G is expected to comprise both negatively charged and hydrophobic residues. Of the three helices in the G′ domain, helix $B_{G'}$ contains the appropriate side chains (Ævarsson *et al*, 1994; Czworkowski *et al*, 1994) and, thus, is a likely candidate to form (part of) the interaction site. Support for this contention comes from the observation that mutations in helix $B_{G'}$ strongly affect the function of EF-G on the ribosome (A Savelsbergh, unpublished results).

L7/12 is required for the full stimulation of the GTPase activity of EF-G, as removal of L7/12 reduces the rate of ribosome-stimulated GTP hydrolysis about 1000-fold (Mohr *et al*, 2002; Diaconu *et al*, 2005). Replacements of several conserved amino acids in the CTD of L7/12, including the single conserved arginine (R73), had little or no effect on single-round GTP hydrolysis by EF-G on the ribosome (Savelsbergh *et al*, 2000b; present results), indicating that none of these amino acids is directly involved in GTP hydrolysis. Rather, L7/12 appears to bring about the GTPase activation of EF-G by inducing a particular conformation of the factor on the ribosome that is required for rapid GTP hydrolysis, and the induction of the active GTPase conformation is not impaired by any of the mutations tested.

As shown by the present results, L7/12 plays a crucial role in Pi release from EF-G. Mutations of V66, I69, or K70 in helix 4 of L7/12 decrease the rate of the first round of Pi release such that it becomes rate-limiting for the turnover reaction. Rates of Pi release on mutant ribosomes are $1–2\,s^{-1}$, that is, 15–30 times lower than the rate of Pi release on native ribosomes, which is $\sim 30\,s^{-1}$ (Savelsbergh *et al*, 2003). This suggests that the nucleotide-binding pocket of the ribosome-bound factor, including the γ-phosphate site, closes upon binding of EF-G·GTP to the ribosome, in keeping with the increased affinity of the nucleotide to ribosome-bound EF-G (Baca *et al*, 1976), and is opened by interactions (or a change of interactions) between the CTD of L7/12 and EF-G, allowing Pi to dissociate. The contact of the L7/12 CTD with EF-G that is established early and leads to GTPase activation may be retained until Pi is released or EF-G·GDP dissociates from the ribosome. GTP hydrolysis enables EF-G to promote a rearrangement of the ribosome (unlocking), which is rate-limiting for both translocation and Pi release, indicating that the active form of the factor is EF-G·GDP·Pi (Savelsbergh *et al*, 2003). The retention of Pi in the nucleotide-binding pocket is important for EF-G function, because if Pi release were uncontrolled, the conformational coupling between EF-G and the ribosome would probably be disturbed or interrupted, interfering with the ribosome rearrangement required for translocation. Thus, controlling GTP hydrolysis and Pi retention (or release) by interactions between EF-G and L7/12 provides a device for timing the functions of EF-G on the ribosome.

Materials and methods

Buffers and reagents

Buffer A: 20 mM Tris–HCl, pH 7.5, 0.6 M NH_4Cl, 20 mM $MgCl_2$, and 5 mM 2-mercaptoethanol; buffer B: 50 mM Tris–HCl, pH 7.5, 30 mM KCl, 7 mM $MgCl_2$, and 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, 70 mM NH_4Cl, 30 mM KCl, 10 mM $MgCl_2$, and 2 mM DTT. GTP, phosphoenolpyruvate, and pyruvate kinase were from Roche Diagnostics. Radioactive compounds were from ICN. All other chemicals were from Merck.

Ribosomes, mRNAs, tRNAs, and factors

Ribosomes from *E. coli* MRE 600 were prepared as described (Rodnina and Wintermeyer, 1995). fMet-$tRNA^{fMet}$, [^{14}C]Phe-$tRNA^{Phe}$, MFTI-mRNA, EF-Tu, EF-G, and initiation factors were prepared and purified as described (Rodnina *et al*, 1994a, b, 1997, 1999).

Preparation of L7/12 mutants

The plasmid used as template for mutagenesis, pGEX-5x-3-L7/12, contained the gene for glutathione *S*-transferase (GST) fused to the gene of L7/12 (Savelsbergh *et al*, 2000b). Mutations were introduced by PCR mutagenesis using *Pfu* polymerase and verified by DNA sequencing. Mutant proteins were expressed in *E. coli* BL21 as GST fusion proteins and purified by affinity chromatography on glutathione-Sepharose 4B as described (Savelsbergh *et al*, 2000b), except that cleavage of the GST fusion proteins 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. Wt L7/12 was expressed and purified in the same way as the L7/12 mutants.

Depletion and reconstitution of ribosomes

Ribosomes were practically completely depleted of proteins L7/12 by NH_4Cl/ethanol treatment (Tokimatsu *et al*, 1981) with some modifications as described previously (Mohr *et al*, 2002). A 450 pmol portion of purified 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 gently 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 15 000 *g* for 30 min, and the ribosomal pellet was dissolved in buffer B. According to immunoblot analysis with anti-L7/12 antibodies (provided by AG Tonevitski, Moscow

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State University), the NH_4Cl/ethanol-treated ribosomes contained no detectable L7/12, that is, the L7/12 content was <2%. The removal of ribosomal proteins other than L7/12 was controlled by precipitating proteins from the NH_4Cl/ethanol supernatant with acetone; according to SDS–PAGE, the supernatant contained, besides L7/12, trace amounts of L10, and no other protein. For reconstitution, ribosomes depleted of L7/12 ('cores') were incubated with a five-fold excess of purified wt or mutant L7/12 for 30 min at 37°C; the extent of reconstitution was >80%, as determined by immunoblot analysis.

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 at 37°C and the reaction was initiated by the addition of [γ-^{32}P]GTP (20 μM). Steady-state kinetic parameters, k_{cat} and K_M, were determined under conditions of initial velocity. Incubation was for 1.5 min (wt L7/12, R73K, and R73M) or 2.5 min (all other mutants). Samples (30 μl) were quenched by adding 1 M $HClO_4$/3 mM potassium phosphate (30 μl) 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 phosphoimager (Bio-Rad).

Single-round GTP hydrolysis by EF-G was measured by mixing native or reconstituted ribosomes (1 μM) with EF-G (3 μM) and [γ-^{32}P]GTP (30 μM) in a quench-flow apparatus (KinTek Corp., Austin, TX, USA). Reactions were quenched with 1 M $HClO_4$/3 mM potassium phosphate and analyzed by extraction of ^{32}P-labeled inorganic phosphate into ethyl acetate in the presence of sodium molybdate (Leder and Bursztyn, 1966; Rodnina *et al*, 1997).

Dissociation of inorganic phosphate

Pi release from EF-G after GTP hydrolysis was measured by the fluorescence change of MDCC-labeled phosphate-binding protein (MDCC-PBP) (Brune *et al*, 1994) in buffer C at 37°C (Mohr *et al*, 2000; Savelsbergh *et al*, 2000a). Ribosomes and EF-G at the indicated concentrations were mixed with GTP (30 μM), MDCC-PBP (2.5 μM), purine nucleoside phosphorylase (0.1 U/ml), and 7-methylguanosine (200 μM) (the latter two components serving as 'Pi mop' to take up trace amounts of contaminating Pi) (Brune *et al*, 1994) in a stopped-flow apparatus (Applied Photophysics), and the fluorescence change of MDCC-PBP upon binding of Pi was monitored. Pi release from EF-Tu was studied in buffer D at 20°C, using ternary complex that was purified by gel filtration on Superdex 75 (Rodnina *et al*, 1994a). Ribosomes were programmed with poly(U) and AcPhe-tRNA^{Phe} in the P site and purified by ultracentrifugation through a sucrose cushion (Gromadski and Rodnina, 2004). Fluorescence was excited at 425 nm and measured after passing a cutoff filter (KV 450, Schott).

Translocation

To prepare initiation complexes, ribosomes (0.5 μM) were incubated with a three-fold molar excess of MFTI-mRNA (GGCAAGGAG GUAAAUAAUGUUUCACGAUU; codons used for the incorporation of M (fMet) and F (Phe) are underlined) and a 1.5-fold excess each of IF1, IF2, IF3, and f[^{3}H]Met-tRNA^{fMet} in buffer C containing GTP (1 mM) for 30 min at 37°C. The ternary complex EF-Tu·GTP·[^{14}C]Phe-tRNA^{Phe} was prepared by first incubating EF-Tu (2 μM) with GTP (1 mM), phosphoenolpyruvate (3 mM), and pyruvate kinase (0.5 mg/l) for 30 min at 37°C and then with [^{14}C]Phe-tRNA^{Phe} (1 μM) for an additional 5 min. Equal volumes each of initiation complex and ternary complex were mixed and incubated for 5 min at 20°C to form pretranslocation complex carrying tRNA^{fMet} in the P site and fMetPhe-tRNA^{Phe} in the A site.

Multiple-turnover translocation was induced by adding EF-G (final concentration 0.5 nM) to pretranslocation complexes present in increasing amounts (0.05–0.7 μM). Steady-state kinetic parameters (k_{cat}, K_M) were determined under conditions of initial velocity. Incubation was for 2 min (wt L7/12, R73K, and R73M), 5 min (V66D), or 4 min (all other mutants). The extent of translocation was measured by reaction with puromycin (Pmn; 1 mM, 10 s, 37°C), monitoring the formation of fMetPhe-Pmn (Rodnina *et al*, 1997). Single-round translocation was measured by fluorescence stopped-flow (Savelsbergh *et al*, 2003), using pretranslocation complex containing fluorescent fMetPhe-tRNA^{Phe} (Prf16/17) (0.5 μM), saturating amounts of EF-G (3 μM), and GTP (1 mM).

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