Stimulation of the GTPase Activity of Translation Elongation Factor G by Ribosomal Protein L7/12*

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Elongation factors (EFs) Tu and G are GTPases that have important functions in protein synthesis. The low intrinsic GTPase activity of both factors is strongly stimulated on the ribosome by unknown mechanisms. Here we report 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. The effect is due to the acceleration of the catalytic step because the rate of GDP-GTP exchange on EF-G, as measured by rapid kinetics, is much faster than the steady-state GTPase rate. The unique, highly conserved arginine residue in the C-terminal domain of L7/12 is not essential for the activation, excluding an "arginine finger"-type mechanism. L7/12 appears to function by stabilizing the GTPase transition state of EF-G.

GTP-binding proteins are involved in a number of important cellular processes, including signal transduction, protein synthesis, and protein export. Generally, GTPases act as molecular switches that alternate between an inactive GDP-bound conformation and an active GTP-bound conformation. The intrinsic GTPase activity of most GTP-binding proteins is low, ranging from 0.02 min^{-1} for Ras-like GTPases to 2–5 min^{-1} for the α subunits of heterotrimeric G proteins. The reaction is thought to proceed through an in-line associative mechanism in which the substrate, GTP, acts as a general base to activate a nucleophilic water (1, 2). The crystal structures of Gα subunits of two heterotrimeric G proteins, $G_t\alpha$ and $G\alpha_{i1}$ (3, 4), revealed two conserved residues, glutamine and arginine, that are crucial for the stabilization of the GTPase transition state. Whereas a glutamine residue in a homologous position is also present in Ras-like GTPases, the corresponding arginine residue is missing, in keeping with the relatively low intrinsic GTPase activity.

In the case of several small GTPases, GTP hydrolysis is accelerated by GTPase-activating proteins (GAPs)[1] that are specific with respect to their target GTP-binding proteins (5). The mechanism by which GAPs accelerate the GTPase reaction has been elucidated recently for Ras (6–8) and Rho (9, 10). Upon interaction with their respective target, both RasGAP and RhoGAP supply a catalytic arginine residue into the active

site of Ras ("arginine finger"), thereby stabilizing the GTPase transition state and increasing the rate of GTP hydrolysis more than 1000-fold. Furthermore, the structure of the active center of the Ras-RasGAP and Rho-RhoGAP complexes appears to be remarkably similar to the putative GTPase transition state structure of Gα (11), suggesting a similar stereochemistry of the GTPase reaction, regardless of whether the catalytic arginine is provided in *cis* or in *trans*.

In several heterotrimeric G proteins, the rate of GTP hydrolysis is enhanced by proteins known as RGS proteins (regulator of G protein signaling) (12, 13). The crystal structure of the complex reveals that RGS proteins bind to the switch regions of Gα and stabilize the GTPase transition state of the G protein (14). Although RGSs bind to similar regions of GTP-binding proteins as GAPs, they do not donate catalytic residues for the GTPase reaction. Recently, yet another mechanism of GTPase stimulation has been found for a Ras-related protein, ARF1 (15). ARFGAP binds to regions of ARF that are distant from the nucleotide binding pocket, and there is no insertion of amino acid side chains from ARFGAP into the catalytic center of ARF.

Elongation factors (EF) Tu and G are large, multi-domain GTPases which belong to a distinct subfamily of GTP-binding proteins (16). For both factors, the molecular mechanisms of GTPase activation and GTP hydrolysis are not known (17). Both EF-Tu and EF-G contain a conserved arginine residue homologous to the catalytic arginine in G$_\alpha$; however, in *Thermus thermophilus* EF-Tu, replacement of this arginine (R59) with threonine did not significantly affect the GTPase (18). The intrinsic GTPase activity of both factors is either very low or absent (19) and is strongly enhanced on the ribosome (20–22), suggesting that the GTPase activity of both factors is increased by contacts with ribosomal components.

Recently, both EF-Tu and EF-G have been visualized on the ribosome by electron cryomicroscopy (23–26). In all reconstructions, a contact of EF-G and EF-Tu with the L7/12 stalk of the 50 S subunit was observed, in keeping with the importance of L7/12 for either factor binding to the ribosome or function on the ribosome, as demonstrated by numerous reports following the initial report by Kischa *et al.* (27). The stalk comprises two dimers of the 12-kDa protein L7/12 (L7 differs from L12 by an acetylated N terminus). The protein consists of three domains. The N-terminal domain is required for dimer formation and for anchoring the protein to the ribosome by binding to ribosomal protein L10, whereas the C-terminal domain is involved in factor binding (28, 29). The hinge region enables independent movement of the C-terminal domains relative to each other and to the N-terminal domains (30, 31). Removal of the hinge region significantly reduces the mobility of the C-terminal domains and, upon reconstitution, yields inactive ribosomes (32). Ribosomes depleted of L7/12 have a decreased ability to interact with elongation factors, and activity is restored by the addition of L7/12 (27, 33).

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[1] The abbreviations used are: GAP, GTPase-activating protein; EF, elongation factor.

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EXPERIMENTAL PROCEDURES

Ribosomes, EF-G, and EF-Tu were prepared as described previously (22, 34, 35). Ternary complexes EF-Tu·GTP·Phe-tRNA^{Phe} were purified by gel filtration on Superdex 75 (36). mant-GDP and mant-GTP (2'-(or 3')-O-(N-methylanthraniloyl)guanosine 5'-diphosphate (or -triphosphate)) were purchased from Molecular Probes. L7/12 was overexpressed from plasmid pT7–6::rpl L (provided by R. Traut, Dept. of Biological Chemistry, School of Medicine, University of California, Davis, CA) in *Escherichia coli* BL21(DE3)pLysS and purified as described previously (32). GTP hydrolysis was measured in Buffer A (Tris-HCl, pH 7.5, 70 mM NH$_4$Cl, 30 mM KCl, and 7 mM MgCl$_2$) at 37 °C using [γ-^{32}P]GTP as described previously (22). Blank values resulting from 3.5% P$_i$ present in [γ-^{32}P]GTP were measured without added factors and subtracted throughout.

Fluorescence stopped-flow measurements were performed on a SX-18MV spectrometer (Applied Photophysics), and the data were evaluated as described previously (37). With the apparatus used, time constants of up to 500 s^{-1} could be measured. The fluorescence of mant-GTP (0.2 μM) and mant-GDP (0.5 μM) was excited at 366 nm and measured after passing KV408 filters (Schott). Experiments were performed in Buffer A at 37 °C by rapidly mixing equal volumes (60 μl each) of mant-GDP or mant-GTP with varying concentrations of nucleotide-free EF-G and monitoring the time courses of the fluorescence change. The data were evaluated by fitting an exponential function with the characteristic time constant, k_{app}, the amplitude, A, and another variable for the final signal, F_∞, according to the equation $F = F_\infty + A \times \exp(-k_{app} \times t)$, where F is the fluorescence at time t. The results of several experiments (typical number, 10 experiments) were used to calculate the average k_{app} and the S.D. shown in Figs. 4B and 5B. Because only single-exponential time courses were observed, the data were treated further in terms of a one-step binding model: EF-G + mant-GXP⇌EF-G·mantGXP. For this model, the bimolecular association rate constant and the dissociation rate constant can be determined from the linear concentration dependence of k_{app}. Values of k_1 and k_{-1} were determined by linear regression using the k_{app} of all data sets, rather than the average values, to take into account the relatively large deviation of individual experiments. Thus, the values of k_1 and k_{-1} are statistically valid, despite the significant S.D. of individual k_{app} values. Calculations were performed using TableCurve software (Jandel Scientific).

Arginine 74 in L7/12 was replaced with methionine (R74M) or lysine (R74K) by polymerase chain reaction mutagenesis with *Pfu* DNA polymerase (Stratagene) using plasmid pT7–6::rpLL. For each mutant, two polymerase chain reaction products were generated overlapping at the R74 mutagenic site. The following primer pairs were used to generate polymerase chain reaction products for the R74K mutant: (*a*) GAGCTGCATGTGTCAGAGGTTTTCAC and GACCCAGGCCAGTGG-CGCCCTTTACTGCTTTGA, and (*b*) TCAAAGCAGTAAAGGGCGCCA-CTGGCCTGGGTC and AATTCTCATGTTTGACAGCTTATCATCGA. Primer pairs used for the R74M mutagenesis were as follows: (*a*) GAG-CTGCATGTGTCAGAGGTTTTCAC and GACCCAGGCCAGTGGCAC-CCATTACTGCTTTGA, and (*b*) TCAAAGCAGTAATGGGTGCCACTG-GCCTGGGTC and AATTCTCATGTTTGACAGCTTATCATCGA. Polymerase chain reaction products were cleaved with *Hin*dIII/*Bst*XI and *Bst*XI/*Sal*I, ligated at the *Bst*XI site, and cloned into the *Hin*dIII/*Sal*I sites of the pT7–6::rpLL vector. The constructs were verified by DNA sequencing. The mutated L7/12 genes were cloned into the *Bam*HI/*Sal*I sites of plasmid pGEX-5x-3 (Pharmacia). Mutant L7/12 proteins were expressed as glutathione *S*-transferase-fusion proteins and purified by affinity chromatography on glutathione-Sepharose 4B (Pharmacia). Glutathione *S*-transferase-fusion proteins were cleaved by factor Xa (Pharmacia). Glutathione-Sepharose 4B was used again to separate glutathione *S*-transferase from L7/12 protein.

RESULTS

Ribosomal Protein L7/12 Stimulates GTP Hydrolysis in EF-G—The ability of isolated L7/12 protein to stimulate GTP hydrolysis by either EF-Tu or EF-G was studied using the binary complexes EF-Tu·GTP and EF-G·GTP and the ternary complex EF-Tu·GTP·Phe-tRNA^{Phe} (Fig. 1). Strong stimulation of GTP hydrolysis upon the addition of L7/12 was observed only with EF-G, whereas practically no GTPase activity was found with either EF-Tu complex. The time course of GTP hydrolysis in the presence of catalytic amounts of EF-G shows a linear increase in the concentration of liberated P$_i$ that significantly exceeds the concentration of the factor (Fig. 2), suggesting
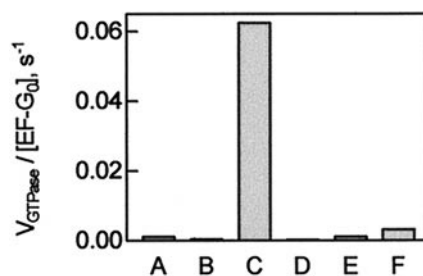

FIG. 1. **Effect of L7/12 on GTP hydrolysis by elongation factors Tu and G.** EF-Tu (0.25 μM) with [γ-^{32}P]GTP (5 μM; *A*), EF-Tu·Phe-tRNA^{Phe}·[γ-^{32}P]GTP (0.25 μM; *B*), or EF-G (0.25 μM) with [γ-^{32}P]GTP (5 μM; *C*), was incubated in the presence of 10 μM L7/12 for 1 min at 37 °C in Buffer A. Controls were performed with L7/12 alone (*D*) or with EF-Tu (*E*) or EF-G (*F*) in the absence of L7/12. V_{GTPase} was calculated as moles of [γ-^{32}P]GTP hydrolyzed per second.
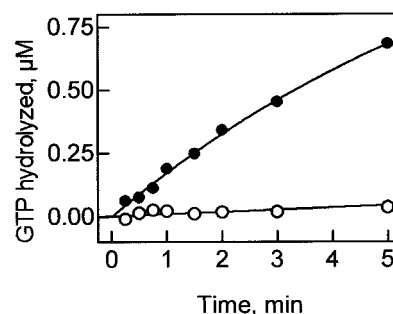

FIG. 2. **Time course of EF-G-dependent GTP hydrolysis in the presence (●) or absence (○) of L7/12.** EF-G (0.2 μM) was incubated with [γ-^{32}P]GTP (5 μM) in the absence or presence of L7/12 (10 μM).

multiple turnover of EF-G during the reaction. No initial burst of GTP hydrolysis was observed. This indicates that the overall rate of reaction is determined by the rate of GTP cleavage, rather than by turnover (see below).

To determine the steady-state kinetic parameters of the L7/12-stimulated GTPase of EF-G, GTP hydrolysis was measured under initial velocity conditions (1 min), in the presence of a catalytic amount of EF-G, with saturating concentrations of GTP as a substrate, and varying concentrations of L7/12. A hyperbolic curve was obtained (Fig. 3) and was fitted by the Michaelis-Menten equation, yielding a K_m for L7/12 of about 40 μM, and the k_{cat} of the stimulated reaction was 0.3 s^{-1}. Whereas this rate is much higher than that observed in the absence of L7/12 (19), it is about 500 times lower than the rate constant of GTP hydrolysis by EF-G on the ribosome at comparable conditions (170 s^{-1}) (22). The addition of the complex of proteins L7/12 with L10 had the same effect as L7/12 alone (data not shown). No GTP hydrolysis was induced on EF-Tu, even at very high concentrations of L7/12 (Fig. 3), although complex formation takes place under these conditions ($K_d = 10$ μM as measured by tryptophan fluorescence of EF-Tu; data not shown).

Kinetics of Nucleotide Exchange in EF-G—The enhancement by L7/12 of turnover GTP hydrolysis by EF-G may be due to the acceleration of either the cleavage of GTP or the exchange of GDP for GTP. To distinguish the two possibilities, the rate constants of nucleotide exchange were measured by stopped-flow using fluorescent derivatives of GDP and GTP, mant-GDP and mant-GTP. Upon binding to EF-G, the fluorescence of mant-GDP increases by about 10% (Fig. 4A). To determine the rate constants of nucleotide binding to EF-G and dissociation from EF-G, time courses were measured at different concentrations of EF-G, and the apparent rate constants were determined by exponential fitting. From the slope of the linear dependence of the apparent rate constant upon EF-G concen-
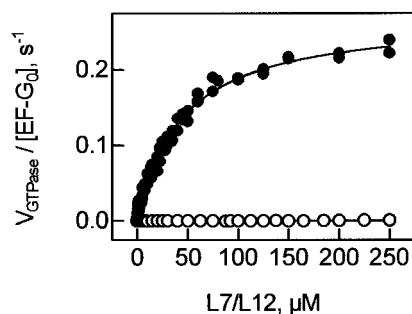
FIG. 3. **Michaelis-Menten titration of EF-G·GTP or EF-Tu·GTP·Phe-tRNA^{Phe} with L7/12.** 0.25 μM EF-G with 20 μM [γ-^{32}P]GTP (●) or 0.25 μM purified EF-Tu·Phe-tRNA^{Phe}· [γ-^{32}P]GTP (○) was incubated at initial rate conditions (1 min at 37 °C in Buffer A) with different concentrations of L7/12. For EF-G, $K_m = 43 \pm 2$ μM, and $k_{cat} = 0.27 \pm 0.01$ s^{-1}.
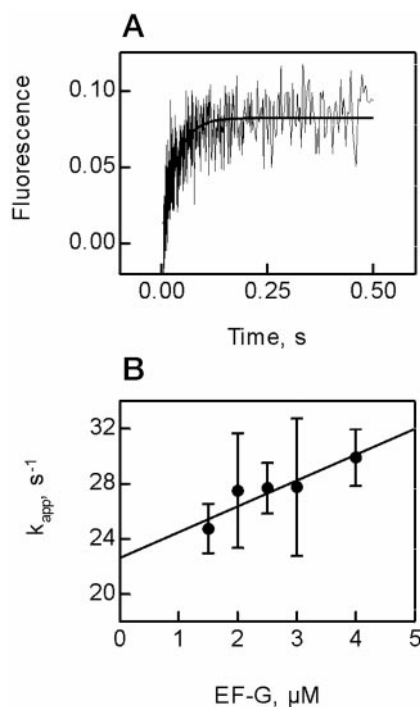

FIG. 4. **Kinetics of EF-G interaction with mant-GDP.** *A*, time course of binding to 3 μM EF-G. The *smooth line* shows the fit to an exponential function with the characteristic time constant, k_{app}, the amplitude, *A*, and another variable for the final signal. *B*, dependence of the k_{app} of binding on the EF-G concentration. Parameters of the linear fit were as follows: $k_1 = 2 \pm 1$ μM^{-1} s^{-1}, and $k_{-1} = 23 \pm 4$ s^{-1}.

tration (Fig. 4*B*), the bimolecular association rate constant k_1 was calculated to be 2 ± 1 μM^{-1} s^{-1} for mant-GDP; from the ordinate intercept, the dissociation rate constant k_{-1} was determined to be 23 ± 4 s^{-1}. With mant-GTP, the increase of fluorescence upon binding to EF-G was about 50% (Fig. 5*A*). From the concentration dependence of the apparent rate constant (Fig. 5*B*), values of $k_1 = 1.2 \pm 0.7$ μM^{-1} s^{-1} and $k_{-1} = 7 \pm 2$ s^{-1} were determined. From these values, we derived equilibrium dissociation constants (K_d) of 12 ± 6 and 6 ± 3 μM, in good agreement with the respective values measured by equilibrium titrations (data not shown).

The rate constants reported above suggest that under the conditions of the GTP hydrolysis experiments (Fig. 3), both the dissociation of EF-G·GDP (about 20 s^{-1}) and the binding of GTP to EF-G (about 6 s^{-1}) (at 5 μM GTP) are much faster than the turnover rate of GTP hydrolysis (0.3 s^{-1}) measured in the presence of L7/12. Hence the stimulation by L7/12 of the turnover GTP hydrolysis by EF-G must be due to the acceleration of
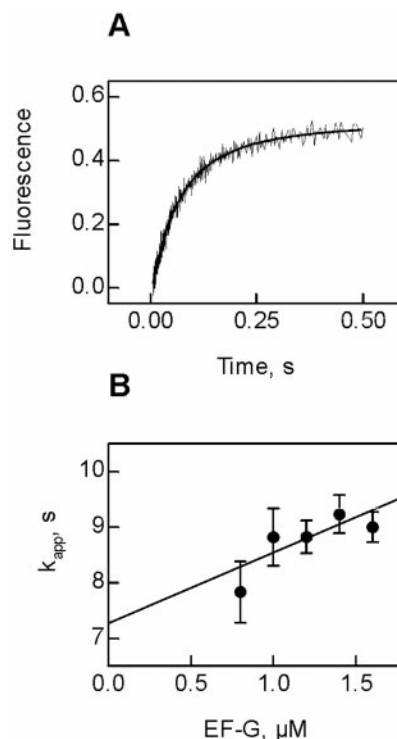

FIG. 5. **Kinetics of EF-G interaction with mant-GTP.** *A*, time course of binding to 1 μM EF-G. The *smooth line* shows the fit to an exponential function with the characteristic time constant, k_{app}, the amplitude, *A*, and another variable for the final signal. *B*, dependence of the k_{app} of binding on EF-G concentration. Parameters of the linear fit were as follows: $k_1 = 1.2 \pm 0.7$ μM^{-1} s^{-1}, and $k_{-1} = 7 \pm 2$ s^{-1}.

the GTP cleavage step.

The concentrations of GDP and GTP in the cell are about 100 and 900 μM, respectively (38). With association rate constants in the range of 1–2 μM^{-1} s^{-1}, the binding of GDP and GTP in the cell is expected to be very rapid, 100–1000 s^{-1}, suggesting that the rate of nucleotide exchange *in vivo* is limited by the dissociation of EF-G·GDP. The rate constant determined for that step, about 20 s^{-1}, is compatible with the overall rate of translation (4–20 s^{-1}) (39).

Arginine 74 in L7/12 Is Not Important for GTPase Activation—Sequence alignments of L7/12 proteins from many different species revealed a unique, highly conserved arginine (R74 in *E. coli*) in the C-terminal domain. Due to the flexible hinge region of L7/12, the C-terminal domain is probably mobile enough to position the arginine into the catalytic center of the elongation factors. It was suggestive, therefore, to assume that L7/12 functions similarly to RasGAP and RhoGAP, namely, by providing a catalytic arginine in *trans* to the catalytic center of the elongation factors. To test this hypothesis, R74 in *E. coli* L7/12 was replaced with lysine or methionine. The stimulation of GTPase activity of EF-G was studied at an intermediate (10 μM) concentration of wild-type or mutant L7/12, to be able to observe changes in either the K_m or k_{cat} of the reaction. Neither mutation significantly affected the stimulatory effect (Fig. 6), suggesting that the arginine at position 74 of L7/12 is not essential for the effect.

DISCUSSION

Our data show that the GTPase activity of EF-G is strongly stimulated by the ribosomal protein L7/12. This effect was not detected previously (40), most likely because the concentration of L7/12 in those experiments (1–2 μM) was too low compared with the K_m of the interaction (40 μM, this study). No stimulation by L7/12 of the GTPase activity of EF-Tu was found, in
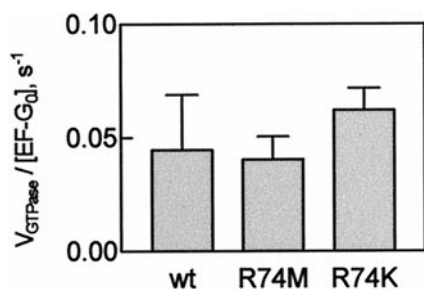
Fig. 6. **GTPase stimulation of EF-G by L7/12 mutants.** Wild-type or mutant L7/12 (10 μM) was incubated with EF-G (0.25 μM) and [γ-^{32}P]GTP (20 μM) for 1 min at 37 °C in Buffer A. Average values from three or four experiments are shown.

agreement with an earlier report (41); thus, our results do not confirm the enhancement by L7/12 of GTP hydrolysis by EF-Tu reported by Donner *et al.* (40). The present result is in keeping with the earlier finding that EF-Tu in the ternary complex is refractory to GTPase stimulation on the ribosome, unless a cognate or near-cognate codon is recognized (20, 21, 42). Thus, GTPase activation of EF-Tu appears to be confined to the ribosomal codon recognition complex, and it is probably not possible to mimic that state with EF-Tu, or the ternary complex, free in solution.

Although L7/12 strongly stimulates the GTPase of EF-G free in solution, the k_{cat} of GTP hydrolysis on the ribosome is substantially higher. There are several possible reasons for this. First, EF-G·GTP on the ribosome may assume an unknown conformation that is more susceptible to the stimulating interaction. Furthermore, additional contacts with the ribosome may contribute to the stimulation. In fact, in addition to the contact with protein L7/12, contacts of EF-G with the ribosome have been established with two regions of 23 S rRNA. In domain II, the loops around residues 1070 and 1100 were shown to take part in EF-G binding (43–45). The 1070 region is also the binding site of protein L11 (46, 47) as well as that of an antibiotic, thiostrepton, that inhibits EF-G function on the ribosome (48). However, the inhibitory effect of thiostrepton is restricted to translocation and EF-G turnover, whereas GTPase activation and GTP hydrolysis are not affected (35). Thus, there is no evidence suggesting a direct involvement of the L11 binding site in GTPase activation of EF-G.

The second region of 23 S rRNA presumed to interact with EF-G (and EF-Tu) is the α-sarcin stem loop in domain VI (positions 2646–2674), although the dimethyl sulfate footprints indicative of the interaction (A2660 and A2662) were observed only when fusidic acid was present to stabilize the EF-G-ribosome complex (43). The isolated α-sarcin stem loop was shown to bind to EF-G in solution (44). In the EF-G-ribosome complex, the α-sarcin stem is close to position 196 in the G domain of EF-G, which lies just above the GTP binding site, whereas the α-sarcin loop region is in the vicinity of position 650 in domain 5 of the factor (49). Whereas the arrangement of EF-G relative to the α-sarcin stem loop would be consistent with an influence on the GTPase site, we did not observe the dimethyl sulfate footprints indicative of the interaction when thiostrepton was present in the complex (35). This suggests that, in the latter complex, EF-G does not interact with the α-sarcin region, whereas the GTPase activity is unaffected. We conclude that the contact of EF-G with the α-sarcin region does not contribute to GTPase activation but is rather a later event in the functional cycle.

Our results indicate that the interaction of L7/12 with the G domain of EF-G induces or stabilizes the GTPase transition state conformation of EF-G. On the ribosome, additional contacts appear to augment the interaction with L7/12. Because

the unique arginine is not essential for the activation, L7/12 does not function by the "arginine-finger" mechanism used by the GAPs of small GTPases. Instead, it may function by a RGS-type mechanism found for heterotrimeric G proteins. In such a mechanism, L7/12 would be expected to bind to the effector region of the factor and stabilize the active conformation of the catalytic center. Alternatively, the interaction between L7/12 and EF-G may occur at a distance from the nucleotide binding site, as observed for the ARF1-ARFGAP complex (15). In such a case, ribosomal elements other than L7/12 may be involved in the interaction with the effector region of EF-G.

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