Mutations in the G-domain of Elongation Factor G from *Thermus thermophilus* Affect both its Interaction with GTP and Fusidic Acid.

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Running title: *Fusidic acid hypersensitive and resistant mutants of EF-G*

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SUMMARY

Two hypersensitive and two resistant variants of EF-G towards fusidic acid are studied in comparison with the wild type factor. All mutant proteins are active in a cell-free translation system and in ribosome dependent GTP hydrolysis. EF-G variants with Thr84Ala or Asp109Lys mutations show strong resistance to antibiotic whereas EF-Gs with Gly16Val or Glu119Lys mutations are the first examples of fusidic acid hypersensitive factors. A correlation between fusidic acid resistance of EF-G mutants and their affinity to GTP analogue is revealed in this study while interactions with GDP show no changes. Thus fusidic acid hypersensitive mutants have a high affinity for uncleavable GTP analog but association of resistant mutants with GTP is decreased. The effects of either fusidic acid sensitive or resistant mutations can be explained by conformational changes in the EF-G molecule, which influence its GTP-binding center. The results presented indicate that fusidic acid sensitive mutant factors have a conformation favorable for GTP binding and subsequent interaction with the ribosome.

Keywords: ribosome; EF-G function; mutagenesis; fusidic acid action
INTRODUCTION

Elongation factors (EF\(^3\)) Tu and G interact consecutively with the ribosome during polypeptide synthesis. EF-Tu in complex with aminoacylated tRNA and GTP delivers the tRNA to the ribosomal acceptor (A) site. After peptide bond formation EF-G catalyzes translocation of the peptidyl-tRNA from the A site to the P site. Simultaneously the messenger RNA is advanced by one codon (1). The function of both factors depends on GTP binding and hydrolysis (reviewed in ref. 2). After GTP hydrolysis, EF-Tu and EF-G dissociate from the ribosome in complex with GDP.

Of the antibiotic inhibitors that influence the action of EF-Tu and EF-G, kirromycin and fusidic acid (FA) have been useful in studies of the translation mechanism. Neither of them inhibits GTP hydrolysis, but both prevent dissociation of EF-Tu and EF-G from the ribosome when GTP is hydrolyzed (2). In contrast to kirromycin, FA binds with high affinity to EF-G on the ribosome only after GTP hydrolysis.

EF-Tu undergoes large conformational changes during its functional cycle (3-5), whereas smaller changes have been identified crystallographically for EF-G (6). As a block, EF-G domains III, IV and V can rotate by 10\(^\circ\), with regard to the other portion of the molecule, i.e. domains G and II. Part of these conformational changes originates from the nucleotide binding site of EF-G and is transmitted through several amino acid residues to the interface between the two structural blocks (6-7).

Numerous mutants conferring kirromycin resistance in EF-Tu (8-9) or FA resistance in EF-G of Salmonella typhimurium (10) and Staphylococcus aureus (6) have been identified and phenotypically characterized in vivo. Based on the tertiary structures of EF-Tu (3, 4,) and EF-G (11-12) it was suggested that antibiotic resistant mutations influence the conformational dynamics of these factors (8-9, 13).

A fusidic acid-resistant allele of the EF-G gene, fusA, containing Leu instead of the Pro413 residue confers a slow growing phenotype. Selection for fast growth of the FA-resistant strain
resulted in the appearance of internal revertants, i.e. alleles encoding EF-G with additional point mutations. Such revertants restore the normal growth rate with or without affecting the resistance level to antibiotic. For instance, the additional G13V mutation occurring in *S. typhimurium* EF-G during selection markedly reduced FA resistance of the strain carrying the *fus* gene with P413L mutation (13). These findings suggest the possibility of constructing EF-G mutated variants highly sensitive to FA (that are unknown so far) for *in vitro* studies for comparison with FA resistant EF-G mutants. Hypersensitive mutants such as these could not be found by phenotypical selection.

To our knowledge, only two FA-resistant EF-G mutants were characterized to some extent *in vitro* (14-15) and no of FA hypersensitive mutants have ever been described. To further study the antibiotic action, we have prepared mutated forms of *T. thermophilus* EF-G with varied responses to FA for comparative studies *in vitro*. 
EXPERIMENTAL PROCEDURES

Materials —All restriction endonucleases and T4 DNA ligase were from Promega (USA). High fidelity *Pfu* Turbo DNA polymerase was purchased from Stratagene (USA) and used according to the manufacturers manual. Mono Q and Phenyl Sepharose were from Pharmacia (Sweden) and HA-Ultrogel from Sigma (USA). Plasmids were maintained in *E. coli* strain XL1 (Promega, USA). For gene expression plasmid pET 11c and strains BL21(DE3) or B834(DE3) (Novagen) were used. The fusidic acid samples were kindly provided by Leo Pharmaceutics (Denmark).

Oligonucleotides —The following oligonucleotides were synthesized by Gene Assembler Plus (Pharmacia) according to the manufacturers manual:

Pr1, 5’-CGG TGGTGCATATGGCGGTCAGGTAG-3’, contains restriction site *Nde*I

Pr2, 5’-GAAGTCTGACGTTGCCGCGGGCGTCGATG-3’ (*Sal*I, T84A)

Pr3, 5’-CTGGCTGGATTTAAAGACCACAGA-3’ (*Dra*I, D109K)

Pr4, 5’-GACGGTCGTTCCGACTGGGC-3’ (*Eco*RI, E119K)

Pr5, 5’-GCGAATTCTATATTGACCCCTTGGAG-3’, primer is complementary to the 3’-end of the gene with a stop codon and *Eco*RI site.

Pr2, Pr3 and Pr4 were used for the T84A, D109K and E119K mutations respectively. Pr1 and Pr5 are 5’-end and 3’-end gene flanking primers. The restriction sites are underlined; non-complementary nucleotides are given in bold letters.

Mutagenesis —All recombinant DNA procedures were according to the published manual (16). The T84A mutation was obtained by polymerase chain reaction (PCR). The plasmid pLS3 carrying the *fus* gene from T. thermophilus was used as a template (17). The first PCR was carried out for 1 min at 94°, 30 sec at 60°, 30 sec at 72°, for twenty cycles. It was necessary to have an additional denaturation step (3 min at 100°) in the second round of PCR. The denaturation and annealing conditions coincided with those used in the first round, and the elongation steps were 1.5 min at 72° for 15 cycles. PCR was performed in 50 µl containing 20
mM Tris/HCl (pH 8.8 at 25°C); 10 mM KCl; 10mM (NH₄)₂SO₄; 2mM MgSO₄; 0.1% Triton X-100; 100 µmoles dNTP; 250 ng of pLS3 and 2 units of Pfu Turbo DNA polymerase. The DNA fragment obtained with Pr1 and Pr2 (about 900 bp) in the first PCR round was used as the 5'-end megaprimer in the second PCR round together with Pr4. The final PCR product was treated with Ndel and EcoRI endonucleases and cloned into the plasmid pET11c at the corresponding sites. As a result, the plasmid pETT84 containing the fus gene with the replacement of Thr84 by Ala was obtained. The mutation was verified by the appearance of a new unique SalI site. The double G16V/T84A mutation was constructed by subcloning a BamHI/EcoRI DNA fragment containing T84A mutation (from plasmid pETT84) into plasmid pET16V treated with the corresponding endonucleases. As a result the plasmid pETGVA was obtained.

EF-G with the D109K or E119K mutations was obtained in a similar way using primers Pr1 and Pr3 for D109K (or Pr1 and Pr4 for E119K) in the first PCR reaction. The purified DNA fragments from the first PCR round were used with the Pr5 primer in the second PCR reactions. After the second PCR round the isolated DNA fragments were digested with Ndel and EcoRI enzymes and cloned into the pET11c plasmid treated with the corresponding endonucleases. By these means the plasmids pETD109 and pETE119 were constructed. The mutant genes were selected by restriction analysis and confirmed by sequencing.

Production and Purification of the Protein—Since all mutant proteins retained their thermostability; a heat denaturation treatment of the postribosomal supernatant was used (60-70°C for 15 min) for coagulation of E. coli proteins. Cell growth and isolation of EF-Gs with the T84A, D109K, E119K, and the double G16V/T84A mutations were performed according to the published procedure (18). EF-G with the G16V mutation was obtained earlier (17). The isolated proteins were stored in 50 mM/Tris HCl, pH 7.6, buffer with 10 mM MgCl₂, 1 mM DTT, 50 mM NH₄Cl, 10% glycerol at -70°C. The protein purity was checked by electrophoresis (19) and concentration was determined with EF-G from E. coli as a standard (20). The yield of purified proteins was 10-15 mg from 12 g of wet cells.
**Poly(U)-directed translation**—Poly(U)-directed polyphenylalanine synthesis was carried out in a cell-free translation system with purified elongation factors Tu and G according to the published procedure (17).

**Nucleotide-dependent interactions**—GDP interactions with EF-G were tested according to the procedure used for the factors from *T. thermophilus* (21). The GDP dissociation constant was determined in the reaction mixture (0.2 ml) in 50 mM Tris-HCl buffer, pH 7.6, 10 mM MgCl₂, 1 mM DTT, 70 mM NH₄Cl containing 300 pmol of EF-G by titration with [³H]GDP (1.1 Ci/mmol) at 37 °C.

GMPPNP binding with mutated proteins (300 pmol) was measured by displacement of [³H]GDP bound to the factor with increasing concentrations of unlabeled GMPPNP. The concentration of [³H]GDP in the mixture was 0.5 µM, and that of GMPPNP varied from 0.5 to 500 µM. Dissociation constant for GMPPNP with EF-G was calculated from titration curves as a concentration of GMPPNP necessary to displace half of the GDP bound to the factor (21).

The binding of EF-G•GDP binary complexes to the ribosome was monitored by nitrocellulose filtration. The reaction mixture (50 µl) contained 50 mM Tris HCl pH 7.6, 70 mM NH₄Cl, 10 mM MgCl₂, 1 mM DTT, 10 µM [³H] GDP (1,1 Ci/mmol), 50 pmol 70S ribosomes and 100 pmol EF-G (21).

The GTPase reaction was followed by measurement of the released inorganic phosphate spectrophotometrically (22). The assay was carried out in the 100 µl reaction mixtures containing 50 mM Tris HCl, pH 7.6, 10 mM MgCl₂, 1 mM DTT, 70 mM NH₄Cl, 0.4 mM GTP, 6 mM phosphoenol pyruvate, 1 µg pyruvate kinase, 20 pmol of *E. coli* 70S ribosomes and 10 pmol of EF-G. The inhibitory effect of fusidic acid (FA) on the turnover of GTP hydrolysis was followed by titration of the reaction mixture with FA (up to 5 mM) at the linear phase of the GTPase reaction.
RESULTS

Selection of the Mutations —The second G13V mutation in *S. typhimurium* EF-G markedly reduces FA resistance of the strain carrying the fus gene with the P413L mutation (13). These data suggest the possibility to obtain a FA sensitive mutant of EF-G for *in vitro* studies. To investigate the effect of the point G16V mutation, the substitution of Gly16 by Val in EF-G from *T. thermophilus* was chosen since it corresponds to the Gly13 residue of *S. typhimurium* EF-G. It was reported that the double A66V/T88A mutation (corresponding residues in *T. thermophilus* EF-G are V69 and T84) confers FA resistance of *E. coli* EF-G (14). Since *T. thermophilus* EF-G already contains Val69 residue, the point T84A mutation was chosen to obtain mutated EF-G for the comparative studies with the factor carrying the G16V mutation.

The side chain of Glu119 is at the interface of domains G and V where a cluster of FA resistant mutations is found and hydrogen bonded to Arg666 (9, 13). Assuming that FA binding influences the motions of EF-G domains V and III (7, 11, 13), the factor with E119K mutation was prepared for *in vitro* studies.

The Asp109 and Phe108 residues are located in the beta strand (residues 102-109) near the helix 115-128 of the G–domain where another cluster of FA resistant mutations is found *in vivo* (7, 10, 13). Since the Y112S (corresponding residue in *T. thermophilus* EF-G is F108) mutation in EF-G from *S. typhimurium* confers its resistance to FA (10, 13), we have assumed that D109K mutation in *T. thermophilus* EF-G might also influence FA interaction with the factor.

Protein isolation —The high thermostability of *T. thermophilus* EF-G simplified its isolation after expression of the fus gene in *E. coli*. All mutated proteins obtained in this study retained thermostability, and were purified to homogeneity using high temperature treatment of protein mixtures in combination with conventional chromatography (see Methods).

Poly(U)-directed translation —EF-G from thermophilic microorganisms can substitute for their counterpart in *E. coli* in a heterologous cell-free translation system with *E. coli* ribosomes.
EF-G species with T84A, D109K and E119K point mutations are active in poly(U)-directed translation (Fig. 1), as well as the G16V mutated EF-G (17). The effect of FA on poly(U)-directed translation catalyzed by EF-G mutants is illustrated in Fig. 1. The activities of both the G16V mutant and wild type factors are completely inhibited at 500 µM of FA, while that of the T84A mutant is reduced only two times compared to activity without FA.

GTP hydrolysis—All mutated factors hydrolyze GTP in the presence of ribosomes (Fig. 2) although the GTPase activity of EF-G with the point mutations T84A, D109K and E119K is somewhat lower than that of the wild type factor. Since FA prevents EF-G dissociation from the ribosome after GTP hydrolysis (23), the inhibitory effect of FA was measured by titration of the GTPase reaction with FA (Fig. 3). The half-inhibition concentrations (K_i) of FA determined in the experiments are listed in Table 1. The data show that the FA sensitivity of T. thermophilus EF-G with the G16V or E119K mutations is close to that of E. coli EF-G (24), and the G16V mutation generates the most FA sensitive factor. Wild type T. thermophilus EF-G appeared to be more resistant to FA action than E. coli EF-G (K_i values are 7.5 and 1 µM respectively). Moreover, T. thermophilus EF-G with T84A, D109K or T84A mutations are even more resistant towards FA as compared to wild type factor. Among them, EF-G with T84A mutation exhibits the highest level of resistance to FA showing the K_i of 440 µM (Table 1).

Nucleotide interactions—EF-G from T. thermophilus forms a relatively stable binary complex with GDP that can be isolated by filtration through a nitrocellulose membrane. The dissociation constant of 0.67 µM was previously reported for GDP in complex with wt EF-G (21). The K_d value determined here by incubation of a constant amount of wild type EF-G at various GDP concentrations by filter binding techniques (Fig. 4) is 1.5 µM. All mutated EF-G variants have similar K_d for GDP and the values are listed in Table 2. This means that the affinity of EF-G for GDP is hardly influenced by FA resistance or sensitivity.

The affinity of mutated EF-G for GMPPNP has been followed by displacement of [^3H]GDP bound to the factor with unlabeled GMPPNP (Fig. 5). The K_d values, calculated from
titration curves as the concentration of GMPPNP necessary to displace the half of bound GDP in the factor (21), are given in Table 2. A striking correlation exists between the inhibitory effect of FA on the GTPase activity of the different EF-G variants and their affinity for GMPPNP. The more FA sensitive is the EF-G variant, the stronger is its affinity to GMPPNP (compare Fig. 3 and 5). Thus, the $K_d$ value for the GTP analog binding to the most resistant EF-G with the T84A mutation is 410 µM. At the same time, the most sensitive, G16V EF-G mutant, binds the GTP analogue much more tightly ($K_d$ is 26 µM), which is less than the $K_d$ for wild type EF-G (compare tables 1 and 2). The affinity of a strongly resistant *S. typhimurium* EF-G, with a P413L mutation, to GTP is 17-fold lower than that of wt EF-G (15). These data suggest that the FA and the GTP-binding centers of EF-G are in a close relationship in a way that the factors hypersensitive to FA show the better ability to bind GTP.

It is interesting that the combination of G16V (highly sensitive) and T84A (strongly resistant) mutations lead to an EF-G with the highest affinity for the GTP analog (13 µM) and its FA sensitivity comes close to that of wt EF-G ($K_i$ are 15 and 8 µM respectively, Table 1). It is likely that the G16V mutation has a more pronounced effect on the GTP binding center of EF-G than the T84A mutation.
DISCUSSION

Fusidic acid resistant mutations—The data given above demonstrate the correlation between FA resistance of EF-G and its affinity to GMPPNP or GTP while GDP binding is not affected. Such a relationship might have a structural explanation.

Thr84 is part of the DxxG consensus motif in the switch II region that is found in all G-proteins (DTPG sequence in many EF-G species (25), DCPG in EF-Tu, and DTAG in Ras-p21). This motif is involved in the conformational change between the GDP and GTP states of G-proteins and the carbonyl group of this threonine is close to the \(\gamma\)-phosphate of GTP (26). It was proposed that replacement of Thr84 by Ala might interfere with conformational changes that are necessary for GTP binding (7, 13). Our results confirm this proposition (Table 2).

Asp109 and Phe108 residues are located in the G–domain (see also Results section). EF-G species from most organisms contain an aspartate residue at the position corresponding to Asp109, but some of them, including E. coli, have a cysteine (25). This Cys113 in E. coli EF-G was found to be important for nucleotide binding (27, 28). The D109K mutation (this work) as well as Y112S in S. typhimurium (7, 13) conferring EF-G resistance to FA is in the \(\beta\)-strand neighboring the helix 115-128 where several FA resistant mutations found in vivo are clustered (10). Neither of these mutations is very far from the GTP/GDP binding site and may affect the GDP/GTP exchange by interference with conformational changes mentioned above (6, 13).

The additional point V291S mutation reduces the resistance level of the S. typhimurium strain with EF-G carrying the P413L mutation, which markedly decreases EF-G affinity to GTP (15). The I286E mutation (I286 in T. thermophilus factor corresponds to V291 in S. typhimurium EF-G) has only a marginal effect on the functional properties of EF-G (Martemyanov & Gudkov, unpublished results). This result suggests that the I286E point mutation does not influence the GTP state of EF-G significantly even though it can serve as a compensatory mutation.

The data discussed above suggest that mutations conferring EF-G resistance towards FA lead to a decreased affinity of EF-G to GTP and consequently to the ribosome. This finding
might explain the slow growth of bacteria (6) carrying a strongly FA resistant EF-G species (P413L and T84A mutants) due to a decreased level of productive complexes of EF-G with GTP and the ribosomes. In such a case, some additional mutations, for instance, I286E and G16V (see below), may compensate the effect of FA resistant mutations restoring GTP affinity to EF-G.

**Fusidic acid sensitive mutations**—Numerous mutations conferring EF-G resistance to FA have been selected and characterized *in vivo* (6, 13). However, FA hypersensitive mutants of EF-G were so far unknown. Two EF-G species with G16V and E119K mutations studied here are FA hypersensitive (Fig. 3), and contrary to FA resistant mutants they possess a markedly increased affinity to GTP analog (Table 2).

Gly16 (Fig. 6) is in the vicinity of the GTP-binding center and very close to Thr84 (the distance between Cα atoms is about 5 Å). As mentioned above, T84 is a part of switch II that is important for GDP/GTP exchange. The structural analysis has led to the conclusion that a substantial conformational rearrangement of this part of the G domain of EF-G may be needed to accommodate the GTP molecule (6-7, 13). The introduction of the bulky side chain of the Val residue into this area might provoke conformational distortions in EF-G that resulted in the high affinity for GTP. It is interesting that the combination of G16V and T84A mutations, which have an opposite effect on their own (see Results), produces a FA sensitive EF-G variant (Table 2). These results support the idea that the affinity of EF-G to GTP and sensitivity of the factor to FA might be functionally connected and depend on the conformational dynamics of EF-G.

The side chains of Glu119 and Arg123 residues located in the G-domain form hydrogen bonds with the side chains of Arg666 and Asn639 residues in domain V respectively (Fig. 6). Thus, the substitution of Glu119 by lysine at the interface of the G-domain and domain V may alter the interaction of these domains because the introduction of a positive charge into this area that is already positively charged (7) will cause repulsion. The ten-degree rotation of domains III, IV and V with respect to domains G and II revealed in the EF-G structure (13) may explain the effect of the mutations at the interface of domains V and G by facilitation of domain motions.
The movement of EF-G domains and conformational changes in the 70S ribosome upon their interaction were recently also visualized by cryo-electron microscopy (29, 30).

**Fusidic acid binding**—The studies of the EF-G function in the presence of FA has resulted in the conclusion that EF-G interaction with the ribosomes and consequent GTP hydrolysis may generate or uncover the FA binding site (31). Moreover, the addition of the ribosomes to EF-G and a GTP analogue decreases the dissociation constant of the analogue from the complex, which was found to be 59 nM (32). This value is about 170 times less than $K_d$ (10 µM) for the GTP interaction with *E. coli* EF-G without ribosomes (21). Thus, the increased affinity of EF-G for GTP analogue caused by the ribosomes is paralleled by the effect of FA hypersensitive mutations in EF-G described here. Hence, it seems likely (and natural enough) that such EF-G variants have a conformation favorable for their association with GTP and the ribosomes.

A number of suggestions have been made with regard to the binding site for FA. Many of the numerous FA resistant mutants are located at interfaces of EF-G domains (6, 13). FA molecule is too small to have contacts with all amino acid residues involved in the mutations (6) since it binds stoichiometrically to the ribosomal complex (24). Moreover, EF-G lacking domain III where many FA resistant mutations are also found (6) is able to bind to the ribosome in the presence of GDP or GTP and fusidic acid (33). These findings indicate that many mutated amino acid residues cannot be involved in the direct contact with the FA molecule but rather affect EF-G conformation.

One possible binding site for FA was proposed between domains V and G (11) where some mutations in domain V are facing numerous mutations in the neighborhood of GTP-binding center and these mutations may inhibit motions between the two blocks of the molecule (domains G and II versus domains III, IV and V). Others FA binding sites were also proposed near the GTP-binding center (12) and in the area of the effector loop (residues 40-65) in one of its possible GDP conformation (6). Evidently, these sites are attractive since all of them can be
explained by conformational changes in GTP binding area and by EF-G domain movements (6, 11, 13), but the actual FA binding site remains unknown.

FA binds with EF-G and retains it on the ribosome after GTP hydrolysis (23, 31, 32). For this reason it was suggested that FA binds to EF-G in its intermediate conformation just after GTP hydrolysis (2). In this connection, it is interesting to note that the yield of the ribosomal complex with EF-G and FA in the presence of GTP is higher than in the presence of GDP (24, 33). It is likely that in the last case conformational adaptation of the EF-G molecule might be necessary for the complex formation in vitro.

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REFERENCES

1. Spirin, A. S. (1999) *Ribosomes*, Kluver Academic/Plenum Publishers, NY, Boston, Dordrecht, London, Moscow, Ed. P. Siekevitz, pp 416

2. Czworkowski, J., and Moore, P. B., (1996) *Prog. Nucl. Acid Res. Mol. Biol.* 54, 293-332.

3. Berchtold, H., Reshetnikova, L., Reiser, C. O. A., Shirmer, N. K., Sprinzl, M. and Hilgenfeld, R. (1993) *Nature*, 365, 126-132.

4. Kjeldgaard, M., Nissen, P., Thirup, S., and Nyborg, J. (1993) *Structure*, 1, 35-50.

5. Abel K., Joder M. D., Hilgenfeld R., and Jurnak F. (1996) *Structure*. 4, 1153-1159.

6. Laurberg, M., Kristensen, O., Martemyanov, K., Gudkov, A. T., Nagaev, I., Hughes, D., and Liljas, A. (2000) *J. Mol. Biol.*, 303, 593-603.

7. Al-Karadaghi, S., Ævarsson, A., Garber, M., Zheltonosova, J., and Liljas, A. (1996) *Structure*. 4, 555-565.

8. Abdulkarim, F., Liljas, L., and Hughes, D. (1994) *FEBS Lett.*, 352, 118-122.

9. Mesters J. R., Zeef L. A., Hilgenfeld R., de Graaf J. M., Kraal B., and Bosh L. (1994) *EMBO J.* 13, 4877-4885.

10. Johanson, U., and Hughes, D. (1994) *Gene* 143, 55-59.

11. Czworkowski, J., Wang, J., Steitz, T.A., and Moore, P.B. (1994) *EMBO J.* 13, 3661-3668.

12. Ævarsson, A., Brazhnikov, E., Garber, M., Zheltonosova, J., Chirgadze, Y., Al-Karadaghi, S., Svensson, L. A. and Liljas, A. (1994) *EMBO J.* 13, 3669-3677.

13. Johanson, U., Ævarsson, A., Liljas, A., and Hughes, D. (1996) *J. Mol. Biol.* 258, 420-432.

14. Richter Dahlfors, A. A. and Kurland, C. G. (1990) *J. Mol. Biol.* 215, 549-557.

15. Macvanin, M., Johanson, U., Ehrenberg, M. and Hughes, D. (2000) *Mol. Microbiol.* 37, 98-107.
16. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

17. Martemyanov, K. A., Liljas, A., and Gudkov, A. T. (1998) *Biochemistry*, (Moscow), 63, 116-120.

18. Martemyanov, K. A., Liljas, A., and Gudkov, A. T. (2000) *Prot. Express. Purific.* 18, 257-261.

19. Laemmli, U. K. (1970). *Nature*, 227, 680-685.

20. Bradford, M. M., (1976) *Anal. Biochem.* 72, 248-254.

21. Arai, K., Arai, N., Nakamura, S., Oshima, T., and Kaziro, Y. (1978) *Eur. J. Biochem.* 92, 521-531.

22. Panusz, H. T., Graczyk, G., Wilmanska, D., and Skazynski, J. (1970) *Anal. Biochem.*, 35, 494-504.

23. Bodley, J. W., Zieve, F. J., and Lin, L. (1970) *J. Biol. Chem.* 245, 5662-5667.

24. Okura, A., Kinoshita, T., and Tanaka, N. (1971) *J. Antibiotics* 25, 655-661.

25. De Vendittis, E., Amatrupda, M. R., Masullo, M., and Bocchini, V. (1993) *Gene*, 136, 41-48.

26. Kjeldgaard, M., Nyborg, Y., and Clark, B. B. C. (1996) *FASEB J.* 10, 1347-1368.

27. Rohrbuach, M. S. and Bodley, J. W. (1976) *J. Biol. Chem.* 251, 930-933.

28. Girshovich, A. S., Bochkareva, E. S., Pozdnyakov, V. A., and Ovchinnikov Yu. A. (1978) *FEBS Lett.* 85, 283-286.

29. Agrawal, R. K., Heagle, A. B., Penczek, P., Grassucci, R. A. and Frank, J. (1999) *Nature Struct. Biol.* 6, 643-647.

30. Stark, H., Rodnina, M. V., Wieden, H. J., van Heel, M., and Wintermeyer, W. (2000) *Cell*, 100, 301-309.

31. Willie, G. R., Richman, N., Godtfredsen, W. O., and Bodley, J. W. (1975) *Biochemistry* 8, 1713-1718.
32. Baca, O. G., Rorbach, M. S., and Bodley, J. W. (1976) *Biochemistry* **15**, 4570-4574.

33. Martemyanov, K. A. and Gudkov, A. T. (2000) *J. Biol. Chem.* **275**, 35820-35824.
LEGENDS TO THE FIGURES

Fig. 1. **Poly(U)-directed cell-free translation in the presence of**: (●) wt *T. thermophilus* EF-G; (▲) EF-G T84A; (■) EF-G E119K; (▼) EF-G D109K; (▲) EF-G T84A + 500 µM FA; (+) EF-G G16V and (○) wt EF-G in the presence of 500 FA. 10 pmol of the 70S ribosomes, 12 pmol of EF-G, and 125 pmol of EF-Tu 200 pmol of [14C]Phe-tRNA were in reaction mixture.

Fig. 2. **GTP hydrolysis in the presence of ribosomes catalyzed by**: (●) wt *T. thermophilus* EF-G; (▲) EF-G T84A; (■) EF-G E119K; (▼) EF-G D109K; (○) no EF-G added. The activity of EF-G with the double G16V/T84A mutation coincides with that of the D109K mutant (the curve not shown). The reaction was carried out with 20 pmol of ribosomes and 10 pmol of EF-G.

Fig. 3. **Inhibition of the ribosome dependent GTP hydrolysis by titration with FA in the presence of**: (○) EF-G G16V; (■) EF-G E119K; (●) wt *T. thermophilus* EF-G; (▼) EF-G D109K; (▲) EF-G T84A. The reaction was carried out with 20 pmol of ribosomes and 10 pmol of EF-G.

Fig. 4. **Binding of GDP to wt EF-G as a function of GDP concentration**. The insert represents the Scatchard plot of the data (r – the ratio of µmol GDP bound/µmol EF-G; l - µmol of free GDP). Analogous plots were used for other mutated forms of EF-G (not shown).

Fig. 5. **Displacement of [3H] GDP bound to the factors with unlabeled GMPPNP**. The reaction was carried out in the presence of [3H] GDP (0.5 µM) and 300 pmol of each factor: (○) EF-G G16V; (■) EF-G E119K; (●) wt *T. thermophilus* EF-G; (▼) EF-G D109K; (▲) EF-G T84A.
Fig. 6. Ribbon model of the tertiary structure of *T. thermophilus* EF-G (A); the enlarged area of the EF-G structure where the mutations were carried out (B). The contour of the GDP molecule is also shown in the G-domain. The atomic coordinates were taken in Protein Data Bank (code 1FNM) supplied by Laurberg *et al.*, (6)
FOOTNOTES

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3The abbreviations used are: EF, elongation factor; GMPPNP, guanosine 5’- [β,γ-imido]triphosphate; wt, wild type; FA, fusidic acid
Table 1. Inhibition effect of FA on the multiple rounds of GTP hydrolysis.

| Components in system                  | $^aK_i$, μM |
|---------------------------------------|-------------|
| Buffer (control)                      | _           |
| Rs, GTP, wt EF-G (*Tth.*)              | 7.5         |
| Rs, GTP, wt EF-G (*Ec*)                | 1.0, (Okura et al., 1971) |
| Rs, GTP, EF-G (I286E)                 | 8           |
| Rs, GTP, EF-G (G16V)                  | 0.4         |
| Rs, GTP, EF-G (E119K)                 | 1.5         |
| Rs, GTP, EF-G (D109K)                 | 190         |
| Rs, GTP, EF-G (T84A)                  | 440         |
| Rs, GTP, (G16V/T84A)                 | 15          |

$^a$Half-inhibition concentration of FA. 20 pmol of *E. coli* 70S ribosomes and 10 pmol of EF-G were in the reaction mixture.
Table 2. Affinity of different EF-G species to GDP and GMPPNP in the absence of the ribosomes.

| Elongation factors | $K_d$ for GDP, $\mu$M | $K_d$ for GMPPNP, $\mu$M |
|--------------------|-----------------------|-------------------------|
| wt EF-G (T.th.)    | 1.5                   | 94                      |
| EF-G (G16V)       | 1.3                   | 26                      |
| EF-G (E119K)      | 1.6                   | 60                      |
| EF-G (T84A)       | 1.8                   | 410                     |
| EF-G (D109K)      | 1.5                   | 180                     |
| EF-G (G16V/T84A)  | 0.62                  | 13                      |

300 pmol of the factors and varied concentration of nucleotides were used in the experiments.

The values are averaged from two experiments.
Martemyanov et al. Fig. 2
Martemyanov et al. Fig. 3

![Graph showing GTPase activity percentage vs. FA concentration in μM.](http://www.jbc.org/Downloaded from)
Martemyanov et al. Fig. 5
Martemyanov et al. Fig. 6
Mutations in the G-domain of elongation factor G from thermus thermophilus affect both its interaction with GTP and fusidic acid
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