Domain IV of elongation factor G from *Thermus thermophilus* is strictly required for translocation

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Abstract Two truncated variants of elongation factor G from *Thermus thermophilus* with deletion of its domain IV have been constructed and the mutated genes were expressed in *Escherichia coli*. The truncated factors were produced in a soluble form and retained a high thermostability. It was demonstrated that mutated factors possessed (1) a reduced affinity to the ribosomes with an uncleavable GTP analog and (2) a specific ribosome-dependent GTPase activity. At the same time, in contrast to the wild-type elongation factor G, they were incapable to promote translocation. The conclusions are drawn that (1) domain IV is not involved in the GTPase activity of elongation factor G, (2) it contributes to the binding of elongation factor G with the ribosome and (3) is strictly required for translocation. These results suggest that domain IV might be directly involved in translocation and GTPase activity of the factor is not directly coupled with translocation.

Key words: Ribosome; Elongation factor G function; Translocation; Mutagenesis

1. Introduction

Elongation factors (EF) Tu and G play a crucial role in the ribosomal elongation cycle. EF-Tu delivers cognate aminoacyl-tRNA into the ribosome and EF-G stimulates peptidyl-tRNA transfer from the A to P ribosomal sites and mRNA movement, i.e. translocation [1]. Recent studies of EFs resulted in a tertiary structure determination of EF-G from *Termus thermophilus* in the complex with GDP and its empty form [2,3], EF-Tu from *T. thermophilus* in GTP forms [4] and the ternary complex of EF-Tu with the uncleavable GTP analog and aminoacylated tRNA [5]. It is interesting that the overall shape of EF-G has been found very similar to that of the EF-Tu complex with the aminoacyl-tRNA and uncleavable GTP analog [6,7]. The most remarkable is that domains III, IV and V of EF-G mimic the tRNA structure in the EF-Tu ternary complex and, in this case, domain IV can be related to the anticodon arm of tRNA. This 'molecular mimicry' is thought to be functionally important [7–10].

To study the functional role of domain IV, we prepared two truncated variants of EF-G from *T. thermophilus* without domain IV. All principal activities of EF-G were investigated and it was demonstrated that the mutated factors possessed a specific ribosome-dependent GTPase activity, whilst their translocation ability was abolished.

2. Materials and methods

2.1. Materials

Restriction endonucleases and T4 DNA ligase were from Promega. DNA polymerase was purchased from New England Biolabs and used according to the manufacturer's directions. Resins for protein isolation, Q-Sepharose FF, Ultrogel Ac34 and HA-Ultrogel were from Pharmacia. Plasmids were maintained in *Escherichia coli* strain XL1 (Promega). For gene expression, plasmid pET 11c and strains BL21(DE3) or B834(DE3) (Novagen) were used [11].

2.2. Oligonucleotides

The following oligonucleotides for mutagenesis were synthesized by Gene Assembler Plus (Pharmacia) according to the manufacturer's manual: Pr1, 5′-CGGTGGTG*CATATG*GCGGTCAAGGTAG-3′, contains restriction site *Nde*I; Pr2, 5′- ATCTCCGTCC*TGATCA*C-AAGGAAGGG-3′, (site *Bcl*I); Pr3, 5′-CCGGTA***GTCGA***CCTG-GGGCTTGC-3′, (site *Sal*I); Pr4, 5′-GTGATC***G***TCGA**C**CCCCAT-CATGC-3′, (site *Sal*I); Pr5, 5′-GG*GAATTC*TTAGTGGTCAAA-GAACA-3′ with a stop codon and *Eco*RI site; Pr6, 5′-CTC-CAC*GTC**G**AC*CGGCTT-3′, (site *Sal*I); Pr7, 5′-CAGAAG***GTCGA***-*C*CCGGTGA-3′, (site *Sal*I). The restriction sites are given in italic, non-complementary nucleotides are given in bold letters.

2.3. Mutagenesis, gene expression and protein purification

Mutagenesis was carried out by polymerase chain reaction (PCR). All other recombinant DNA procedures were according to the published manual [12]. Two independent PCR reactions were carried with primers (either Pr1 and Pr3 or P4 and pr5) as in reference [13]. The two PCR products were purified with a 'Wizard PCR preps kit' (Promega) and digested either with *Nde*I and *Sal*I or with *Sal*I and *Eco*RI. The obtained fragments were simultaneously ligated into the pET11c plasmid treated with *Nde*I and *Eco*RI. In this way, the expression plasmid pETΔIVC was obtained with the DNA fragment encoding the EF-GΔIVC protein. The second plasmid, pETGΔIV, was constructed in a similar way using primers Pr1, Pr6 and primers Pr2, Pr7. This plasmid carries the gene for the EF-GΔIV protein (see Section 3).

Cloned genes were expressed according to the published procedure [11]. A heat denaturation approach was used due to the high thermostability of the produced proteins [13]. The purity of the isolated proteins was checked by SDS electrophoresis [14].

2.4. Nucleotide binding and GTP hydrolysis

Complex formation of EF with the ribosomes and uncleavable GTP analog was tested by the filter binding technique [15]. To test weak interactions of the truncated factors with ribosomes and GMPPNP, the sedimentation technique was used [16]. After centrifugation, aliquots of 50 μl were carefully withdrawn, mixed with 20 μl of the sample electrophoresis buffer and loaded on the gel for SDS electrophoresis. The distribution of EF-G in the fractions was registered by a HP Jet Scanner after gel staining by Coomassie G-250. Band intensities were quantified with the help of the Kodak 1D program.

Uncoupled GTPase activity of EF-G with the ribosomes was followed spectrophotometrically by measuring released inorganic phosphate as described in [17].

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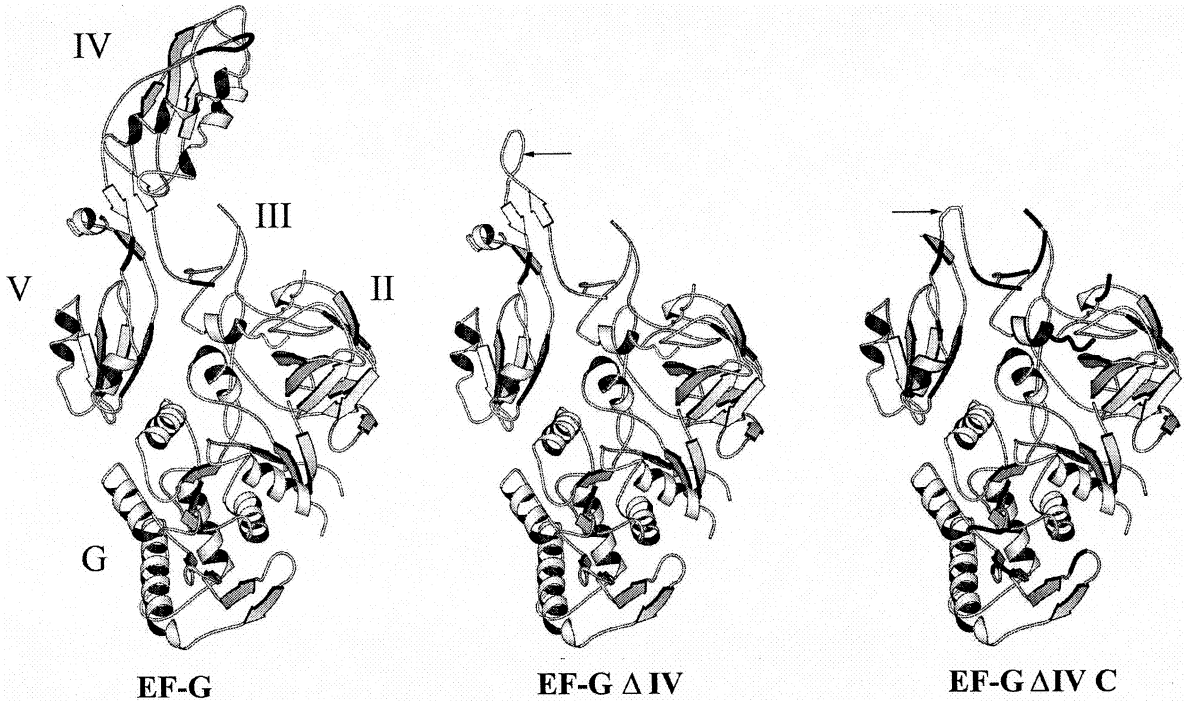

Fig. 1. Ribbon models of the tertiary structures of intact *T. thermophilus* EF-G and its truncated variants. Arrows indicate new connections after domain IV excision (see text). The helix between domains IV and V is a C-terminal helix. The models were drawn with the help of RASMOL program using PDB coordinates (PDB ID. 1DAR).

2.5. Translation in vitro

Poly(U)-directed polyphenylalanine synthesis was carried out in a cell-free translation system with purified EF-Tu and EF-G according to the published procedure [18]. The stimulation of non-enzymatic translation by EF-G and deletion mutants was tested as described [19]. Translocation activities of the factors were also checked by competence of the ribosomal complex to puromycin. In this case, the pre-translocation complex was prepared as described in [20]. The puromycin reaction was carried out with 40 pmol of the factors, 45 pmol of the ribosomes and 1 mM (final concentration) of puromycin as in [21].

3. Results

3.1. Structural characteristics of the truncated EF-G

The EF-G tertiary structure [2,3] was considered in mutagenesis planning. It was noticed that Val-481 and Pro-604, as well as Asp-492 and Pro-599, are spatially close in the EF-G tertiary structure. It was difficult to predict the stability of the truncated factors. For this reason, the residues between these neighboring amino acids were deleted and two truncated variants of EF-G were independently obtained.

The first mutated EF-G with deleted residues 493–598 is designated as EF-GΔIV. This protein contains an intact C-terminal helical fragment, two short β-strands of the domain IV and its residues Asp-492 and Pro-599 are directly connected. In the second construct, a new stop codon was inserted instead of Tyr-676. As a result, the EF-GΔIVC protein was obtained with deleted residues 482–603 and one foreign amino acid (Asp) inserted after Val-481. The C-terminal residues 676–691 constituted the C-terminal helix were also excised (Fig. 1).

The EF-GΔIVC and EF-GΔIV proteins contain 554 and 585 amino acids (including N-terminal Met) with molecular weights of 61 900 and 65 509, respectively.

Both truncated factors produced in the cells were stable up to 70°C. At this temperature, the EF-G from *E. coli* is completely inactivated and precipitated [22]. For this reason, a heat denaturation step was used for protein purification.

3.2. Binding to the ribosomes and GTPase activity

The mutated factors under study displayed very similar functional activities and all given data below are related to the both of them.

Table 1
Ribosomal complex formation with EF-G and uncleavable GTP analog

Mixture composition[a]	EF-G[b] ratio in fractions	Complex formation[c] (%)
EF-G, GMPPNP, 70S Rs	31.2	100
EF-G, GMPPNP, 70S Rs, 75 μM thiostrepton	1.2	0.2
EF-G, GMPPNP	1.2	–
EF-GΔIV, GMPPNP, 70S, RS	5.6	14.5
EF-GΔIV, GMPPNP, 70S Rs, 75μM thiostrepton	3.4	7.5
EF-GΔIV, GMPPNP	1.1	–

[a] *T. thermophilus* EF-G and *E. coli* 70S ribosomes were used.
[b] The ratios of the EF-G amount in the bottom/supernatant fractions are given in arbitrary units. The S.D.s are ±0.1.
[c] The amount of wild-type EF-G bound to the ribosomes was taken as 100%.

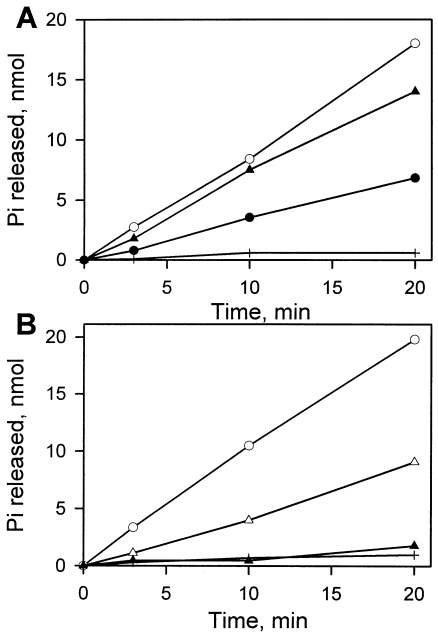

Fig. 2. Kinetics of GTP hydrolysis by EF-G and EF-GΔIV on the 70S ribosomes and ribosomal subunits. (A) The 70S ribosomes presented in all cases, (o) EF-GΔIV, (▼) *E. coli* EF-G, (●) *T. Thermophilus* EF-G, (+) no EF-G; (B) (o) EF-GΔIV with the 70S ribosomes, (▽) EF-GΔIV with 50S subunits, (▲) 50S with no EF-G and (+) EF-GΔIV with 30S subunits. Values for the phosphate released are given for 20 μl aliquots.

The binding studies of the truncated factors have shown their decreased affinity to the 70S ribosomes. In contrast to the wild-type EF-G, the ribosomal complexes with the mutant proteins and uncleavable GTP analog (GMPPNP) could not be detected by the filter binding technique. The existence of the complexes was demonstrated by centrifugation and electrophoresis analysis. The complex formation was inhibited by thiostrepton. In case of EF-GΔIV, the inhibition effect was less pronounced than for intact EF-G (Table 1). The binding of the truncated factors to isolated 50S subunits was even weaker (not shown).

The GTPase activity of the truncated factors specifically depended on the 70S ribosomes or the 50S subunits but did not depend on the 30S subunits. The EF-GΔIV and EF-G from *E. coli* displayed similar rates of uncoupled GTP hydrolysis and these rates were a little higher than in the case of

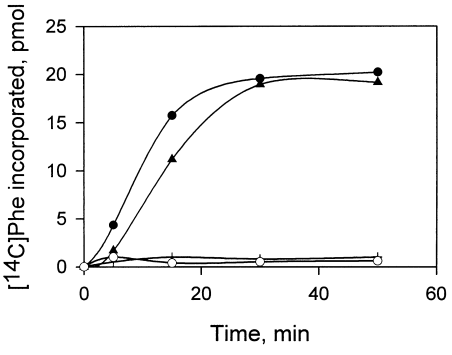

Fig. 3. Poly(U)-directed cell-free translation. (●) With wild-type *T. thermophilus* and (▼) *E. coli* EF-G, (o) with the truncated factor and (+) 30S without EF-G. Values are given for 10 μl aliquots.

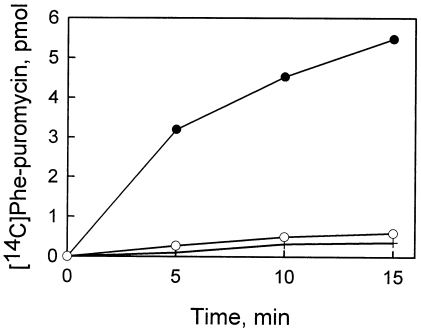

Fig. 4. The yield of Phe-puromycin in reaction with pre-translocation ribosomes promoted by: (●) wild-type EF-G, (o) by EF-GΔIV, (+) no EF-G added. The yield was calculated for the total reaction mixture.

intact *T. thermophilus* EF-G (Fig. 2A). The rate of GTP hydrolysis with truncated factors and the 50S subunit was slower than with a 70S ribosome (Fig. 2B).

The fusidic acid (FA) inhibited the ribosome-dependent GTP hydrolysis promoted by wild-type EF-G and EF-GΔIV. A half-inhibition effect is less than 50 μM FA (titration curve not shown).

3.3. Translation activity

The EF-G from *T. thermophilus* was capable to substitute the EF-G from *E. coli* in the poly(U)-directed translation system with *E. coli* ribosomes [13]. The translation activity of *T. thermophilus* EF-G was similar to that of *E. coli* EF-G, but the truncated proteins were not active in the poly(U)-directed translation system (Fig. 3).

The translocation ability of the mutant proteins was also tested in the non-enzymatic translation system [19]. The truncated factors virtually had no stimulation effect in this system. During 5 h of incubation, 18 pmol of poly-Phe was synthesized in the system supplemented with wild-type EF-G and only about 3 pmol (a comparable value for 'factor-free' system) in the presence of EF-GΔIV. EF-GΔIV was also not active in a puromycin reaction with pre-translocation ribosomes. The yield of Phe-puromycin was comparable with its formation in the experiment without the EF-G (Fig. 4), even at a 5-fold excess of the EF-GΔIV over ribosomes.

4. Discussion

The progress in the structural studies of EF-Tu and -G have led to the observation of the molecular mimicry between EF-G and the ternary complex of the EF-Tu with tRNA. In this case, EF-G domain IV is a particularly interesting part of the factor. Its relationships to the anticodon arm of the tRNA in the ternary complex raises the question whether this is an important EF-G part for translocation.

Years ago, it was shown [15] that EF-Tu and -G from *T. thermophilus* can substitute their counterparts from *E. coli* in reactions with *E. coli* ribosomes. This was confirmed by the EF-G produced in *E. coli* from the cloned *fus* gene of *T. thermophilus* [13].

Both wild-type EF-G and EF-GΔIV promoted GTP hydrolysis with the 70S and 50S particles. Isolated 30S subunits do not stimulate GTPase activity, but they do so in the 70S ribosome by improving the interaction of the factors with

50S subunits [23,24]. The EF-G interacts with both subunits of the ribosome and domain IV has contacts with the 30S subunit [25]. Hence, the decreased affinity of EF-GΔIV to the ribosome (Table 1) can be explained by the absence of domain IV. This may also explain the increased GTPase activity of the EF-GΔIV (Fig. 2A). In such an event, incomplete contacts of the truncated factor with the ribosome may prevent some conformational adjustments (both in the ribosome and in EF-G) which occur in the case of intact EF-G. Consequently, it may facilitate dissociation of EF-GΔIV after GTP hydrolysis and the hydrolysis turnover will be increased.

These results also suggest that (1) either the 50S subunit has a somewhat different conformation in the 70S ribosome or (2) some conformational changes could occur in the 70S ribosome and/or in the intact EF-G upon its binding to the ribosome, but in the case of EF-GΔIV, this does not happen. These suggestions are in agreement with the data and discussions available in the literature on conformational changes in the ribosomes and EFs upon their interaction [4,23,25–29].

Analysis of FA resistant mutations in EF-G placed them in the G domain and at the interface between domains G, II, III and V [9]. Since FA inhibits GTP hydrolysis catalyzed by a truncated factor, we conclude that these domains are intact in EF-GΔIV and domain IV deletion does not influence the FA mode of action markedly.

The truncated factor was not active in poly(U)-directed translation (Fig. 3). EF-GΔIV also had no stimulatory effect on the non-enzymatic translation (see the Section 3), though this system is known as a sensitive test for the translocation ability of EF-G [19]. In contrast to the wild-type EF-G, no activity was found in the EF-GΔIV-dependent puromycin reaction with isolated pre-translocation ribosomes (Fig. 4). These data indicate convincingly that factors lacking domain IV do not promote translocation in all tested translation systems. The essential role of domain IV for translocation was demonstrated also by mutation of a loop at the distal end of this domain in the *T. thermophilus* EF-G. The insertion of six amino acids into the loop greatly decreased the translocation ability of the EF-G, while its GTPase activity was not affected [30].

Hence, from the data obtained, it can be concluded that (1) domain IV contributes to the EF-G binding to the ribosomes, (2) EF-GΔIV possesses a specific ribosome-dependent GTPase activity (multiple turnover) and (3) EF-G lacking domain IV is not active in translocation, so that the GTP hydrolysis catalyzed by the EF-GΔIV is uncoupled with translocation.

Recent studies of *E. coli* EF-G with a deleted domain IV have shown that domain IV was not strictly required for translocation. This mutant factor was found irreversibly bound to the ribosome and for this reason, its activities were restricted to a single round of GTP hydrolysis as well as to a single translocation [31]. Its irreversible binding to the ribosome after GTP hydrolysis seems very unusual. If we assume that an *E. coli* truncated factor will dissociate normally, then, its function would be virtually the same as of wild-type *E. coli* EF-G. The truncated *E. coli* factor produced in *E. coli* cells was insoluble and had to be renatured from urea before usage. This difference may relate to the functional features observed for truncated factors from *E. coli* and *T. thermophilus*.

Several models of the EF-G function were proposed in recent publications [10,32,33]. Since the ribosome is capable of spontaneous translocation [34,35] and EF-G promotes translocation with an uncleavable GTP analog, the above results support the view that EF-G catalyzes translocation by promoting some re-arrangement in the ribosomes (not exactly known yet). In this case, domain IV of the EF-G mimicking the anticodon arm of tRNA may interact with the tRNA and 30S subunit [36] and, by its movement into the acceptor site, could stimulate tRNA displacement together with mRNA, i.e. translocation. At the same time, the GTP binding domain interacts with the 50S subunit, GTP is hydrolyzed and the EF-G dissociates from the ribosome. Therefore, we accept that the GTP binding and its hydrolysis modulate the EF-G actions basically in the same way as they do in the case of all members of GTP binding proteins [37].

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