Review

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Converting GTP hydrolysis into motion: versatile translational elongation factor G

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Abstract: Elongation factor G (EF-G) is a translational GTPase that acts at several stages of protein synthesis. Its canonical function is to catalyze tRNA movement during translation elongation, but it also acts at the last step of translation to promote ribosome recycling. Moreover, EF-G has additional functions, such as helping the ribosome to maintain the mRNA reading frame or to slide over non-coding stretches of the mRNA. EF-G has an unconventional GTPase cycle that couples the energy of GTP hydrolysis to movement. EF-G facilitates movement in the GDP-P_i form. To convert the energy of hydrolysis to movement, it requires various ligands in the A site, such as a tRNA in translocation, an mRNA secondary structure element in ribosome sliding, or ribosome recycling factor in post-termination complex disassembly. The ligand defines the direction and timing of EF-G-facilitated motion. In this review, we summarize recent advances in understanding the mechanism of EF-G action as a remarkable force-generating GTPase.

Keywords: protein synthesis; reading frame maintenance; ribosome bypassing; ribosome recycling; translocation.

Introduction

Translational GTPases are protein factors that help the ribosome to synthesize proteins in the cell. Like most members of the GTPase superfamily, they share an evolutionarily conserved GTP-binding (G) domain and act as molecular switches that alternate between the active GTP-bound and inactive GDP-bound form. Translational GTPases, such as initiation factors IF2, eIF2 and eIF5B (where 'e' denotes eukaryotic origin), elongation factors EF-Tu, eEF1A, EF-G, eEF2, SelB and eEFSec, termination factors RF3 and eRF3 as well as unconventional eukaryotic GTPBP1 and GTPBP2, comprise a GTPase subfamily (Leipe et al., 2002; Atkinson, 2015). They all have distinct roles and act at one specific step of translation. One notable exception is EF-G (in bacteria, or a/eEF2 in archaea and eukaryotes). The canonical role of the factor is to help the ribosome to translocate along the mRNA during the elongation phase of translation. In addition, EF-G is involved in ribosome recycling, the last step of translation at which the ribosome after termination dissociates into small and large ribosomal subunits (SSU and LSU, respectively) to prepare for translation of the next mRNA (Janosi et al., 1996). Moreover, recent experiments suggest that EF-G can carry out non-conventional reactions such as suppressing ribosomal frameshifting or carrying out pseudo-translocation using an mRNA element instead of tRNA, which helps the ribosome to slide along a non-coding mRNA region (Klimova et al., 2019), suggesting a remarkable multitude of modes of action for this unique GTPase.

Translational GTPases have many features that are typical for GTPases (Bourne et al., 1991). Most of them are inactive in the GDP-bound form. Nucleotide exchange may occur spontaneously or with the help of a specialized guanine nucleotide exchange factor (GEF). Most translational GTPases have only very low intrinsic GTPase activity and are activated by interactions with the ribosome, except for eIF2 which is activated by a protein (eIF5). The functional cycle of translational GTPases is terminated by GTP hydrolysis, but in contrast to other GTPases that are activated by the interactions with a GTPase-activating protein (GAP), translational GTPases are activated upon binding of their G-domain to ribosomal RNA (rRNA). The best-studied example of a canonical translational GTPase is EF-Tu (Rodnina, 2018). EF-Tu is inactive in the GDP-bound form and GDP-to-GTP exchange is facilitated

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by EF-Ts. In the GTP-bound form EF-Tu is active; it binds aminoacyl-tRNA (aa-tRNA) and delivers it to the ribosome where it remains tightly bound to both the aa-tRNA and the ribosome until GTP is hydrolyzed. GTP hydrolysis leads to a large conformational change of EF-Tu resulting in a loss of interactions with the aa-tRNA and dissociation from the ribosome (Rodnina, 2018).

For a long time, it seemed that EF-G follows the same canonical switch mechanism. Based on experiments with non-hydrolyzable GTP analogs, it was assumed that EF-G binds to the ribosome and catalyzes translocation in the GTP-form only and GTP hydrolysis subsequent to the tRNA movement is required for dissociation of EF-G (Kaziro, 1978). This simple picture began to totter when rapid kinetic experiments showed that GTP hydrolysis precedes translocation and strongly accelerates the reaction, indicating that the mechanism of EF-G function does not follow the general GTPase switch model (Rodnina et al., 1997). Experiments of the last decade indicated how EF-G converts the energy of GTP hydrolysis in facilitating movement, akin to ATP-consuming motor proteins. In this review, we summarize how EF-G accomplishes its different functions and how it couples the energy of GTP hydrolysis to molecular movement. EF-G provides a remarkable example of how a dynamic protein can perform versatile functions powered by the energy of GTP hydrolysis.

EF-G as a GTPase

EF-G consists of five domains (Ævarsson et al., 1994; Czworkowski et al., 1994). Domain 1 is the G-domain that binds GTP/GDP. Domain 2 is shared by most translational GTPases and together with the G-domain forms a structural superdomain. Domains 3–5, which form another superdomain, are specific to EF-G and its eukaryotic homolog eEF2 and are essential for their function as tRNA translocases (Figure 1). The two superdomains move relative to each other, allowing EF-G to change its structure substantially (Peske et al., 2000; Lin et al., 2015). EF-G

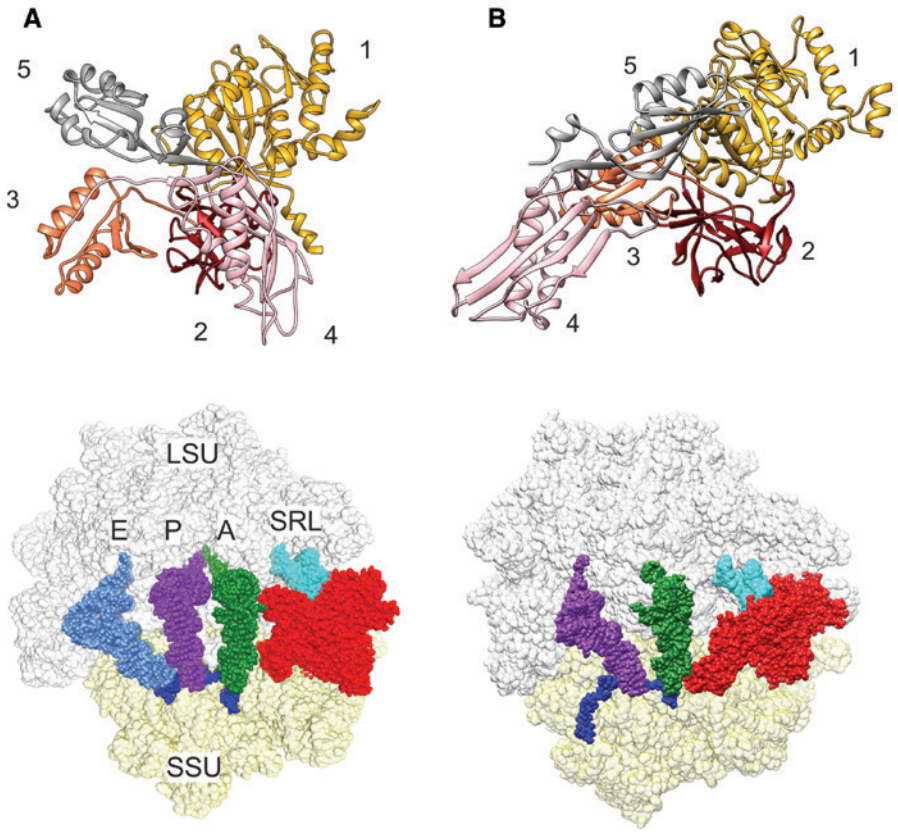

Figure 1: Conformations of EF-G on the ribosome.
(A) Compact conformation. (B) Elongated conformation. Upper panel shows the detailed arrangement of EF-G domains 1–5. The tRNAs bound to the A, P, and E site of the ribosome are colored green, magenta and light blue, respectively. The mRNA is shown in dark blue, EF-G in red. Images were prepared from PDB 4WPO and 4V7D (Brilot et al., 2013; Lin et al., 2015).

can adopt two very distinct GTP-bound conformations: a compact one, in which domain 4 is in close proximity of the G-domain and domain 2, and an elongated one with domain 4 protruding away from the G-domain (Lin et al., 2015; Salsi et al., 2015). Single-molecule FRET measurements using two fluorophores attached to EF-G suggested that EF-G predominantly adopts a compact conformation in solution (Salsi et al., 2015), whereas crystal structures favor the elongated conformation regardless of the bound nucleotide (Czworkowski et al., 1994; Czworkowski and Moore, 1997). EF-G switch 1 and 2 regions in the G-domain, which act as guanine-nucleotide sensors in most GTPases (Wittinghofer and Vetter, 2011), remain disordered in the GTP and GDP forms in solution (Hansson et al., 2005). On the ribosome, EF-G exhibits a great degree of conformational flexibility and the preferred EF-G conformation may be dictated by the state of the ribosome (see below), rather than by the bound guanine nucleotide.

The affinity of EF-G to guanine nucleotides is in the micromolar range and similar for GTP and GDP (Wilden et al., 2006). Given that GTP is prevalent in the cell, EF-G can spontaneously and rapidly [at a rate of about 300 s^{-1} (Wilden et al., 2006)] exchange GDP for GTP without a nucleotide exchange factor. Occasionally, EF-G can bind to the ribosome in the GDP-bound form and exchange GDP with GTP while bound to the ribosome (Hauryliuk et al., 2008). In the absence of the bound nucleotide, EF-G is inactive. Experiments with a non-hydrolyzable GTP analog, GDPNP, suggest that in the complex with the ribosome, GDPNP binding to EF-G is greatly stabilized (Wilden et al., 2006), probably by the interaction with the sarcin-ricin loop (SRL) of 23S rRNA. This correlates with the ordering of the switch 1 and 2 regions of EF-G (Tourigny et al., 2013). With GTP, this complex is short-lived, because GTP hydrolysis by EF-G on the ribosome is very rapid (100–250 s^{-1}) (Rodnina et al., 1997; Savelsbergh et al., 2003; Pan et al., 2007).

EF-G does not hydrolyze GTP on its own and depends on the ribosome to activate the GTPase. The mechanism of GTP hydrolysis is expected to be evolutionarily conserved, as crystal structures show similar contacts between the SRL and all translational GTPases studied so far, except for RF3. The exact pathway for GTPase activation is best studied for EF-Tu [for review, see (Fischer et al., 2016; Maracci and Rodnina, 2016)] (Figure 2). The reaction proceeds through the nucleophilic attack of a water molecule on the γ-phosphate of GTP. The negative charge developing on the β-γ bridging oxygen is probably stabilized by the side chain of a universal lysine residue (Lys24 in EF-Tu; Lys22 in EF-G, *Escherichia coli* numbering) and multiple main-chain interactions with the P-loop residues of EF-Tu,

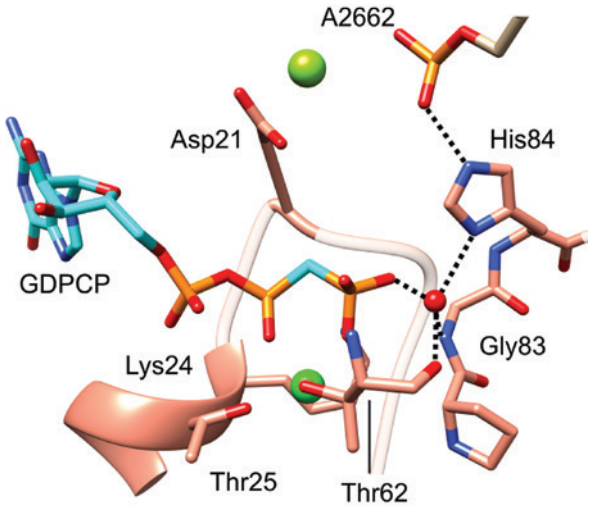

Figure 2: The activated GTPase site of a translational GTPase bound to the ribosome using EF-Tu as example.
The SRL is represented by residue 2662 of the 23S rRNA. The hydrolytic water molecule (red sphere) and Mg^{2+} ions (green sphere) coordinated to Lys21 and Lys24 are indicated. The Mg^{2+} ion coordinated to Asp21 is not seen in the EF-Tu structure, but is found in EF-G (PDB 4V90) (Chen et al., 2013; Tourigny et al., 2013). The EF-Tu structure is from PDB 4V5L (Voorhees et al., 2010).

as shown for other GTPases (Allin and Gerwert, 2001). The reaction on the ribosome depends on the presence of His84 of EF-Tu (His91 in EF-G) in the switch 2 region and the side chain of Asp21 of EF-Tu (Asp19 in EF-G), as well as the phosphate group of A2662 in the SRL of the 23S rRNA (Daviter et al., 2003; Maracci et al., 2014; Koch et al., 2015). A2662 is responsible for the stabilization of a conformation where His84 is rotated towards the nucleotide (Voorhees et al., 2010). Asp21 favors the movement of the negatively charged phosphate group of A2662 toward His84 (Aqvist and Kamerlin, 2015a). Asp21 further stabilizes the transition state by coordinating a Mg^{2+} ion close to the crucial A2662; this Mg^{2+} ion is also found in the structure of EF-G (Chen et al., 2013; Tourigny et al., 2013). Thus, the ribosome appears to accelerate GTP hydrolysis by rearranging the catalytic site of EF-Tu/EF-G into a conformation that provides the optimal electrostatic stabilization. This is consistent with the exceptionally high entropic contribution to catalysis (Aqvist and Kamerlin, 2015b), the lack of pH-dependence, and the kinetic solvent isotope effect of the reaction with EF-Tu (Maracci et al., 2014).

GTP hydrolysis by EF-G is much faster than tRNA movement (250 s^{-1} vs. 40 s^{-1}, respectively) (Savelsbergh et al., 2003; Belardinelli et al., 2016a). This raises the question of how and in which conformation EF-G acts to promote tRNA movement. Rapid kinetic studies suggested that, although GTP hydrolysis is very rapid, the release of

inorganic phosphate (P_i) is delayed and occurs at the same rate as tRNA translocation on the SSU (Savelsbergh et al., 2000). This suggests that both reactions are rate-limited by a preceding ribosome rearrangement ('unlocking') that allows the ribosome to move along the mRNA. Thus, the active form of EF-G appears to be the GDP-P_i-bound form, rather than the GTP-bound form as is the case with other GTPases. Consistent with this notion, the rate of translocation is greatly reduced when EF-G is blocked in the GTP-bound form, i.e. by replacing GTP by non-hydrolyzable GTP analogs or with a GTPase-deficient EF-G mutant, and follows a different pathway than with GTP (Cunha et al., 2013; Belardinelli et al., 2016a).

EF-G in tRNA-mRNA translocation

The canonical function of EF-G is to promote tRNA-mRNA translocation after peptide bond formation has taken place. During translocation, the two tRNAs bound to the ribosome, deacylated tRNA in the P site and peptidyl-tRNA in the A site, move together with their codons into the E and P sites, respectively. The E-site tRNA then dissociates spontaneously. In the absence of EF-G, translocation is extremely slow. This spontaneous, thermally-driven reaction is an equilibrium process in which the tRNAs make rapid, spontaneous excursions in both forward and backward directions (Shoji et al., 2006; Konevega et al., 2007; Fischer et al., 2010). Preferential directionality is determined by the affinities of the tRNAs for their respective binding sites (Shoji et al., 2006; Konevega et al., 2007; Bock et al., 2013). EF-G accelerates the reaction by four orders of magnitude. GTP hydrolysis accelerates translocation by a further 40-fold (Rodnina et al., 1997; Munro et al., 2010) and alters the translocation pathway (Belardinelli et al., 2016a), suggesting that the energy of GTP hydrolysis is utilized to facilitate forward movement.

In the pre-translocation (PRE) state, the SSU and LSU can move relative to each other. The ribosome oscillates between the non-rotated and rotated conformations [for review see (Belardinelli et al., 2016b; Noller et al., 2017; Jobe et al., 2019)]. At the same time, the tRNAs in P and A sites move towards E and P sites, respectively, on the LSU, while still bound to the P and A sites on the SSU, and fluctuate between these hybrid and the classical states (Noller et al., 2017). EF-G can bind to either state of the ribosome (Holtkamp et al., 2014a), however, it is possible that there is a preference for a certain structure of EF-G (Lin et al., 2015). Crystal structures revealed a complex of the non-rotated ribosome with EF-G and GDP (Lin et al., 2015). The structure of the ribosome with EF-G in the compact state may represent an early interaction intermediate, as binding of the elongated conformation of EF-G appears unfavorable due to a potential steric clash between domain 4 of EF-G and the anticodon stem-loop (ASL) of the A-site tRNA. Alternatively, the compact GDP-bound EF-G may represent a very late intermediate of EF-G dissociation, which in the Lin et al. structure may be stabilized through the fusion between EF-G and the ribosomal protein L9. In fact, kinetic data suggest that EF-G dissociation is a multiphasic process (Belardinelli et al., 2016a), which could justify the existence of such an intermediate. EF-G binding accelerates the transition from the non-rotated to the rotated state of the ribosome and stabilizes the rotated state; this action does not require GTP hydrolysis, as the rates of EF-G-catalyzed rotation are similar with GTP and a non-hydrolyzable GTP analog (Sharma et al., 2016) (Figure 3A). Most of the structural and single-molecule work suggests that – except for the initial binding complex – the elongated conformation of EF-G is favored on the ribosome.

EF-G binding facilitates the subsequent rearrangements of the pre-translocation complex which can be grouped in several steps based on their kinetics (Savelsbergh et al., 2003; Holtkamp et al., 2014; Adio et al., 2015; Belardinelli et al., 2016a) (Figure 3B). After binding (step 1) EF-G hydrolyzes GTP and adopts the GDP-P_i-bound conformation (step 2). This uncouples the movements of the head and body domains of the SSU. The body domain starts moving backward towards a non-rotated state, whereas the SSU head domain moves further in the forward direction (Belardinelli et al., 2016a) in a motion called swiveling (Schuwirth et al., 2005). The ASL domains of tRNAs move with the SSU head. As a result, the ASLs remain in contact with P and A sites on the SSU head domain, but occupy the E and P positions, respectively, on the SSU body domain. This state of the tRNA is called chimeric (Ramrath et al., 2013; Zhou et al., 2013, 2014). On the LSU, the 3′ ends of the tRNAs are in positions that are close to but not fully adjusted in E and P sites; therefore, the resulting tRNA positions are denoted as chimeric pe/pe and ap/ap (Holtkamp et al., 2014; Zhou et al., 2014). The next rearrangement (step 3) unlocks the tRNA-mRNA complex on the SSU. This is a critical step of translocation that limits the rate of tRNA movement and of P_i release from EF-G (Savelsbergh et al., 2000). The 3′ end of the A-site peptidyl-tRNA moves into the P-site, where it becomes reactive towards puromycin, which is a diagnostic test for translocation on the LSU (Holtkamp et al., 2014). The SSU head starts moving backwards, but it takes two more steps until it returns into its non-rotated position (Belardinelli et al., 2016a). Steps 2 and 3 can take place without GTP hydrolysis and even

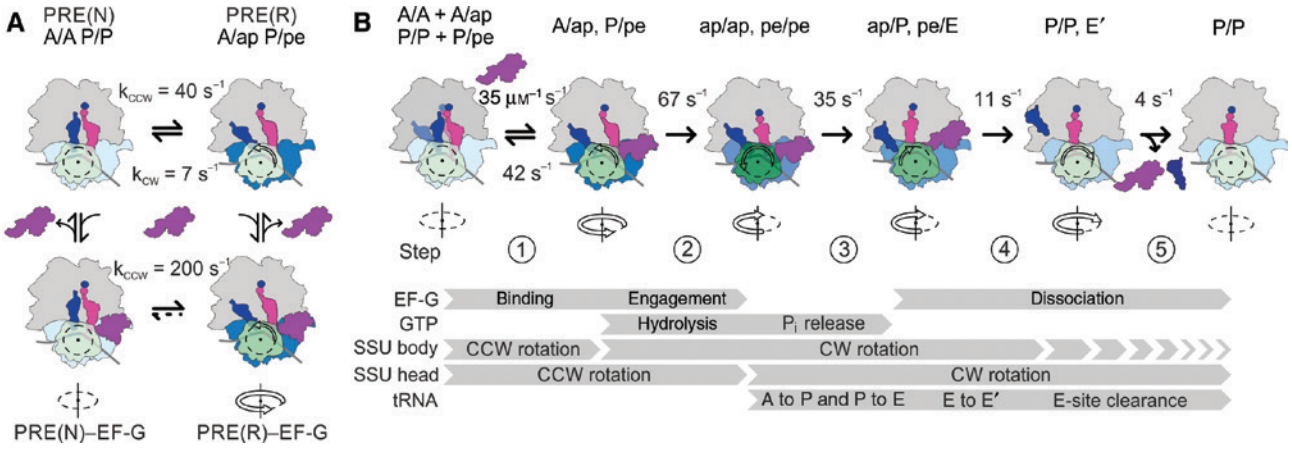

Figure 3: Role of EF-G in tRNA translocation.
(A) EF-G binding to the ribosome in the PRE state. EF-G binding accelerates formation of the rotated state and arrests fluctuations to the non-rotated state; the rates of transitions are from (Sharma et al., 2016). The rotation states of the SSU relative to the LSU (gray) are indicated by color intensity of the SSU body (light blue for non-rotated (N), dark blue for rotated (R)). The swiveling motions of the SSU head are depicted by a color gradient from light green (classical non-swiveled SSU head position) to dark green (maximum degree of rotation relative to the SSU body) (Belardinelli et al., 2016b). Peptidyl- and deacylated-tRNA in the PRE complex are shown in magenta and blue, respectively. EF-G (purple) is depicted in both compact (Lin et al., 2015) and elongated conformation (Ramrath et al., 2013; Zhou et al., 2014). The tRNA states in the R state of the ribosome are denoted as A/ap, P/pe, because although the A-site tRNA moves towards the P site on the LSU, it is not puromycin-reactive and thus not fully accommodated in the P site. A dashed line indicates a very low rate constant of backward reaction.
(B) Kinetic mechanism of translocation. The assignment of the tRNA states is based on structural studies (Brilot et al., 2013; Ramrath et al., 2013; Zhou et al., 2013, 2014, 2019) and the sensitivity to puromycin (Holtkamp et al., 2014; Peng et al., 2019). The rates of EF-G binding and dissociation (step ①) are ensemble rate constants obtained for a mixture of N and R states in which the PRE(R) state is predominant (Sharma et al., 2016). All other rate constants for the kinetically defined steps ②, ③, ④, and ⑤ are from ensemble kinetics studies at 37°C (Belardinelli et al., 2016b). Kinetics of GTP hydrolysis and P_i release are from (Rodnina et al., 1997; Savelsbergh et al., 2005). Ribbons below the kinetic scheme indicate the movements of different elements of the complex during translocation.

without EF-G, but at much reduced rates. Interaction of EF-G with the ribosome lowers the energy barrier for tRNA-mRNA movements, which we described as energizing (Adio et al., 2015). Step 3 depends on interactions with the tip of domain 4 of EF-G, as mutations at the tip reduce the translocation rate by 40-fold (Peng et al., 2019).

The unlocking step 3 is facilitated by EF-G in the GDP-P_i form. Unlocking precedes tRNA movement and P_i release (Savelsbergh et al., 2003) and can occur also when P_i release is inhibited by mutations in ribosomal protein L12 (Savelsbergh et al., 2005) or when the GDP-P_i-like state is trapped using vanadate as a P_i mimic (Savelsbergh et al., 2009). Single-molecule experiments show that after GTP hydrolysis EF-G undergoes a small (~10°) global rotational motion relative to the ribosome that exerts a force to unlock the ribosome (Chen et al., 2016). The initial stroke comes during step 2 but is likely to continue through step 3. Force measurements using optical-tweezers suggest that the force developing during the EF-G power stroke is modest, 13 pN (Liu et al., 2014). Estimations from alternative techniques give much higher values, up to 85 pN (Yao et al., 2013; Xu et al., 2019); the reasons for such large discrepancies are unclear. During the power stroke, EF-G most likely does not push the tRNA directly, because both tRNA movement and P_i release occur as distinct independent steps that follow and are rate-limited by the unlocking step (Peske et al., 2000; Savelsbergh et al., 2003, 2005). Rather, the power stroke by EF-G changes the conformation of the ribosome, which then allows the tRNAs to move and/or tilts the reaction coordinate in a way that favors forward movement.

The exact nature of unlocking is not clear. Because domain 4 of EF-G comes in direct contact with the A-site tRNA, early models suggested that the energy of GTP hydrolysis may be required to disrupt the interactions of the key A-site sensing 16S rRNA residues A1492 and A1493 with the codon-anticodon complex (Savelsbergh et al., 2003; Ramrath et al., 2013) or by opening the G530-A1492 latch that controls the SSU head and body domains closure. This model is currently somewhat disfavored, because viomycin, an antibiotic that greatly stabilizes tRNA binding in the A site (Peske et al., 2004), does not alter the initial power stroke (Chen et al., 2016) and because EF-G remains in contact with the A-site tRNA even after the interactions of the tRNA with the ribosome are disrupted (Zhou et al., 2019). Alternatively, EF-G may (indirectly) facilitate opening of the G1338-U1341 ridge between the P and E sites on the SSU, which would allow the P-site

tRNA to move and the A-site tRNA to follow (Schuwirth et al., 2005). G1338 and A1339 interact with P-site tRNA. Mutations of G1338 to C or U and of the neighboring A1339 confer a significant decrease in translation activity (Abdi and Fredrick, 2005), although the exact effect on translocation has not been examined. The impact of EF-G on other interactions, for example, on 16S rRNA C1397 and A1503, which can intercalate with mRNA bases (Zhou et al., 2013), is also conceivable.

After the initial power-stroke by EF-G, the tRNAs move into their E- and P-site positions on the SSU and the E-site tRNA moves towards exiting the ribosome (step 4) (Belardinelli et al., 2016a) (Figure 3). During these steps, domain 4 likely escorts the A-site peptidyl-tRNA into the P site. These motions can actively push the tRNA as a result of a rearrangement brought about by P_i release or proceed through a Brownian ratchet mechanism (Liu et al., 2014; Chen et al., 2016). The finding that translocation can proceed even if P_i release is impaired (Savelsbergh et al., 2005) argues in favor of a Brownian ratchet mechanism, rather than a second force-generation step. The role of EF-G at this step would be to act as a doorstop to prevent the tRNA from backward movement. Finally, in step 5 the SSU head completes its backward movement and the deacylated tRNA and EF-G-GDP leave the ribosome, thereby bringing to the end one elongation cycle and one round of EF-G action (Belardinelli et al., 2016a).

In summary, the roles of EF-G in translocation are the following:

- EF-G accelerates the formation of the rotated state of the ribosome and prevents it from returning to the non-rotated state. The binding can occur in the GTP or GDP-bound form and does not require GTP hydrolysis.
- After GTP hydrolysis, EF-G uncouples the motions of the SSU head and body domains from the movement of the tRNA-mRNA complex, which appears to exert a power stroke and accelerates translocation.
- After P_i release, EF-G helps the ribosome to complete translocation by either actively moving the tRNAs or as Brownian ratchet acting as a doorstop that prevents spontaneous backward tRNA movement.

This illustrates that the functional cycle of EF-G on the ribosome is very different from that of a canonical GTPase in that EF-G uses the energy of GTP hydrolysis for a power stroke-like motion acting on the ribosome and the tRNAs. It is tempting to compare EF-G to motor proteins, such as kinesin or myosin, which use the energy of ATP binding or hydrolysis to promote movement. There appear to be similarities between EF-G and myosin concerning the key role of the P_i-bound state (Sweeney and Houdusse, 2010; Geeves, 2016; Mijailovich et al., 2017). However, many aspects of EF-G function are either different from myosin or not clear enough to allow for an informed comparison. For example, in contrast to myosin, the free energy changes accompanying GTP hydrolysis and P_i release for EF-G are not known with certainty, which makes it difficult to understand how the energy is utilized. The transition from the ADP-P_i to the ADP state of myosin results in a large change in the free energy, which is the source of its ability to perform mechanical work. This appears different in EF-G, because the presumed force-generating unlocking step precedes and is independent of P_i release (Savelsbergh et al., 2005, 2009). Alternatively, EF-G can be compared to kinesin where ATP binding is the force-generation step (Cross, 2016). While this is not true for EF-G, a GTP-like GDP-P_i state appears to be the active conformation that generates movement (Savelsbergh et al., 2003). Finally, while in myosin or kinesin the power stroke is directly coupled to movement, in EF-G this coupling appears indirect through changing the dynamic conformational landscape of the ribosome. Understanding the exact timing and structural mechanism of force generation by EF-G is a challenging goal for future studies.

Reading frame maintenance

EF-G-catalyzed translocation occurs in precisely defined steps of one codon at a time. A larger or smaller step inevitably results in a change of the reading frame, which alters the sequence of the protein and results in production of faulty, non-functional polypeptides. The frequency of these spontaneous frameshifting events is very low, below 1 in 100 000 translated codons, and the question is how the translational machinery achieves such a high translocation fidelity.

First answers have come recently from biochemical and structural experiments. When the translating ribosome arrives at a so-called slippery sequence of the mRNA, i.e. where the same tRNA can read codons in both the 0- and the –1 frame, it can in fact slip into the –1-frame (Peng et al., 2019). This can happen if there is not enough EF-G to rapidly translocate the tRNAs. In contrast, when EF-G is present in excess, frameshifting is negligible. This suggests that the ribosome is inherently prone to frameshifting, and EF-G helps to maintain the correct reading frame (Peng et al., 2019). Crystal structures show how this is achieved: residues at the tip of domain 4 interact with the A-site tRNA and prevent its spontaneous movement towards the –1-frame when traversing from the A to the

P site (Zhou et al., 2014, 2019). In fact, mutations of the contact residues in EF-G (Q507 and H583) – which disrupts these interactions – increase frameshifting, in agreement with the structural data (Peng et al., 2019).

The detailed kinetic analysis reveals that in addition to the direct action on the A-site tRNA, EF-G controls frameshifting indirectly by coordinating and synchronizing motions within the translocating ribosome (Peng et al., 2019). Mutations of Q507 or H583 do not affect EF-G binding to the ribosome (step 1 in Figure 4) and facilitate the movement of the P-site tRNA to the E site and its release from the ribosome. This implies that the grip of the ribosome on the codon-anticodon complex is weakened, which may favor the slippage of the A-site peptidyl-tRNA into the –1-frame. The following steps 3–5 are very slow with mutant EF-G, which provides time for slippage before translocation is completed. For different amino acid substitutions at position Q507, the frameshifting efficiency strongly correlates with the rate of translocation. This correlation allows the estimation of the intrinsic rates of tRNA movement into the –1-frame, which turns out to be about 10 s^{-1} at a slippery sequence, compared to about 40 s^{-1} for tRNA movement promoted by wild-type EF-G (Peng et al., 2019). Thus, EF-G helps the tRNA to remain bound in the 0-frame not only by escorting it through the ribosome, but also by controlling the speed of translocation. With wild-type EF-G, translocation is rapid compared to slippage, whereas any pausing at a slippery sequence opens a kinetic time window during which the ribosome can slip into a different frame.

Pseudo-translocation during ribosome sliding over the mRNA

Although the ribosome usually moves along the mRNA one codon at a time, in special cases it can slide over

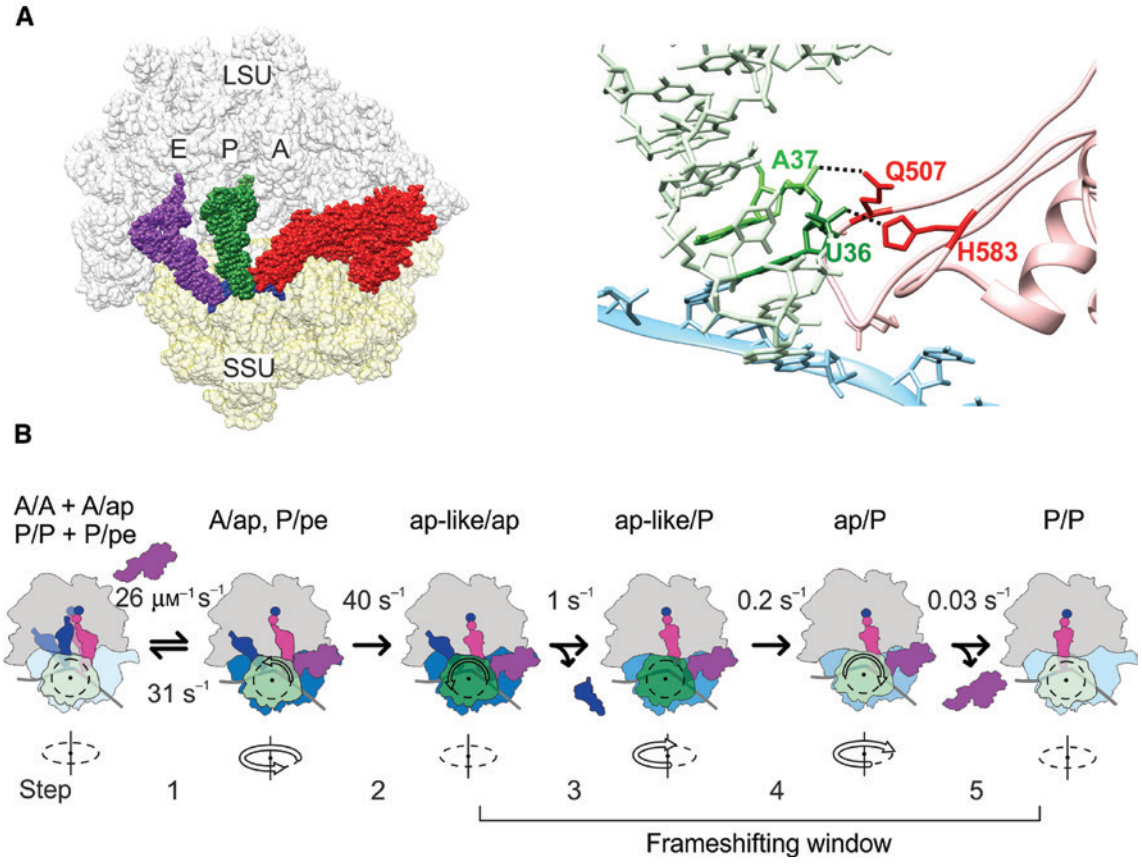

Figure 4: EF-G in reading frame maintenance.
(A) Left, structure of the post-translocation state with EF-G domain 4 interacting with the ASL of the A-site tRNA. Right, zoomed-in view of the interactions of residues Q507 and H583 of EF-G domain 4 with tRNA (corresponding to Q500 and H573 in the *Thermus thermophilus* EF-G structure). From PDB 4V5F (Gao et al., 2009). (B) Effect of Q507D mutation on the kinetics of translocation (compare to Figure 3). Steps 3–5 are very slow with mutant EF-G and the deacylated tRNA dissociates before the ribosomal subunits move towards their non-rotated state (Peng et al., 2019).

non-coding mRNA regions, for example, in the 3′ untranslated regions of eukaryotic mRNAs (Guydosh and Green, 2014; Miettinen and Bjorklund, 2015). The best-studied example of such sliding in bacteria is the bypassing of a non-coding gap during translation of the gene-60 mRNA of bacteriophage T4 (Huang et al., 1988; Samatova et al., 2014; Chen et al., 2015). The ribosome translates 46 codons from the 5′ open reading frame of the mRNA, then takes off and slides over 50 nt of a non-coding region to resume translation of the 3′ part of the open reading frame. Until recently, it was unclear why this takes place. Biochemical and single-molecule FRET studies now revealed that EF-G plays a key role in sliding (Klimova et al., 2019). Before taking off, the ribosome assumes a hyper-rotated conformation with the SSU and LSU rotated even further than in the canonical rotated state (Klimova et al., 2019). The unique feature of the take-off complex is a short mRNA stem-loop (SL) that forms in the A site (Agirrezabala et al., 2017) (Figure 5).

Bypassing is initiated by EF-G by facilitating a pseudo-translocation event in which the A-site SL mimics the tRNA (Klimova et al., 2019). This likely disrupts the codon-anticodon interactions of the P-site peptidyl-tRNA, but the nascent peptide anchors the tRNA to the LSU, thereby preventing premature drop-off (Samatova et al., 2014; Agirrezabala et al., 2017). The ribosome starts moving along the mRNA which is no longer constrained by interactions with the tRNA. The movement towards the 3′ end and landing is accompanied by multiple cycles of EF-G binding and GTP hydrolysis, as the kinetics of docking at the landing site is identical to the kinetics of GTP hydrolysis by EF-G (Klimova et al., 2019). On average, EF-G hydrolyzes 90 molecules of GTP to traverse the non-coding gap, or 1.8 molecules of GTP per nucleotide. The role of GTP hydrolysis during sliding is not entirely clear. It might help for the directionality of movement, akin to the doorstop function in canonical translocation, or for maintaining the rotated conformation of the ribosome required for sliding. Unlike in translocation, mutations of Q507 and H583 do not inhibit sliding (Klimova et al., 2019).

EF-G in ribosome recycling

After termination of protein synthesis, ribosomes in bacteria are recycled from post-termination complexes by the combined action of EF-G, ribosome recycling factor (RRF), and initiation factor 3 (IF3). RRF consists of two domains connected by a flexible linker (Selmer et al., 1999; Hirokawa et al., 2002). Its overall shape resembles that of tRNA; on the ribosome, it occupies the A site (Gao et al., 2005). This prompted initial suggestions that EF-G performs recycling by translocating RRF. However, kinetic experiments have shown that the mRNA does not translocate during recycling (Peske et al., 2005). Instead, EF-G together with RRF catalyze the dissociation of the ribosomal subunits (Figure 6). This reaction does not require IF3; rather, IF3 facilitates ribosome recycling by preventing re-association of the ribosomal subunits and by accelerating the dissociation of the tRNA from the SSU P site (Karimi et al., 1999; Peske et al., 2005). Time-resolved cryo-EM studies demonstrate that RRF and EF-G jointly attack the intersubunit bridge B2a, thereby promoting the dissociation of the ribosome into subunits (Fu et al., 2016).

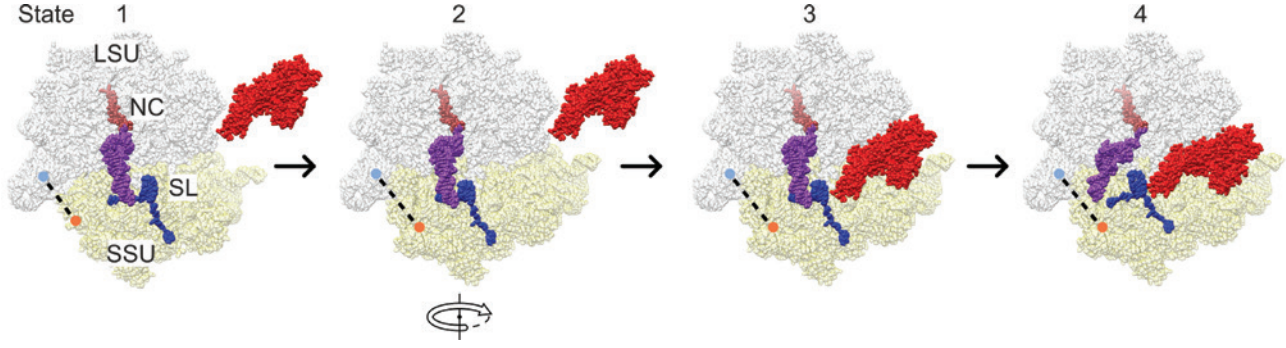

Figure 5: Model of pseudo-translocation catalyzed by EF-G to initiate ribosome sliding.
In state 1, ribosomes are in a non-rotated (rolled) state stalled at the take-off codon (Agirrezabala et al., 2017). The P-site tRNA is shown in magenta, the nascent-peptide chain (NC) in brown. The mRNA (blue) forms a short SL in the A site. The dashed line between arbitrary chosen points on the SSU (orange) and LSU (blue) serves to follow the degree of subunit rotation. Upon activation of sliding, the SSU hyper-rotates relative to LSU, which is indicated by the distance change between the two landmark points; the direction of rotation is indicated by an arrow (state 2). Binding of EF-G (state 3) and the subsequent pseudo-translocation on the SSU (to state 4) results in the loss of codon-anticodon interactions in the P site and initiates ribosome sliding along the mRNA. State 1 is from PDB 5NP6 (Agirrezabala et al., 2017), all other states are hypothetical, based on biochemical and single-molecule FRET experiments (Klimova et al., 2019).

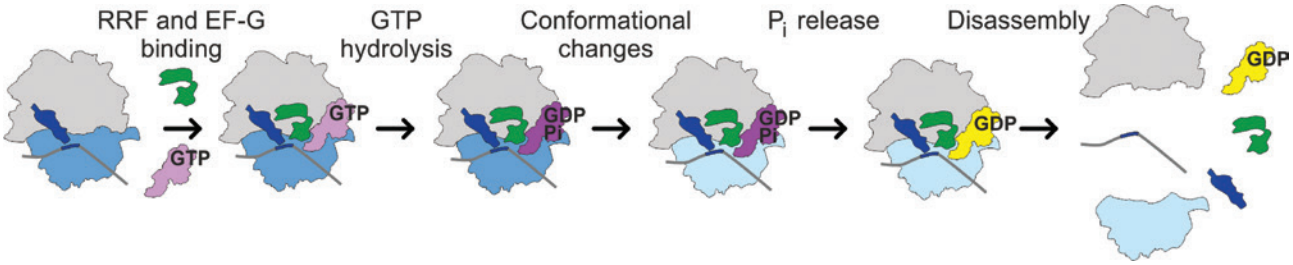

Figure 6: EF-G in ribosome recycling.
Post-termination complex is predominantly in the rotated state with the tRNA in a hybrid P/E state (Dunkle et al., 2011). Binding of RRF (green) and EF-G-GTP (lilac) forms a recycling complex. EF-G hydrolyzes GTP (indicated by dark lilac), which induces a conformational change of the SSU and RRF. Ribosome splitting into subunits occurs after (as shown) or concomitant with P_i release. Disassembly of the SSU-tRNA-mRNA complex is facilitated by IF3 (not shown).

As in translocation, EF-G domain 4 is important, but RRF contacts a different face of EF-G than the tRNA (Gao et al., 2007). The exact order of events, i.e. whether the tRNA and/or mRNA dissociates prior to or after subunit dissociation, remains controversial (Chen et al., 2017; Iwakura et al., 2017), presumably owing to the use of model mRNAs that may or may not represent an authentic post-termination mRNA context. However, as all experiments reported so far used model mRNAs, rather than natural post-termination complexes, in the following we refer to the model supported by high-resolution kinetic and structural studies (Karimi et al., 1999; Peske et al., 2005; Savelsbergh et al., 2009; Borg et al., 2016; Fu et al., 2016).

GTP hydrolysis by EF-G on post-termination complexes is rapid (75 s^{-1} at 4°C) and independent of the presence of RRF (Savelsbergh et al., 2009). Taking into account the temperature dependence, this rate is comparable to that of EF-G-dependent GTP hydrolysis on pre-translocation complexes (250 s^{-1} at 37°C); this supports the notion that the rate of GTP hydrolysis by EF-G is independent of the functional state of the ribosome (Rodnina et al., 1997). However, unlike translocation, which can slowly proceed with non-hydrolyzable GTP analogs, ribosome recycling is strictly dependent on GTP hydrolysis. P_i release from the EF-G-GDP-P_i complex is delayed relative to GTP hydrolysis during both translocation and ribosome recycling, but the rate of P_i release is 10 times slower on post-termination complexes with RRF than on pre-translocation complexes. Moreover, ribosome disassembly is inhibited by vanadate, an analog of P_i, indicating that ribosome recycling requires P_i release (Savelsbergh et al., 2009). This is again different in translocation, where the tRNA-mRNA movement *per se* does not depend on P_i release (Savelsbergh et al., 2005).

P_i release presumably induces a conformational change of EF-G that is essential for ribosome disassembly to take place. On the other hand, single-round P_i release is still faster than subunit dissociation [3 s^{-1} vs. 0.2–0.3 s^{-1} (Savelsbergh et al., 2009)]. In the simplest model, this suggests that GTP hydrolysis and P_i release precede subunit dissociation. Alternatively, not every attempt of EF-G to split the ribosome may be successful. In such a scenario, P_i release and subunit dissociation can take place simultaneously, but because some attempts are unproductive, ribosome splitting appears slow. The rate of ribosome disassembly depends on the concentration of magnesium (Mg^{2+}) ions, which prevent ribosome splitting by stabilizing intersubunit bridges (Selmer et al., 2006). At low Mg^{2+} concentration, where the rate of subunit dissociation is much faster, the stoichiometry of GTP hydrolysis by EF-G to subunit dissociation is very close to 1, but the ratio rapidly increases to >15 GTP molecules hydrolyzed per splitting event when the conditions become suboptimal (e.g. the ratio between RRF and EF-G) (Borg et al., 2016). A loose coupling between the energy-generating rearrangements of EF-G and the movements on the ribosome seems to represent the nature of the ribosome as a Brownian machine.

Conclusions

EF-G is a non-canonical GTPase with several distinct functions. Unlike canonical GTPases, GTP binding does not stabilize its switch 1 and 2 regions. Rather, the switch regions are stabilized by binding to the ribosome, which also activates GTP hydrolysis by EF-G. The resulting GDP-P_i conformation is the active form of the factor in which it performs its functions. The initial steps of the EF-G cycle – ribosome binding and GTP hydrolysis – take place regardless of the functional state of the ribosome. Subsequent steps are modulated by the ligand in the A site of the ribosome. With tRNA in the A site, EF-G promotes ribosome rearrangement leading to translocation. If an mRNA element occupies the A site instead of the tRNA,

EF-G initiates a pseudo-translocation event, whereas the presence of RRF re-routes the pathway towards subunit splitting. EF-G domain 4 plays a key role in coupling the GTPase cycle to motion, but the contacts of EF-G appear to be specific for the A-site ligand. After P_i release, EF-G switches into the GDP form and dissociates from the ribosome, similar to other GTPases where the formation of the GDP-bound state terminates function. The GTPase superfamily entails many very different proteins with versatile roles in the cell, and their evolution led to specification and adaptation of each GTPase to its particular function. Its role in facilitating movement led EF-G to evolve towards ATPases, while maintaining its core GTPase features. The growing understanding of how EF-G works provides insights into molecular motions in Brownian machines.

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