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Translation initiation without IF2-dependent GTP hydrolysis

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ABSTRACT

Translation initiation factor IF2 is a guanine nucleotide-binding protein. The free energy change associated with guanosine triphosphate hydrolase (GTPase) activity of these proteins is believed to be the driving force allowing them to perform their functions as molecular switches. We examined role and relevance of IF2 GTPase and demonstrate that an *Escherichia coli* IF2 mutant bearing a single amino acid substitution (E571K) in its 30S binding domain (IF2-G3) can perform *in vitro* all individual translation initiation functions of wild type (wt) IF2 and supports faithful messenger RNA translation, despite having a reduced affinity for the 30S subunit and being completely inactive in GTP hydrolysis. Furthermore, the corresponding GTPase-null mutant of *Bacillus stearothermophilus* (E424K) can replace *in vivo* wt IF2 allowing an *E. coli infB* null mutant to grow with almost wt duplication times. Following the E571K (and E424K) mutation, which likely disrupts hydrogen bonding between subdomains G2 and G3, IF2 acquires a guanosine diphosphate (GDP)-like conformation, no longer responsive to GTP binding thereby highlighting the importance of interdomain communication in IF2. Our data underlie the importance of GTP as an IF2 ligand in the early initiation steps and the dispensability of the free energy generated by the IF2 GTPase in the late events of the translation initiation pathway.

INTRODUCTION

Translational initiation factor IF2, an essential bacterial protein (1), belongs to a family of universally conserved P-loop GTPases together with elongation factors EF-Tu and EF-G (2). However, aside from their overlapping ribosomal localization and some structural similarity, these proteins differ in their affinity for GTP and guanosine diphosphate (GDP), requirement for a nucleotide exchange factor, mechanism of guanosine triphosphate hydrolase (GTPase) control and activation, timing of Pi release, functional role of GTP hydrolysis (3).

The activity of IF2 during the early events of translation initiation is modulated by its guanine nucleotide ligand; IF2-GTP has a higher affinity for the 30S ribosomal subunit than IF2-GDP (4,5) and docking of the 50S ribosomal subunit to the 30S initiation complex (30S IC) is faster and more efficient in the presence of GTP (6–8). Furthermore, binding in place of GTP of the alarmone ppGpp, whose level increases under unfavorable metabolic conditions, hinders IF2 functions, resulting in feedback inhibition of translation initiation (9,10).

However, less clear is the role of the IF2-dependent GTP hydrolysis ensuing during the transition from 30S IC to 70S IC (11,12 and references therein). X-ray crystallography of eIF5B, the archaeal IF2 homolog and nuclear magnetic resonance spectroscopy of bacterial IF2-G2 (the guanine nucleotide binding domain of IF2) indicate that GTP hydrolysis induces or is accompanied by a large conformational change (13,14), whereas cryo-electron microscopy reconstructions show that the corresponding GTP and GDP forms of IF2 represent intermediate states of the translation initiation pathway (15–17).

Traditionally, GTP hydrolysis was considered necessary to drive two non-mutually exclusive functions: the IF2-dependent adjustment of the initiator tRNA in the P-site (e.g. 18,19) and IF2 recycling off the ribosomes (e.g. 20,21). Substitutions of H448 and H301 in the IF2-G2 domain of the *Escherichia coli* (22) and *Bacillus stearothermophilus* (23,24) IF2, respectively, result in the loss of the GTPase activity and give rise to dominant

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lethal phenotypes, which have been attributed to a failure of the factor to dissociate from the ribosomes (22). However, because mutations in the C-terminal domain of the factor (IF2-C2), which strongly reduce the IF2 affinity for fMet-tRNA, can suppress the lethality caused by the GTPase inactivation, an alternative interpretation was offered to explain this lethal phenotype (25). According to this hypothesis, the presence of the GTPγ-phosphate prevents the conformational transition of IF2, which allows the dissociation of the interaction between IF2 and the acceptor end of fMet-tRNA, which keeps the factor anchored to the 70S ribosome and prevents initiation dipeptide formation (25). Furthermore, a strict GTP requirement for the adjustment of fMet-tRNA in the P-site was observed only when fMet-tRNA binding was not directed by a template occupying the ribosomal messenger RNA (mRNA) channel (26). Similar conclusions were also reached following fast kinetic analyses, which showed that dipeptide formation occurs also in the absence of GTP hydrolysis (i.e. in the absence of any guanine ligand or in the presence of GDP). In addition, 70S A-site binding of an EF-Tu-aminoacyl-tRNA-GTP complex was neither hindered nor slowed down in the presence of IF2-GDP or of ligand-free IF2. In light of the overlapping binding sites of IF2 and EF-Tu on the ribosome, these findings suggested that ribosome clearance of IF2 does not require IF2-dependent GTP hydrolysis (11,25). However, at variance with these results, Antoun *et al*. (27) reported that in the presence of GTP the IF2-dependent formation of the first peptide bond is ~2 orders of magnitude faster than in the presence of GDP or 5′-guanylyl-imidodiphosphate (GDPNP) and concluded that GTP hydrolysis is strictly necessary to accelerate the recycling of IF2 from the 70S ribosome and for initiation dipeptide formation. More recently, it was also reported that GTP hydrolysis by IF2 drives the ribosomes to acquire an elongation-competent conformation and commits them to enter the elongation cycle, whereas blocking the GTPase yields 70S complexes unable to enter elongation (28).

To evaluate role and importance of the GTPase of IF2, here we have examined the properties of two mutants (one in *E. coli* and one in *B. stearothermophilus* IF2), which are completely inactive in GTP hydrolysis as a result of equivalent single amino acid substitutions. Our results demonstrate that these IF2 mutants can perform all functions of wild type (wt) IF2 *in vitro* and allow near-normal growth of cells lacking wt IF2. We conclude that the free energy generated by IF2-dependent GTP hydrolysis is dispensable both *in vitro* and *in vivo* and suggest that the IF2-fMet-tRNA interaction has an important role in modulating the affinity of IF2 for the ribosome, similar to that played by the aIF5B-eIF1A interaction in eukaryotes.

MATERIALS AND METHODS

Binding of mant-GTP/GDP to IF2, the kinetic analysis of 30S IC-50S ribosomal subunit association and IF2-dependent i) binding of fMet-tRNA to the 30S ribosomal subunits; ii) GTPase activity; iii) initiation dipeptide formation and iv) mRNA translation *in vitro* are described in detail in 'supplementary information'. *In vivo* complementations were performed essentially as described (24) using an *E. coli* BL21strain whose chromosomal *infB* gene had been inactivated by insertion of a kanamycin cassette and whose viability is ensured by the *in trans* expression of an *infB* gene carried by pMAK705, a plasmid bearing a thermosensitive origin of replication. This strain was transformed with a pGEX plasmid vector carrying *B. stearothermophilus* wt or E424K *infB* under control of the *lac* promoter; because transcription from this promoter is leaky, no isopropyl β-D-1-thiogalactopyranoside (IPTG) induction is necessary for the expression of these genes. After incubation at 42°C to inhibit the replication of pMAK705 containing *E. coli infB*, surviving transformants were selected to isolate cells in which *B. stearothermophilus* wt IF2 or IF2 E424K had replaced the wt factor of *E. coli*. Several colonies able to grow at 42°C and able to withstand several passages at this non-permissive temperature were isolated and analyzed. The presence of the pGEX carrying *B. stearothermophilus infB* and of the kanamycin cassette interrupting the chromosomal *infB* gene as well as the absence of pMAK705 were verified by PCR amplification using appropriate primers (Figure S2). The presence of *B. stearothermophilus* IF2 E424K and the absence of *E. coli* IF2 were confirmed by Western blot analyses using monoclonal antibodies directed against *B. stearothermophilus* or *E. coli* IF2, respectively (Figure 6C).

RESULTS

Lethality resulting from a GTPase-inactivating mutation in IF2-G2 is suppressed by E571K mutation in IF2-G3

As mentioned previously, selection for intragenic suppressors of dominant lethality due to the loss of the GTPase activity of IF2 resulting from His residue mutations (H448 in *E. coli* and H301 in *B. stearothermophilus*) yielded a large number of C-terminal domain (IF2-C2) mutants displaying a 7–10-fold reduced affinity for fMet-tRNA (23–25). To obtain intragenic suppressors affected in other parts of the molecule, we subjected *B. stearothermophilus infB* to random mutagenesis, targeting only the regions encoding IF2-G2 and IF2-G3, the domains responsible for the IF2-ribosome interaction (15–17). The cells capable of surviving the over-expression *in trans* of the GTPase-deficient IF2 H301Y mutant were selected and three lethality-suppressor mutations were identified and characterized. All three displayed a reduced affinity for the ribosomal subunits: two of them (S387P and E424K) for the 30S subunit and the other (G420E) for both subunits (24). In particular, our attention was attracted by a remarkable feature of the E424K mutant in the IF2-G3 domain; in spite of being totally inactive in GTP hydrolysis, it appeared to be able to support initiation dipeptide formation, an IF2 activity considered to be IF2 GTPase-dependent, at least according to the literature (27).

Thus, in this study, we introduced in *E. coli* the mutation, which corresponds to the E424K substitution

in *B. stearothermophilus*; a G was changed to A in *E. coli infB* to yield an E571K substitution both in wt IF2 and in the IF2 carrying the dominant-lethal H448S substitution. In this way we obtained a single (E571K) and a double (H448S/E571K) mutant of IF2. As expected, the expression of the H448S mutant causes a rapid growth arrest (Figure 1A) and a reduction in the number of viable cells by 3 or 4 orders of magnitude within 20–30 min after induction (Figure S1 in Supplementary information). In contrast, induction of the GTPase-deficient double mutant (H448S/E571K) affects neither the growth of the cells (Figure 1A) nor the viable counts (Figure S1 in Supplementary information), demonstrating that the E571K mutation is capable of suppressing lethality. Also the cells expressing IF2 bearing the E571K single mutation continued to grow like those expressing wt IF2 (Figure 1A) and without any reduction in the viable counts (Figure S1 in Supplementary information).

The IF2 E571K mutant has a reduced affinity for the 30S subunit and no GTPase activity

The *in vitro* phenotypes of the IF2 E571 mutant were analyzed in individual reactions of the translation initiation pathway. Compared with wt IF2, the mutant displays a reduced (~6.5-fold) affinity for the small ribosomal subunit (Figure 1B), and wt affinity for the 50S ribosomal subunit (not shown). These findings are consistent with the location of the E571K substitution in IF2-G3, the 30S binding domain of IF2 (Figure 1C and D).

In addition to its reduced affinity for the 30S subunit, the IF2 E571K mutant was completely inactive in ribosome-dependent GTP hydrolysis, even when offered in excess amounts (Figure 2A) or under long time of incubation (Figure 2B). Because a possible reason for this defect could be a failure in substrate binding, the kinetics of mant-GTP and mant-GDP interaction with wt and mutant IF2 were analyzed by fluorescence stopped-flow experiments. As seen from Figure 3A, the fluorescence intensity of both mant-nucleotides increases rapidly upon their mixing with either type of IF2 and in both cases the binding kinetics reflected a single step interaction mechanism, the resulting tracings of the fluorescence changes being fitted to a single exponential function. However, the fluorescence amplitude of mant-GTP increases somewhat more with wt IF2 than with the mutant protein, whereas the opposite occurs with mant-GDP (Figure 3A and B). This finding suggests that the two types of IF2 molecules may have somewhat different affinities for the two nucleotides and/or that the environments in which the mant-nucleotides are bound are somewhat different, a hypothesis, which could account for the failure of IF2 E571K to hydrolyze GTP. The k_{app} of binding shows a linear dependence on the mant-nucleotides concentration and the k_{on} values calculated from the slopes of the linear regressions are very similar for wt and mutant IF2 for the binding of both GTP ($k_{1wt} = 0.22\,\mu M^{-1}\,s^{-1}$ and $k_{1mut} = 0.17\,\mu M^{-1}\,s^{-1}$) and GDP ($k_{1wt} = 1.49\,\mu M^{-1}\,s^{-1}$ and $k_{1mut} = 1.80\,\mu M^{-1}\,s^{-1}$). The differences are slightly larger for the k_{off}, which are $k_{-1\,wt} = 6.7\,s^{-1}$ and $k_{-1mut} = 14.5\,s^{-1}$ for GTP and $k_{-1wt} = 12.2\,s^{-1}$ and $k_{-1mut} = 8.5\,s^{-1}$ for GDP (Figure 3C). The affinities calculated as k_{off}/k_{on} or from the hyperbolic fitting of the amplitude dependencies are $K_{dwt} = 30\,\mu M$, $K_{dmut} = 85\,\mu M$ for GTP and $K_{dwt} = 8\,\mu M$, $K_{dmut} = 4\,\mu M$ for GDP. Overall, these data indicate, or confirm in the case of wt IF2 (4), the higher affinity of the factor for GDP than for GTP and also show that affinity for GTP of wt IF2 is ~3-fold higher than that of the mutant; the opposite is true for GDP for which the E571K mutant has ~ 2-fold higher affinity than the wt IF2. Thus, at equal molar concentrations the mutation in IF2 would increase by ~6-fold the preferential binding to GDP compared with GTP.

The IF2 E571K mutant is active in 30S IC and initiation dipeptide formation

The activities of wt and mutant IF2 in promoting the binding of fMet-tRNA to mRNA-programmed 30S ribosomal subunits to yield a 30S IC were compared in two types of experiments that measure fMet-tRNA binding before (Figure 4A) and after (Figure 4B) the conformational change responsible for its adjustment (locking) in the P-site (Figure 4C) (29). In the first case, the fluorescence resonance energy transfer (FRET) signal generated by the proximity between 30S-bound $IF3_{166Atto555}$ (acceptor) and fluorescein-labeled (at position 8) fMet-tRNA (donor) was followed by fluorescence stopped-flow analysis. As seen from Figure 4A, the fluorescence intensity increases with the same k_{obs} with both wt and mutant IF2, but reaches a ~30% lower level with the mutant compared with the wt factor (Figure 4A).

On the other hand, in the presence of wt IF2 the level of fMet-tRNA bound in the 'locked' 30S IC in the presence of wt IF2 depends on the nature of the factor's ligand, being highest with GTP, lowest with GDP and intermediate with the GDPCP, as expected (4) (Figure 4B). In contrast, aside from being ~60% lower than that obtained with wt IF2, the level of 30S IC formation promoted by IF2 E571K is barely influenced by the nature of the guanine nucleotide present, being similar in all cases to that attained with wt IF2 in the presence of GDP (Figure 4B).

Wild-type and mutant IF2 were also directly compared for their capacity to stimulate initiation dipeptide formation (fMet-Phe in this case) and to support overall mRNA translation, two activities regarded as being dependent upon the GTPase of IF2 (27). In spite of its failure to hydrolyze GTP, the IF2 E571K proved to be active in both functions (Figure 5A). The somewhat reduced activity displayed by the mutant in these two tests cannot be attributed to its lack of GTPase activity because the same reduction in activity compared with wt IF2 was observed also in fMet-tRNA binding (Figure 4A,B), a translation initiation step in which GTP hydrolysis is not involved. Indeed, when directly compared with one another, 30S IC formation, initiation dipeptide formation and protein synthesis as a whole yield almost identical IF2 E571K-dependence curves and reach the same plateaus at a level ~35% that obtained in the presence of wt IF2 (Figure 5A).

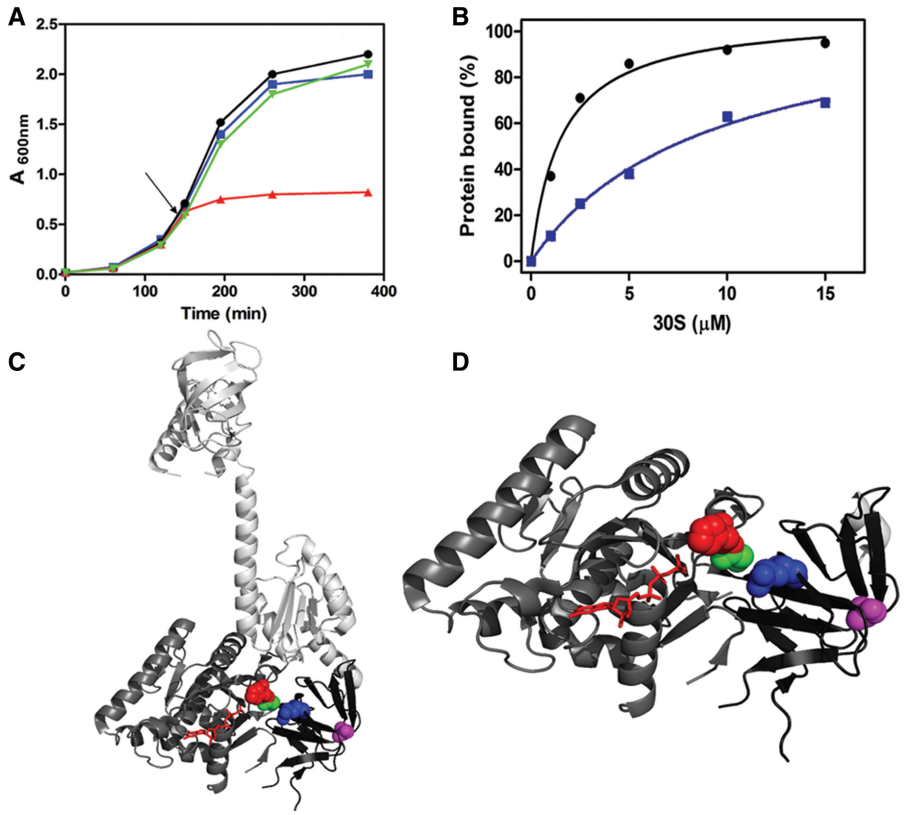

Figure 1. Over-expression and 30S binding of wt and mutant *E. coli* IF2. (**A**) Growth of *E. coli* BL21 cells bearing the pETM11 expression vector encoding wt IF2 (black), IF2 H448S mutant (red), IF2 H448S/E571K double mutant (green) and IF2 E571K mutant (blue); the arrow indicates the time of induction by IPTG; (**B**) binding of *E. coli* wt IF2 (black) and IF2 E571K mutant (blue) to homologous 30S ribosomal subunits; (**C**) 3D structure of the G2 (gray), G3 (dark gray) and C1-C2 (light gray) domains of IF2 based on the structure of its archaeal homolog aIF5B (13); the amino acids mutagenized or mentioned in Discussion are shown as space-filling structures and indicated as follows with the numbers corresponding to *E. coli*/*B. stearothermophilus*: IF2H448/H301 (red), E571/E424 (blue), G447/G300 (green). The position corresponding to A709 of *Saccharomyces cerevisiae* eIF5B is shown in magenta, whereas bound GTP is shown as a red stick model. (**D**) Enlargement of the G2–G3 domains of IF2 showing the location of the mutated residues and GTP. The color code is the same as in panel (C).

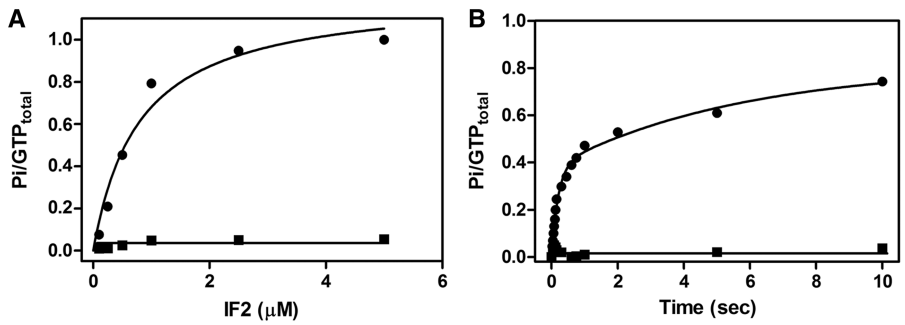

Figure 2. Ribosome-dependent GTPase activity of wt and mutant IF2. GTPase activity of (•) wt IF2 and (■) IF2E571K (**A**) measured manually after 5-min incubation at 37°C in the presence of ligand-free 70S ribosomes as a function of IF2 concentration and (**B**) measured by quenched flow as a function of time after mixing 50S ribosomal subunits with complete 30S IC. Experimental details can be found in Supplementary information.

Comparison of the kinetics of 30S IC-50S docking and initiation dipeptide formation reveals that in the presence of wt IF2, subunit association is ~10 times faster than dipeptide formation ($k_{app} = 10\,s^{-1}$ versus $1\,s^{-1}$), whereas with the IF2 E571K the two reactions proceed at approximately the same rate ($k_{app} = 0.3\,s^{-1}$ and $0.2\,s^{-1}$); this finding suggests that 30 S IC-50S docking has become rate-limiting for dipeptide formation in the presence of the mutant (Figure 5B). Because in the initiation pathway fMet-tRNA binding to the 30S subunit and subunit association precede both dipeptide formation and mRNA translation as a whole, it can be surmised that the diminished activities of the mutant compared with wt IF2 are not due to the GTPase defect but stem

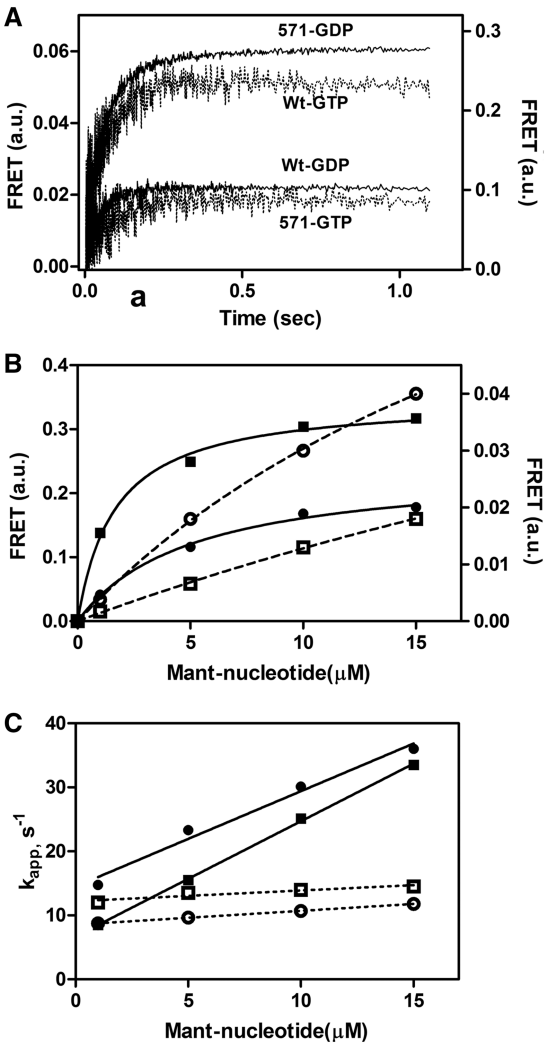

Figure 3. Binding of Mant-GTP and Mant-GDP to wt and mutant IF2. (**A**) Kinetics of Mant-GTP (dotted tracing) and Mant-GDP (continuous tracing) binding to wtIF2 and IF2 E571K mutant (as indicated in the figure) analyzed by fluorescence stopped-flow using as observable the FRET signal generated by exciting the intrinsic Trp residue of the factor; (**B**) FRET fluorescence amplitude obtained upon binding Mant-GTP (dotted line) and Mant-GDP (continuous line) to wt IF2 (○, •) and IF2 E571K mutant (□, ■) as a function of increasing concentrations of the nucleotides; (**C**) variation of the k_{app} of Mant-GTP (dotted line) and Mant-GDP (continuous line) binding to wt IF2 (○, •) and IF2 E571K mutant (□, ■) as a function of increasing concentrations of the nucleotides. Experimental details can be found in Supplementary information.

from its reduced affinity for the 30S ribosomal subunit and possibly from the slower 30S IC-50S association.

The IF2 E571K mutant supports faithful mRNA translation

The standard method to measure *in vitro* translation by quantifying the incorporation of a radioactive amino acid precursor into an acid-insoluble product (Figure 5A) does not distinguish between faithful and non-faithful translation. Because IF2 plays an important role in determining translational fidelity by selectively recognizing fMet-tRNA and kinetically favoring its binding to 30S subunits (30–32), it seemed important to ascertain if the GTPase-defective mutant of IF2 is still able to ensure a correct translational start. Thus, an Enzyme-linked immunosorbent assay with monoclonal antibodies directed against the protein encoded by the mRNA template was used to determine not only the level but also the nature of the translation product. The results obtained demonstrate that IF2 E571K-dependent protein synthesis initiates faithfully, yielding the expected product with an efficiency of ~50% that obtained with wtIF2 (Figure 5C).

A GTPase-defective IF2 mutant supports the growth of cells lacking wt IF2

The data presented so far demonstrate that the IF2 E571K mutant is capable of promoting all activities performed by wt IF2, at least *in vitro*. To determine if an IF2 GTPase-defective mutant can also substitute wt IF2 *in vivo*, we used an *E. coli infB* null strain whose survival depends on the presence of wt IF2 constitutively expressed *in trans* from a plasmid with a thermosensitive origin of replication (24,33,34). Because *B. stearothermophilus* IF2 can replace *E. coli* IF2 *in vivo* (24), in these experiments we used the *B. stearothermophilus* IF2 E424K mutant that corresponds to the *E. coli* E571K mutant and displays an identical phenotype, including complete loss of GTPase activity (Figure S2 in Supplementary information). This choice was made because DNA sequence differences between the two bacterial *infB* genes and the availability of species-specific anti-IF2 monoclonal antibodies allow the easy detection of the two *infB* genes and of their products in the cells.

Thus, the aforementioned *E. coli* strain was transformed with a second plasmid carrying *B. stearothermophilus infB* gene (either wt or E424K mutant) under the control of a leaky *lac* promoter. Colonies that had lost the vector encoding *E. coli* wt IF2 and produced exclusively *B. stearothermophilus* IF2, either wt IF2 or E424K mutant were then isolated by a series of passages at the non-permissive temperature. The *E. coli* cells expressing *B. stearothermophilus* but no *E. coli* IF2 were found to grow at 37°C with nearly the same generation time, regardless of having a wt IF2 (52 ± 5 min) or the GTPase-defective IF2 mutant (75 ± 8 min) (Figure 6A). The cells expressing the mutant factor are able to grow also at lower temperature (30°C), albeit more slowly than those expressing wt IF2 (69 ± 9 min versus 147 ± 15 min) (Figure 6B). However, at even lower temperature (≤25°C) the difference becomes larger, indicating that the GTPase-defective IF2 confers a cold-sensitive phenotype to the cells.

It was reported that a >2-fold reduction in the cellular level of IF2 severely affects the growth rate and that a complete growth arrest occurs upon a 5–10-fold IF2 deprivation (1). However, our results demonstrate that not even a trace amount of wt IF2 is present in the test cells. In fact, PCR analysis using species-specific primers clearly indicated that these *E. coli* cells contain only the heterologous *infB* gene (Figure S3 in Supplementary information), whereas immunological analyses demonstrated the exclusive presence of *E. coli* IF2 in the control cells (Figure 6C, upper panel) and the exclusive presence of

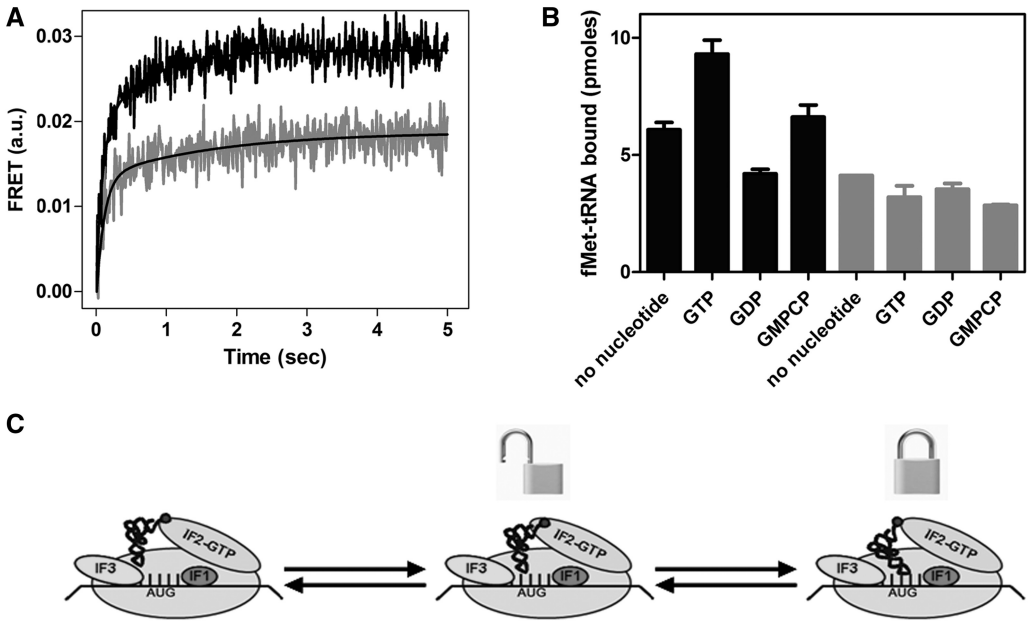

Figure 4. IF2-dependent formation of 30S IC. (**A**) Kinetics of fMet-tRNA binding to 022 mRNA-programmed 30S ribosomal subunits in the presence of wtIF2 (black tracing) and IF2 E571K mutant (gray tracing) as measured by fluorescence stopped-flow using the FRET signal between fluorescein-labeled initiator tRNA and Alexa555-labeled IF3. (**B**) Levels of fMet-tRNA bound at equilibrium to 022 mRNA-programmed 30S ribosomal subunits in the presence of wt IF2 (black bars) and IF2 E571K mutant (gray bars) in the presence or absence of the guanine nucleotides as indicated. Binding was measured by nitrocellulose filtration. (**C**) Schematic representation of the steps involved in the formation of the 30S IC. The first of the two steps was determined by fluorescence stopped-flow analysis, whereas the locked 30S IC were detected by nitrocellulose filtration. Experimental details can be found in Supplementary information.

B. stearothermophilus IF2 (wt or mutant) in the test cells (Figure 6C, lower panel).

Taken together, these results demonstrate that the *E. coli* cells expressing exclusively a mutant IF2 molecule incapable of hydrolyzing GTP can grow almost normally, at least under optimal conditions, and allow us to conclude that also *in vivo* the free energy generated by the IF2-dependent GTPase is not necessary.

DISCUSSION

To determine whether in bacteria translation initiation could function efficiently in the absence of the free energy generated by the IF2-dependent hydrolysis of GTP, the properties of two structurally and functionally equivalent IF2 mutants carrying a single amino acid substitution (E571K and E424K in *E. coli* and *B. stearothermophilus*, respectively) both totally inactive in GTP hydrolysis (Figure 2A and B, S2) were investigated. Both mutants were identified by selecting suppressors of the dominant lethal phenotype caused by the expression of IF2 H448S and IF2 H301Y mutants, respectively (Figure 1, S1).

The *E. coli* IF2 E571K mutant was studied *in vitro,* whereas the IF2 E424K mutant of *B. stearothermophilus* was used for the *in vivo* experiment in *E. coli*, because immunological tests relying on species-specific monoclonal antibodies can selectively distinguish this factor from its enterobacterial homolog (Figure 6C).

The E571K mutation causes a complete loss of the GTPase activity without preventing GTP binding (Figure 2) and increases the affinity for GDP (Figure 3). At variance with the GTPase-defective H448S mutant (22), the E571K mutant does not produce any growth defect or lethality (Figure 1A) giving a first indication that IF2 GTPase-defective mutants are not necessarily toxic. The different phenotypes of the H448S and E571K mutants, which have in common the inactivation of the GTPase, can be explained by the fact that both are conformational mutants (unpublished data from our laboratory) but that the conformations resulting from the amino acid substitutions are different and yield proteins with functionally different characteristics. Furthermore, the IF2 E571K mutant was shown to support *in vitro* all the partial reactions of translation initiation, as well as faithful mRNA translation (Figures 4 and 5). The somewhat reduced activity of the E571K mutant compared with wt IF2 (35–50%) is unlikely due to its failure to hydrolyze GTP because it can be entirely accounted for by its GDP-like conformation, which lowers its affinity for the small ribosomal subunit; the higher concentrations of the mutant factor (compared with wt) required to elicit the maximum activity (e.g. Figure 5C) are likely necessary to overcome this defect.

The data presented here also demonstrate that a *B. stearothermophilus* IF2 E424K mutant, corresponding in both structure and phenotype to *E. coli* E571K, can support *in trans* the growth of an *E. coli infB* null mutant. In LB medium, at 37°C the doubling time of the cells expressing exclusively the GTPase-inactive IF2 mutant is only slightly longer (75 versus 52 min) than those expressing wt IF2 (Figure 6A); however, the

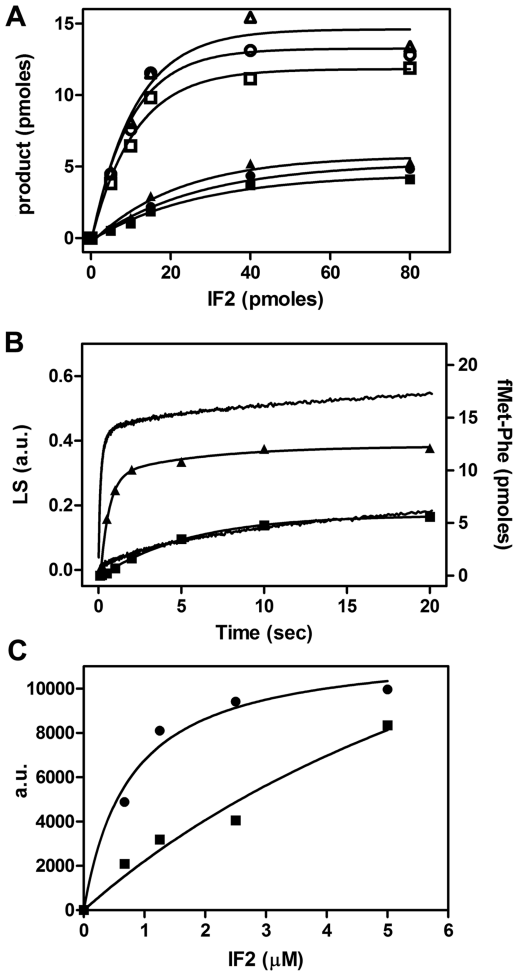

Figure 5. *In vitro* activities of wt and mutant IF2. (**A**) IF2-dependent formation of 30S IC (○, ●), fMet-Phe initiation dipeptide formation (□, ■) and 022mRNA translation (△, ▲) in the presence of increasing amounts of wt IF2 (open symbols) and IF2 E571K mutant (closed symbols); (**B**) Kinetics of 50S docking to 30S IC (measured by stopped flow light scattering) and fMet-Phe initiation dipeptide formation (measured by quenched flow) in the presence of wt IF2 (▲ and continuous tracing) and IF2 E571K mutant (■ and dotted tracing); (**C**) Translation of 027IF2Cp(**A**) mRNA in the presence of increasing concentrations of wtIF2 (●) and IF2 E571K mutant (■). The product synthesized after 30-min incubation at 37°C was quantified by ELISA using monoclonal antibodies directed against the protein encoded by the mRNA. Experimental details can be found in Supplementary information.

B. stearothermophilus IF2 mutant confers a cold-sensitive phenotype with longer generation times at lower temperature (Figure 6B).

Overall, the present findings underlie the importance of the structure of GTP-bound IF2 for the translation initiation steps occurring before GTP hydrolysis, such as the interaction with the 30S subunit, the recruitment of fMet-tRNA to the 30S subunit, the transition from 'unlocked' to 'locked' 30S IC and the docking of the 50S subunit to the 30S IC (Figure 4). However, our results demonstrate that even without the free energy generated by IF2-dependent hydrolysis of GTP, protein synthesis can proceed normally and faithfully, both *in vitro* and *in vivo*, despite the fact that the IF2 contribution to translational fidelity (30–32) was reported to be sensitive to the nature of its ligand (GTP, GDP or none) (35).

An approach similar to that applied by us in studying the function of the GTPase of bacterial IF2 has been used in the case of yeast aIF5B (36,37). As in our case, the authors reasoned that if GTPase inactivation causes aIF5B/IF2 to remain ribosome-bound, mutations causing a weakening of the factor-ribosome interaction should suppress the defects caused by the loss of the GTPase activity. Indeed, suppressor mutants having these expected properties were found in yeast (36,37) and in both *B. stearothermophilus* (24) and *E. coli* (present results). Nevertheless, some important structural and functional differences between the yeast and the bacterial system should be mentioned.

First of all, the long α-helix (H12) of eIF5B, which acts as a rigid lever causing the C-terminal domain IV to swing upon GTP hydrolysis (13), is not continuous in bacterial IF2 so that the corresponding domain (IF2-C2) tumbles freely and independently from the rest of the molecule (14). Furthermore, eukaryotic eIF5B and eIF1A directly interact with each other; the C-terminus of eIF1A docks eIF5B to the 48S complex, whereas GTP hydrolysis by eIF5B allows the dissociation of both eIF5B and eIF1A from the ribosome (38).

The inactivation of the GTPase of eIF5B causes slow growth but not lethality in yeast and the identified suppressor mutations weaken the eIF5B–40S interaction, either directly, disrupting the contact between helix h5 of 18S ribosomal RNA and A709 of the factor (Figure 1C and D), or indirectly by targeting eIF1A and weakening the eIF1A-eIF5B interaction (36–38).

In contrast, the bacterial phenotype resulting from the expression of GTPase-inactive IF2 mutants is much more severe, causing cell lethality and the intragenic suppressors identified so far have a 7–10-fold lower affinity either for the ribosomal subunits (Figure 1B) or for the acceptor end of fMet-tRNA (23, 25 and unpublished results). Furthermore, unlike domain IV of eIF5B, which binds eIF1A, IF2-C2 does not contain the C-terminal helices H13 and H14 responsible for the eIF1A–eIF5B interaction and binds instead to the initiator tRNA; also doubtful is the occurrence of an IF2-IF1 contact on the ribosome (39). Finally, although the eukaryotic suppressor mutations affect directly the ribosomal binding of eIF5B but do not affect its GTPase activity, the E571K and E424K likely represent 'conformational' mutants, which cause the complete inactivation of the GTPase activity of IF2 and affect indirectly its interaction with the ribosome.

In conclusion, in light of the present results and of what is known from the literature, it is tempting to draw a parallel between the eukaryotic aIF5B–eIF1A and the bacterial IF2-fMet-tRNA interplay in modulating, together with the guanine nucleotide ligand, the ribosomal affinity of the respective initiation factors.

As to the molecular basis of the phenotypes displayed by the E571K and E424K mutants, this can be traced back to a structural alteration caused by these substitutions (in G3), which are expected to prevent hydrogen bonding with the backbone amide of G447 or G300

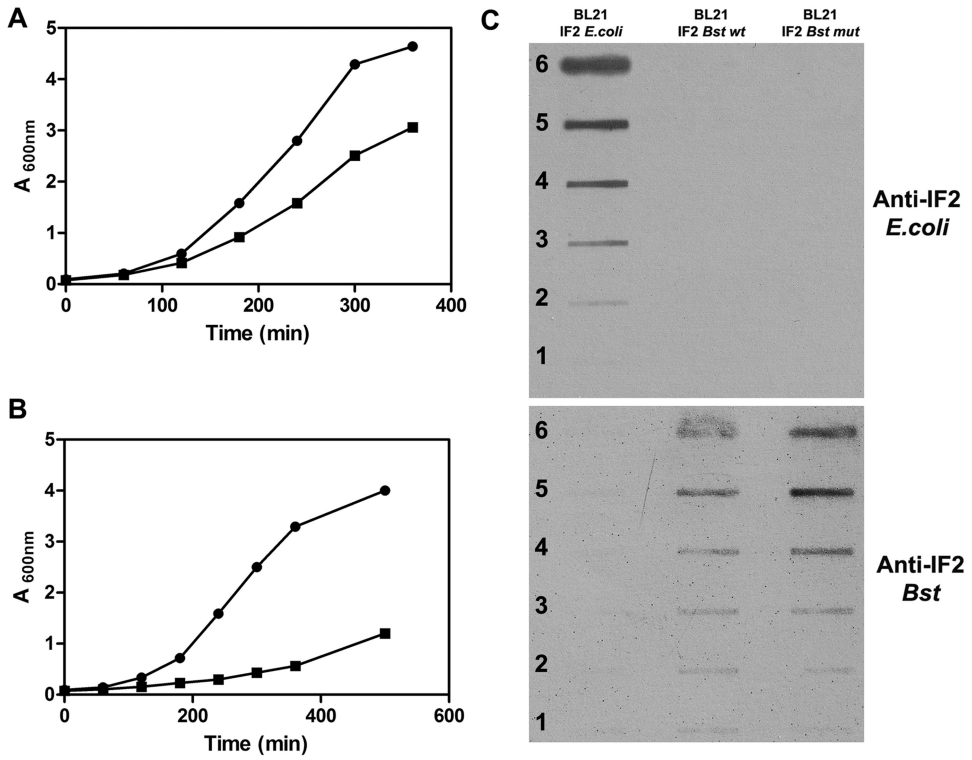

Figure 6. The GTPase-defective IF2 mutant supports growth of cells devoid of wt IF2. Growth curves in LB of *E. coli* cells in which the homologous wt IF2 is replaced by *B. stearothermophilus* (•) wt IF2 and (■) GTPase-defective IF2 E424K mutant at (**A**) 37°C and (**B**) 30°C; (**C**) immunological detection by slot-blot analysis of IF2 in extracts of *E. coli* with an inactivated chromosomal *infB* gene expressing in trans either *E. coli* IF2, *B. stearothermophilus* wtIF2 or IF2 E424K mutant as indicated. The extracts were obtained from saturated cultures, and the individual slots were loaded with increasing amounts of the corresponding extracts (from bottom to top, slots 1–6 contain 0.6, 1.2, 2.5, 5, 10 and 20 μg of total protein). The slot-blotted filters were exposed to monoclonal antibodies directed against *E. coli* (upper filter) and *B. stearothermophilus* (lower filter) IF2.

(in G2) (Figure 1C and D), thereby causing the disruption of the correct G2–G3 interdomain communication and likely constraining the factor into a GDP-like conformation. In turn, this structural alteration likely modifies the position of H448 or H301, whose correct spatial orientation is critical for GTP hydrolysis (40) and inactivates the GTPase activity. In fact, although capable of GTP binding, the mutants have a ~6-fold increased affinity for GDP compared with wt IF2 (Figure 3) and, unlike the latter, are no longer functionally responsive to the stimulation of GTP and GDPNP (Figure 4B). A GDP-like conformation is also likely responsible for the reduced affinity of the factor for the 30 S subunit (Figure 1B), for its reduced capacity to form a 'locked' 30S IC (Figure 4B and C) and for the slower rate of 30S IC-50S subunit docking (Figure 5B). Also the 35–50% activity reduction of the mutant in promoting initiation dipeptide formation and mRNA translation (Figure 5) likely stem from these conformationally induced defects.

From the affinity of IF2 for GTP (4) and from the cellular concentration of this nucleotide under optimal nutritional conditions (9), it can be predicted that in the cell the factor exists mainly in its GTP form. Because IF2, either ligand-free or bound to the non-hydrolyzable GDPCP and GDPNP nucleotides, can perform *in vitro* most of its function, it can be surmised that IF2-dependent GTP hydrolysis is not necessary, at least during most of the initiation pathway. The present data lead us beyond this point, demonstrating that the free energy generated by GTP hydrolysis during the 30S IC to 70S IC transition is not strictly necessary for the subsequent IF2 functions. Because these late steps, which entail the dissociation of the IF2-fMet-tRNA interaction and the formation of the initiation dipeptide, can occur in the absence of guanine nucleotides or in the presence of GDP (11) but are inhibited by GDPNP and GDPCP (18–21,26), we suggest that the hydrolysis of GTP likely reflects the need of the factor to free itself from the γ-phosphate of GTP, which becomes toxic during the very late events of translation initiation. In the absence of GTP hydrolysis the factor remains blocked in a conformation, which does not allow fMet-tRNA to dissociate from IF2 and participate in formation of the first peptide bond. In support of this premise is the data of a small angle x-ray scattering (SAXS) analysis, which suggests that IF2C2, the fMet-tRNA binding domain of IF2, is stretched out to ensure contact with an initiator tRNA in the GDPNP-bound IF2, whereas it is moved and retracted in the GDP-bound form (41).

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figures 1–3, Supplementary Methods and Supplementary References [42–47].

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REFERENCES

1. Cole,J.R., Olsson,C.L., Hershey,J.W., Grunberg-Manago,M. and Nomura,M. (1987) Feedback regulation of rRNA synthesis in *Escherichia coli*. Requirement for initiation factor IF2. *J. Mol. Biol.*, **198**, 383–392.
2. Brown,E.D. (2005) Conserved P-loop GTPases of unknown function in bacteria: an emerging and vital ensemble in bacterial physiology. *Biochem. Cell. Biol.*, **83**, 738–746.
3. Rodnina,M.V., Stark,H., Savelsbergh,A., Wieden,H.J., Mohr,D., Matassova,N.B., Peske,F., Daviter,T., Gualerzi,C.O. and Wintermeyer,W. (2000) GTPases mechanisms and functions of translation factors on the ribosome. *Biol. Chem.*, **38**, 377–387.
4. Pon,C.L., Paci,M., Pawlik,R.T. and Gualerzi,C.O. (1985) Structure-function relationship in *Escherichia coli* initiation factors. Biochemical and biophysical characterization of the interaction between IF2 and guanosine nucleotides. *J. Biol. Chem.*, **260**, 8918–8924.
5. Caserta,E., Tomsic,J., Spurio,R., La Teana,A., Pon,C.L. and Gualerzi,C.O. (2006) Translation initiation factor IF2 interacts with the 30S ribosomal subunit via two separate binding sites. *J. Mol. Biol.*, **362**, 787–799.
6. Pavlov,M.Y., Zorzet,A., Andersson,D.I. and Ehrenberg,M. (2011) Activation of initiation factor 2 by ligands and mutations for rapid docking of ribosomal subunits. *EMBO J.*, **30**, 289–301.
7. Grunberg-Manago,M., Dessen,P., Pantaloni,D., Godefroy-Colburn,T., Wolfe,A.D. and Dondon,J. (1975) Light-scattering studies showing the effect of initiation factors on the reversible dissociation of *Escherichia coli* ribosomes. *J. Mol. Biol.*, **94**, 461–478.
8. Antoun,A., Pavlov,M.Y., Tenson,T. and Ehrenberg,M. (2004) Ribosome formation from subunits studied by stopped-flow and Rayleigh light scattering. *Biol. Proced. Online*, **6**, 35–54.
9. Milon,P., Tischenko,E., Tomsic,J., Caserta,E., Folkers,G., La Teana,A., Rodnina,M.V., Pon,C.L., Boelens,R. and Gualerzi,C.O. (2006) The nucleotide binding site of bacterial translation initiation factor IF2 as a metabolic sensor. *Proc. Natl Acad. Sci. USA*, **103**, 13962–13967.
10. Mitkevich,V.A., Ermakov,A., Kulikova,A.A., Tankov,S., Shyp,V., Soosaar,A., Tenson,T., Makarov,A.A., Ehrenberg,M. and Hauryliuk,V. (2010) Thermodynamic characterization of ppGpp binding to EF-G or IF2 and of initiator tRNA binding to free IF2 in the presence of GDP, GTP and ppGpp. *J. Mol. Biol.*, **402**, 838–846.
11. Tomsic,J., Vitali,L.A., Daviter,T., Savelsbergh,A., Spurio,R., Striebeck,P., Wintermeyer,W., Rodnina,M.V. and Gualerzi,C.O. (2000) Late events of translation initiation in bacteria: a kinetic analysis. *EMBO J.*, **19**, 2127–2136.
12. Grigoriadou,C., Marzi,S., Pan,D., Gualerzi,C.O. and Cooperman,B.S. (2007) The translation fidelity function of IF3 during transition from the 30S initiation complex to the 70S initiation complex. *J. Mol. Biol.*, **373**, 551–561.
13. Roll-Mecak,A., Cao,C., Dever,T.E. and Burley,S.K. (2000) X-ray structures of the universal translation initiation factor IF2/eIF5B: conformational changes on GDP and GTP binding. *Cell*, **103**, 781–792.
14. Wienk,H., Tishchenko,E., Belardinelli,R., Tomaselli,S., Dongre,R., Spurio,R., Folkers,G.E., Gualerzi,C.O. and Boelens,R. (2012) Structural dynamics of bacterial translation initiation factor IF2. *J. Biol. Chem.*, **287**, 10922–10932.
15. Allen,G.S., Zaviakov,A., Gursky,R., Ehrenberg,M. and Frank,J. (2005) The cryo-EM structure of a translation initiation complex from *Escherichia coli*. *Cell*, **121**, 703–712.
16. Myasnikov,A.G., Marzi,S., Simonetti,A., Giuliodori,A.M., Gualerzi,C.O., Yusupova,G., Yusupov,M. and Klaholz,B.P. (2005) Conformational transition of initiation factor 2 from the GTP- to GDP state visualized on the ribosome. *Nature Struct. Mol. Biol.*, **12**, 1145–1149.
17. Simonetti,A., Marzi,S., Myasnikov,A.G., Fabbretti,A., Yusupov,M., Gualerzi,C.O. and Klaholz,B.P. (2008) Structure of the 30S translation initiation complex. *Nature*, **455**, 416–420.
18. Hershey,J.W. and Thach,R.E. (1967) Role of guanosine 5′-triphosphate in the initiation of peptide synthesis, I. Synthesis of formylmethionyl-puromycin. *Proc. Natl Acad. Sci. USA*, **57**, 759–766.
19. Ohta,T., Sarkar,S. and Thach,R.E. (1967) The role of guanosine 5′-triphosphate in the initiation of peptide synthesis. 3. Binding of formylmethionyl-tRNA to ribosomes. *Proc. Natl Acad. Sci. USA*, **58**, 1638–1644.
20. Lockwood,A.H., Sarkar,P. and Maitra,U. (1972) Release of polypeptide chain initiation factor IF2 during initiation complex formation. *Proc. Natl Acad. Sci. USA*, **69**, 3602–3605.
21. Fakunding,J.L. and Hershey,J.W. (1973) The interaction of radioactive initiation factor IF-2 with ribosomes during initiation of protein synthesis. *J. Biol. Chem.*, **248**, 4206–4212.
22. Luchin,S., Putzer,H., Hershey,J.W., Cenatiempo,Y., Grunberg-Manago,M. and Laalami,S. (1999) In vitro study of two dominant inhibitory GTPase mutants of *Escherichia coli* translation initiation factor IF2. Direct evidence that GTP hydrolysis is necessary for factor recycling. *J. Biol. Chem.*, **274**, 6074–6079.
23. Guenneugues,M., Caserta,E., Brandi,L., Spurio,R., Meunier,S., Pon,C.L., Boelens,R. and Gualerzi,C.O. (2000) Mapping the fMet-tRNA(f)(Met) binding site of initiation factor IF2. *EMBO J.*, **19**, 5233–5240.
24. Caserta,E., Ferrara,C., Milon,P., Fabbretti,A., Rocchetti,A., Tomsic,J., Pon,C.L., Gualerzi,C.O. and La Teana,A. (2010) Ribosomal interaction of *Bacillus stearothermophilus* translation initiation factor IF2: characterization of the active sites. *J. Mol. Biol.*, **396**, 118–29.
25. Gualerzi,C.O., Brandi,L., Caserta,E., Garofalo,C., Lammi,M., La Teana,A., Petrelli,D., Spurio,R., Tomsic,J. and Pon,C.L. (2001) Initiation factors in the early events of mRNA translation in bacteria. *Cold Spring Harb. Symp. Quant. Biol.*, **66**, 363–376.
26. La Teana,A., Pon,C.L. and Gualerzi,C.O. (1996) Late events in translation initiation. Adjustment of fMet-tRNA in the ribosomal P-site. *J. Mol. Biol.*, **256**, 667–675.
27. Antoun,A., Pavlov,M.Y., Andersson,K., Tenson,T. and Ehrenberg,M. (2003) The roles of initiation factor 2 and guanosine triphosphate in initiation of protein synthesis. *EMBO J.*, **22**, 5593–5601.
28. Marshall,R.A., Aitken,C.E. and Puglisi,J.D. (2009) GTP hydrolysis by IF2 guides progression of the ribosome into elongation. *Mol. Cell*, **35**, 37–47.
29. Milon,P., Carotti,M., Konevega,A.L., Wintermeyer,W., Rodnina,M.V. and Gualerzi,C.O. (2010) The ribosome-bound initiation factor 2 recruits initiator tRNA to the 30S initiation complex. *EMBO Rep.*, **11**, 312–316.
30. Gualerzi,C.O. and Wintermeyer,W. (1986) Prokaryotic initiation factor 2 acts at the level of the 30 S ribosomal subunit: a fluorescence stopped-flow study. *FEBS Lett.*, **202**, 1–6.
31. Canonaco,M.A., Calogero,R.A. and Gualerzi,C.O. (1986) Mechanism of translational initiation in prokaryotes. Evidence for a direct effect of IF2 on the activity of the 30 S ribosomal subunit. *FEBS Lett.*, **207**, 198–204.
32. Hartz,D., McPheeters,D.S. and Gold,L. (1989) Selection of the initiator tRNA by *Escherichia coli* initiation factors. *Genes Dev.*, **3**, 1899–1912.

33. Hamilton,C.M., Aldea,M., Washburn,B.K., Babitzke,P. and Kushner,S.R. (1989) New method for generating deletions and gene replacements in *Escherichia coli*. *J. Bacteriol.*, **171**, 4617–4622.
34. Guillon,J.M., Mechulam,Y., Schmitter,J.M., Blanquet,S. and Fayat,G. (1992) Disruption of the gene for Met-tRNA(fMet) formyltransferase severely impairs growth of *Escherichia coli*. *J. Bacteriol.*, **174**, 4294–4301.
35. Wang,J. and Gonzalez,R.L. Jr (2009) How initiation factor 2 regulates the fidelity of translation initiation. *Biophysical J.*, **96(Suppl. 1)**, 580a.
36. Shin,B.S., Maag,D., Roll-Mecak,A., Arefin,M.S., Burley,S.K., Lorsch,J.R. and Dever,T.E. (2002) Uncoupling of initiation factor eIF5B/IF2 GTPase and translational activities by mutations that lower ribosome affinity. *Cell*, **101**, 1015–1025.
37. Shin,B.S., Kim,J.R., Acker,M.G., Maher,K.N., Lorsch,J.R. and Dever,T.E. (2009) rRNA suppressor of a eukaryotic translation initiation factor 5B/initiation factor 2 mutant reveals a binding site for translational GTPases on the small ribosomal subunit. *Mol. Cell. Biol.*, **29**, 808–821.
38. Fringer,J.M., Acker,M.G., Fekete,C.A., Lorsch,J.R. and Dever,T.E. (2007) Coupled release of eukaryotic translation initiation factors 5B and 1A from 80S ribosomes following subunit Joining. *Mol. Cell. Biol.*, **27**, 2384–2397.
39. Kapralou,S., Fabbretti,A., Garulli,C., Spurio,R., Gualerzi,C.O., Dahlberg,A.E. and Pon,C.L. (2008) Translation initiation factor IF1 of *Bacillus stearothermophilus* and *Thermus thermophilus* substitute for *Escherichia coli* IF1 *in vivo* and *in vitro* without a direct IF1–IF2 interaction. *Mol. Microbiol.*, **70**, 1368–1377.
40. Voorhees,R.M., Schmeing,T.M., Kelley,A.C. and Ramakrishnan,V. (2010) The mechanism for activation of GTP hydrolysis on the ribosome. *Science*, **330**, 835–838.
41. Rasmussen,L.C.V., Oliveira,C.L.P., Pedersen,J.S., Sperling-Petersen,H.U. and Mortensen,K.K. (2011) Structural transitions of translation initiation factor IF2 upon GDPNP and GDP binding in solution. *Biochemistry*, **50**, 9779–9787.
42. Brandi,L., Fabbretti,A., Milon,P., Carotti,M., Pon,C.L. and Gualerzi,C.O. (2007) Methods for identifying compounds that specifically target translation. *Meth. Enzymol.*, **431**, 229–267.
43. Milon,P., Konevega,A.L., Peske,F., Fabbretti,A., Gualerzi,C.O. and Rodnina,M.V. (2007) Transient kinetics, fluorescence, and FRET in studies of bacterial initiation. *Meth. Enzymol.*, **430**, 1–30.
44. Caserta,E., Ferrara,C., Milon,P., Fabbretti,A., Rocchetti,A., Tomsic,J., Pon,C.L., Gualerzi,C.O. and La Teana,A. (2010) Ribosomal interaction of *Bacillus stearothermophilus* translation initiation factor IF2: characterization of the active sites. *J. Mol. Biol.*, **396**, 118–29.
45. Parmeggiani,A. and Sander,G. (1981) Properties and regulation of the GTPase activities of elongation factors Tu and G, and of initiation factor 2. *Mol. Cell. Biochem.*, **35**, 129–158.
46. Gromadski,K.B., Wieden,H.J. and Rodnina,M.V. (2002) Kinetic mechanism of elongation factor Ts-catalyzed nucleotide exchange in elongation factor Tu. *Biochemistry*, **41**, 162–169.
47. Milon,P., Konevega,A.L., Gualerzi,C.O. and Rodnina,M.V. (2008) Kinetic checkpoint at a late step in translation initiation. *Mol. Cell*, **30**, 712–720.