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 hydrolysis (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 underline 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 hydrolyase (GTPase) control and activation, timing of Pi release, functional role of GTP hydrolysis (3).

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 GTPγS-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-aminocyclo-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 BL21 strain 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 E571K 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 changes being fitted to a single exponential function.

The differences are slightly larger for the mant-GDP (Figure S1 in Supplementary information). The activities of wt and mutant IF2 in promoting the formation of fMet-tRNA in 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 IF3166Atto555 (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*~on~ and *k*~off~ 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).
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 ($k_{\text{app}} = 10 \text{s}^{-1}$ versus $1 \text{s}^{-1}$), whereas with the IF2 E571K the two reactions proceed at approximately the same rate ($k_{\text{app}} = 0.3 \text{s}^{-1}$ and $0.2 \text{s}^{-1}$); this finding suggests that 30S 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...
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 wt IF2 (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
**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
in vitro, 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

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

Overall, the present findings underline 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 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 30S 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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