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Cooperative and Critical Roles for Both G Domains in the GTPase Activity and Cellular Function of Ribosome-Associated *Escherichia coli* EngA▽

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To probe the cellular phenotype and biochemical function associated with the G domains of *Escherichia coli* EngA (YfgK, Der), mutations were created in the phosphate binding loop of each. Neither an S16A nor an S217A variant of G domain 1 or 2, respectively, was able to support growth of an *engA* conditional null. Polysome profiles of EngA-depleted cells were significantly altered, and His_6-EngA was found to cofractionate with the 50S ribosomal subunit. The variants were unable to complement the abnormal polysome profile and were furthermore significantly impacted with respect to in vitro GTPase activity. Together, these observations suggest that the G domains have a cooperative function in ribosome stability and/or biogenesis.

GTP-binding proteins constitute a superfamily that regulates a diverse range of cellular processes such as translation and cell cycle control (7). In recent years, there has been an accumulation of evidence to suggest that many prokaryotic GTPases are associated with ribosome function, particularly ribosome biogenesis (5). Studies of Era, YjeQ, YlqF, and Obg (CgtA) have revealed links to the ribosome, ribosomal proteins, or rRNA (5). Polysome profiles of mutants of Era, YjeQ, YlqF, *Escherichia coli* ObgE/$CgtA_E$, and *Caulobacter crescentus* $CgtA_C$ have all shown an accumulation of the 30S and 50S subunits at the expense of 70S ribosomes (9, 13, 14, 16, 22, 26). Direct associations with the ribosome or its subunits have been shown for all of these proteins as well as for *Vibrio harveyi* $CgtA_V$ (14, 16, 23, 26, 27).

In addition to the above-mentioned proteins, there is also emerging evidence for a role for EngA in ribosome function (1, 21). The EngA protein is a broadly conserved bacterial GTPase that lacks a human orthologue and has been shown to be indispensable for a variety of gram-positive and gram-negative organisms (11, 12, 17, 18); its physiological role in the cell, however, is currently unresolved. Evidence for a link to the ribosome includes the results of a large-scale study wherein EngA copurified with five ribosomal proteins (6). Furthermore, the defective polysome profile of a mutant of *RrmJ*/(*FtsJ*), an *E. coli* heat shock methyltransferase, was rescued by overexpression of EngA or $CgtA_E$ (25). The only phenotype that has been shown for EngA-depleted cells, however, is cell filamentation in the *E. coli* and *Bacillus subtilis* conditional nulls (12).

The GTP-binding superfamily is very diverse and widely distributed. Interestingly, EngA and its orthologues are the only members of the superfamily that are known to contain two GTP-binding domains. A 1.9 Å X-ray structure of *Thermotoga maritima* EngA shows that the two domains are folded on either side of a C-terminal KH-like domain (21). The results of a study of *T. maritima* EngA suggested that G domain 2 does not make a contribution to the overall GTPase activity of the protein in vitro, since an Asn-to-Asp mutation in the G4 motif of this domain did not alter the observed activity. In the same study, a truncation variant suggested that G domain 2 possessed half of the activity of the full-length protein (21). This apparent paradox and a paucity of in vivo data regarding the significance of the two G domains, in particular with respect to ribosome function, prompted the work described here.

Here, we report the creation of a precise deletion in *E. coli engA* complemented with an ectopic copy at the *araBAD* locus under arabinose control. We found that variants in G domain 1 and G domain 2 (S16A and S217A, respectively) were unable to support life in the *engA* null, suggesting that the GTPase activities of both domains are indispensable to the critical cellular function of EngA. Polysome profiles of EngA-depleted cells revealed a decrease in the level of 70S ribosomes and an accumulation of ribosomal subunits compared to fully complemented or wild-type cell results. That EngA cofractionated with the 50S subunit further suggests a link to ribosome function. S16A and S217A variants were unable to restore wild-type levels of 70S ribosomes in the null strain, suggesting that both G domains are important for both viability and function. Furthermore, steady-state kinetic studies of the pure recombinant protein revealed that mutations targeting either G domain had a significant and cooperative impact on the GTPase activity of the protein as a whole.

Construction of a strain suitable for depletion of EngA. In order to probe the cellular function of *engA*, a strain was created wherein the gene deletion was complemented from a distant locus on the chromosome under the control of the tightly regulated P_{BAD} promoter. (Strains and plasmids used in the study are shown in Table 1.) Strain EB1208 was constructed

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype	Source
Strains		
BL21-AI	F$^-$ *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm araB*::T7RNAP-*tetA*	Invitrogen
BL21(DE3)	F$^-$ *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3)	Novagen
EB1208	*araBAD*::*engA*-Kan^r	This study
EB1209	*araBAD*::*engA*-Kan^r *engA*::Cm^r	This study
EB1262	EB1209 harboring plasmid K15A-pDEST14	This study
EB1263	EB1209 harboring plasmid S16A-pDEST14	This study
EB1486	EB1209 harboring plasmid K216A-pDEST14	This study
EB1456	EB1209 harboring plasmid S217A-pDEST14	This study
EB1487	EB1209 harboring plasmid *engA*-pDEST14	This study
Plasmids		
pDEST14	Gateway T7 expression vector	Invitrogen
pJM1407	(His_6)*engA*-pET28a	This study

by insertion of a second copy of *engA* at the *araBAD* locus as previously described (8). The conditional null, EB1209, was then created by replacing native *engA* with a chloramphenicol resistance cassette (10). Growth of EB1208 and EB1209 was characterized on solid rich media in the presence or absence of 2% L-arabinose. The *engA* diploid strain displayed similar levels of growth in the presence and in the absence of arabinose; the conditional null, however, was dependent on arabinose to form single colonies (Fig. 1A). In the absence of an inducer, growth was only observed for this strain in the area of heavy inoculation and was likely due to a low level of leaky expression from P_{BAD}.

Both GTP-binding domains of EngA are essential for cellular function. The tandem repeat of the GTP-binding domain seen in this enzyme is unprecedented among bacterial GTPases and presents a special challenge to understanding the function and mechanism of action of the EngA protein. To dissect the importance of each G domain, single-residue mutations were made in the phosphate-binding loop of each and tested for in vivo complementation of the lethal phenotype of the *engA* null. The 1.9 Å resolution crystal structure of EngA from *T. maritima* shows Lys14 and Ser15 of the G1 motif of G domain 1 positioned to form hydrogen bonds to two free phosphates in the active site (21). Furthermore, the equivalent lysine in Ras, a small eukaryotic GTPase, is important for binding phosphate whereas the serine has a critical role in coordinating a catalytic Mg^{2+} ion (4). Thus, these two residues, Lys15 and Ser16 in G domain 1 and Lys216 and Ser217 in G domain 2 of *E. coli* EngA, were selected for mutation to alanine. The gene *engA* (SwissProt annotation) was cloned into pDEST14 by use of a Gateway cloning kit (Invitrogen Canada, Burlington, Ontario, Canada). Variants K15A, S16A, and S217A were created using a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), while K216A was constructed by amplifying *engA* in two segments using internal primers that contain the desired mutation followed by overlap extension PCR to amplify the entire gene.

The ability of these sequences to complement the lethal phenotype of the deletion was checked on solid rich media in the presence or absence of arabinose. The deletion mutant was in a genetic background that does not produce T7 RNA polymerase, but its lethal phenotype could be complemented by the leaky expression from the T7 promoter of pDEST14 carrying wild-type *engA*, presumably from nonspecific recognition of promoter elements by *E. coli* RNA polymerase. K15A was the only variant that supported growth in the absence of arabinose (Fig. 1B). The inability of either S16A or S217A to complement the deletion provides the first in vivo evidence that both of the GTP-binding domains are critical to the cellular function of EngA. Western blot analysis demonstrated that wild-type EngA and all four variants were expressed from pDEST14 at similar levels (Fig. 1C). The previously reported observation that mutation of the N residue of the G4 motif NKXD in G domain 2 results in activity similar to that seen with the wild-type enzyme (21) may be due to the possibility that this Asn in

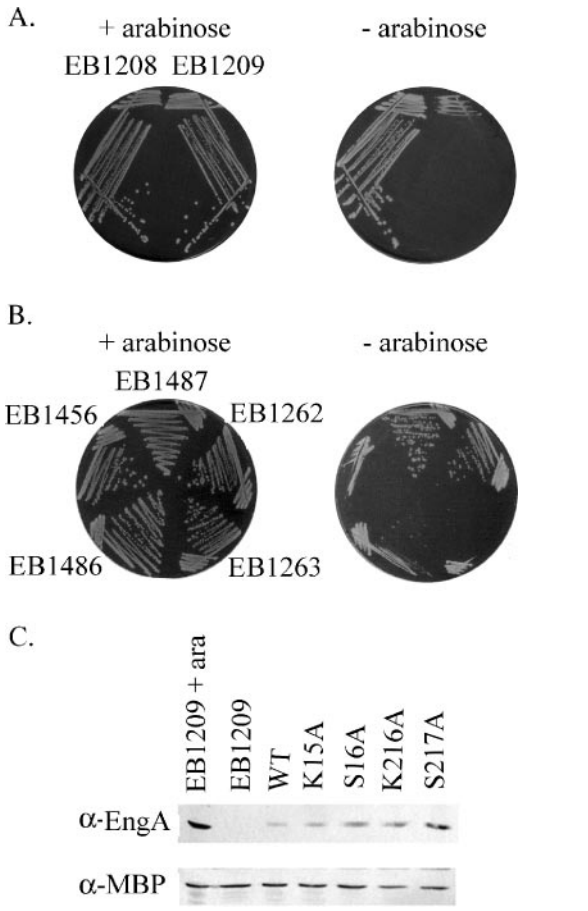

FIG. 1. Complementation of the lethal phenotype of EB1209 by G-domain mutants. (A) The *engA* diploid strain (EB1208) and the arabinose-inducible null (EB1209) grown on LB-agar-kanamycin in the presence (left) or absence (right) of arabinose. (B) Strains were grown on LB-agar-ampicillin in the presence (left) or absence (right) of 2% (wt/vol) L-Ara. Shown on each plate is EB1209 harboring pDEST14 carrying wild-type *engA* (EB1487), K15A (EB1262), S16A (EB1263), K216A (EB1486), or S217A (EB1456). (C) Analysis of the expression levels of EngA and its variants from pDEST14 by Western blotting. Lysates obtained from cultures that were grown to an optical density at 600 nm of 0.8 were subjected to Western blot analysis using α-EngA (Cocalico Biologicals, Reamstown, PA) or α-maltose binding protein (New England Biolabs, Beverley, MA) rabbit polyclonal primary antibody and donkey α-rabbit horseradish peroxidase-conjugated secondary antibody. The first and second lanes contain EB1209 grown in the presence or absence of 1% L-Ara, respectively. The remaining lanes contain EB1209 harboring pDEST14 carrying wild-type *engA* (lane 3), K15A (lane 4), S16A (lane 5), K216A (lane 6), or S217A (lane 7).

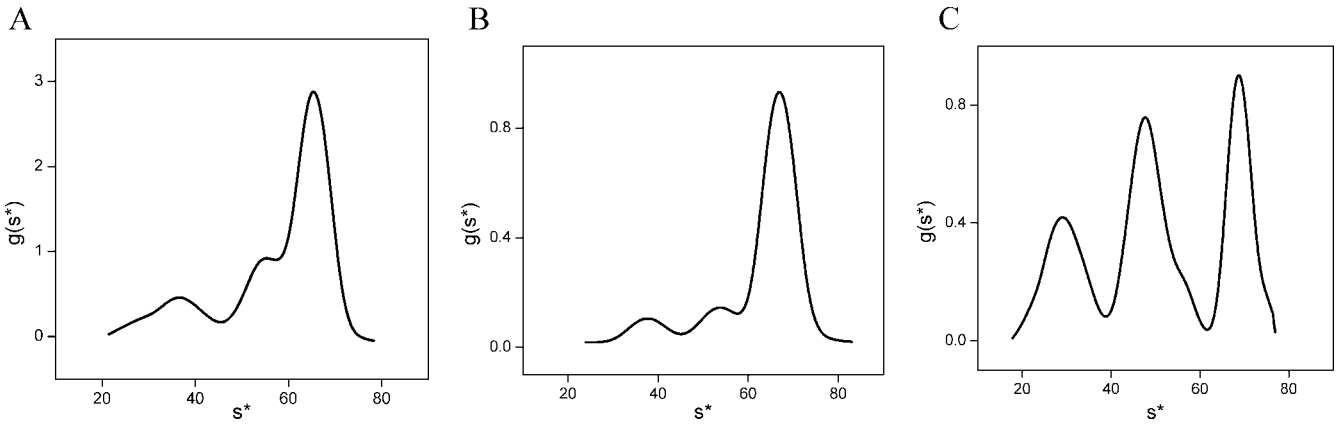

FIG. 2. Ribosome profiles of EB1209 with or without complementation. Cells were subjected to one cycle of depletion by growth in the absence of arabinose before growing to an optical density at 600 nm of ~0.20. Ribosomes were obtained from clarified lysates by centrifugation over a 35% sucrose cushion in buffer A [20 mM Tris, 10.5 mM $Mg(OAc)_2$, 300 mM NH_4Cl, 0.5 mM EDTA, 3 mM β-mercaptoethanol, 10 μg/ml RNase-free DNase I]. The ribosomal pellet was analyzed by sedimentation velocity on a Beckman Coulter model XL-I analytical ultracentrifuge. The sedimentation time derivative method (24) was employed, using Microcal Optima v. 6.0 analysis software, to find g(S*), the Gaussian distribution of molecules, at each sedimentation coefficient (S*). Note that S* is a variation of the Svedberg coefficient (S), which is specified at 20°C in water. The effects of temperature and buffer composition on the sedimentation rate were adjusted with the program Sednterp v. 1.01 (D. B. Philo, J. P. Hayes, and T. M. Laue). The profiles of *E. coli* MG1655 (A), EB1209–1% L-Ara (B), and EB1209 (C) are shown.

G domain 2, like the Lys of G domain 1 seen here, does not make a significant enough contribution to binding to abolish GTPase activity upon mutation.

EngA-depleted cells have an altered polysome profile. Sedimentation velocity analytical ultracentrifugation was used to examine the distribution of 30S, 50S, and 70S ribosomes and proved to be a robust and reproducible method of polysome profiling. Furthermore, the interference optics used in this methodology provide a signal that is faithfully integrated to quantify the distribution of ribosomal subunits. The profiles were carried out on cells that were partially depleted of EngA by growth in the absence of arabinose. After being subcultured once, EB1209 had doubling times of approximately 30 min in the presence of arabinose and 60 min in the absence of arabinose. EB1209 grown in the presence of inducer had a profile similar to that of wild-type *E. coli* MG1655, with the 70S ribosomes composing at least 60% of total ribosome (Fig. 2A and B and Table 2). Upon depletion of EngA a shoulder was observed on the middle peak which may represent a mixture of immature and mature 50S subunits (Fig. 2C). This shoulder was not seen in the profiles of wild-type or complemented cells (Fig. 2A and B). Furthermore, compared to the results seen with wild-type *E. coli*, an approximately twofold increase was observed for the partially depleted null in the level of the 30S subunit (14.4% to 26.4%) and also the 50S subunit (23.8% to 45.4%). This was accompanied by a twofold decrease in the level of 70S (61.7% to 28.1%) (Table 2). The finding that EngA is essential for the production of wild-type levels of ribosomes suggests that the protein may be a novel ribosome assembly factor.

TABLE 2. The distribution of 30S, 50S, and 70S ribosomes in EB1209 containing *engA* or a variant

Strain	% Ribosome[a]		
	30S	50S	70S
Wild-type MG1655	14.4 ± 2.8	23.8 ± 4.5	61.7 ± 3.5
EB1209–1% Ara	13.7 ± 1.8	20.3 ± 1.3	66.0 ± 2.4
EB1209	26.4 ± 1.7	45.4 ± 1.8	28.1 ± 1.3
engA-pDEST14	12.5 ± 1.4	21.9 ± 1.9	65.6 ± 2.3
K15A	20.1 ± 1.0	35.5 ± 1.8	44.4 ± 0.9
S16A	29.9 ± 5.9	41.2 ± 1.1	28.9 ± 4.9
K216A	27.6 ± 6.9	47.0 ± 3.1	25.4 ± 4.1
S217A	23.2 ± 0.9	39.5 ± 5.7	37.3 ± 4.7

[a] Values shown represent the average results ± standard deviations of triplicate experiments.

EngA cofractionates with the large ribosomal subunit. To further investigate the link between EngA and the ribosome, cells overexpressing His_6-EngA were fractionated using a sucrose gradient. A_{254} was monitored to produce a polyribosome trace, and the fractions were subsequently immunoblotted with anti-His antibody (Fig. 3) as described before (15). EngA was detected in the peak of the polyribosome trace corresponding to the 50S subunit. EngA that was not associated with ribosomes was found in the early fractions corresponding to soluble (unbound) protein. Taken together, these findings place EngA in a growing group of small bacterial GTPases, such as CgtA/Obg, YjeQ, YlqF, and Era, which have been implicated in ribosome function. More study will be required to investigate this phenomenon and the hypothesis that EngA has a role in ribosomal assembly.

Both G domains of EngA can be linked to the polysome defect. The distribution of ribosomes was examined in EB1209 harboring pDEST14 encoding either wild-type EngA or one of the variants thereof, K15A, S16A, K216A, or S217A. Sedimentation velocity analysis showed that *engA*-pDEST14 was able to restore a wild-type polysome profile (approximately 12% 30S, 22% 50S, and 65% 70S); S16A, K216A, and S217A mutations, however, resulted in profiles that are similar to that of the uncomplemented *engA* null (approximately 25% 30S, 45% 50S, and 30% 70S) (Table 2). This suggests that inactivation of either domain interferes with the role of EngA in the forma-

tion or stability of 70S ribosomes. K15A provided partial complementation of the defective polysome profile (Table 2) consistent with the observation that this mutation complements growth (Fig. 1B). K216A did not display any such complementation, indicating that this lysine may be more important in G domain 2. Since inactivation of either domain has the same impact on 70S levels as deletion of the entire gene, the G domains clearly have a cooperative and critical role in the maintenance or assembly of ribosomes.

Active-site substitutions in either G domain impair GTPase activity. Untagged EngA and the variants were overexpressed from pDEST14 in BL21-AI cells and purified by Q-Sepharose Fast Flow anion exchange chromatography (Amersham Biosciences, Baie d'Urfe, Quebec, Canada). Wild-type (0.5 μM) or variant (10 μM) EngA was incubated with various GTP concentrations and 1 μCi [α-^{32}P[GTP in assay buffer (50 mM Tris, 5 mM $MgCl_2$ and 400 mM KCl, pH 8.0) for 10 to 120 min at 22°C. Quenched reactions were resolved by anion exchange chromatography on a Waters 600 high-performance liquid chromatography system (Milford, MA) as previously described (2). The kinetic constants k_{cat} and K_m were determined from a plot of initial velocity versus [GTP] by use of SigmaPlot 2000 software to fit the data to a single rectangular two-parameter hyperbolic function.

At steady state, a k_{cat} of 70 h^{-1} and a K_m of 143 μM were observed for *E. coli* EngA (Table 3), which is comparable to the k_{cat} of 50 h^{-1} and K_m of 110 μM reported for the *T. maritima* enzyme (21). Purified recombinant variant enzymes S16A, K216A, and S217A had K_m constants of approximately 5 mM and turnovers of approximately 25 h^{-1} (Table 3). Since the major impact was on the K_m, this indicates that the primary effect of the mutations was a reduction in the productive binding of EngA to GTP.

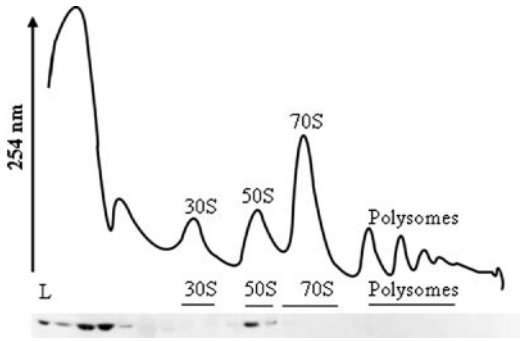

FIG. 3. His_6-EngA cofractionates predominantly with the large ribosomal subunit. Midexponential phase *E. coli* BL21(DE3) bacteria harboring (His_6)*engA*-pET28a were induced with 50 μM IPTG (isopropyl-β-D-thiogalactopyranoside) for 30 min at 22°C. The culture was incubated with 200 μg/ml chloramphenicol (FisherBiotech) for 30 s before harvesting. Cleared lysates (13 units of optical density at 260 nm) containing 100 μg/ml chloramphenicol were layered onto a 10 ml gradient of 7% to 47% sucrose in buffer B (10 mM Tris-Cl [pH 7.5], 10 mM $MgCl_2$, 100 mM NH_4Cl). Sedimentation of the ribosomes and fractionation of the gradient were carried out as previously described (27). Shown is the profile of absorbance at 254 nm resulting from fractionation. The positions of the 30S and 50S subunits, the 70S monosomes, and the polysomes are indicated. Below the polyribosome trace is an anti-His immunoblot of trichloroacetic acid-precipitated fractions showing the amount of His_6-EngA detected. L, 1/100th of the total sample loaded onto the gradient.

TABLE 3. Kinetic characterization of wild-type EngA and P-loop mutants[a]

Strain	k_{cat} (h^{-1})	K_m (μM)	Decrease in k_{cat}/K_m[b]
Wild type	70	143	1
K15A	7.5	695	45
S16A	20	4,900	118
K216A	31	6,655	104
S217A	21	4,770	109

[a] Values shown represent the average results of duplicate experiments.
[b] Severalfold decrease relative to wild-type results.

The specificity constant k_{cat}/K_m, which describes the coming together of enzyme and substrate to form a productive complex, is the apparent rate constant at a low substrate concentration (20). This is a particularly useful parameter for describing the variants, since the estimated cellular concentration of GTP, <1 mM (3), is much lower than the K_m of the variants. The k_{cat}/K_m constants were approximately 118-, 104-, and 109-fold lower in S16A, K216A, and S217A, respectively (Table 3), i.e., less than 1% of that of wild-type EngA. Thus, inactivation of one G domain had a negatively cooperative impact on the other domain. Interestingly, the K15A variant was the least impacted (45-fold reduction in k_{cat}/K_m) and it is the only variant that provided partial complementation (Fig. 1B and Table 2). Since we have observed that the GTPase activity of a single G domain cannot be isolated, this is consistent with strong positive cooperativity whereby binding or hydrolysis in one G domain considerably stimulates the other.

Concluding remarks. The data presented here are consistent with unique but cooperative roles for the two G domains in the function of EngA, since each was shown to be important for cell viability and for normal polysome profiles. There are several structural differences between the N- and C-terminal G domains which suggest that each has a unique character (21). The most striking is the positioning of the GTP-binding sites in relation to the uncharacterized C-terminal KH-like domain (21). In G domain 1, the conserved motifs of nucleotide binding are right at the interface, while in G domain 2, these motifs are distal to the interface. It has recently been shown that G domain 1 undergoes a rearrangement upon GDP binding which exposes a positively charged face on the KH-like domain (19). The structure suggests that the two G domains communicate differently with the KH-like domain. The remarkable level of cooperativity observed in this enzyme is also suggestive of intramolecular regulation whereby the two activities are exquisitely coordinated to achieve function.

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