The *Escherichia coli* *trmE* (*mnme*) gene, involved in tRNA modification, codes for an evolutionarily conserved GTPase with unusual biochemical properties

Hugo Cabedo, Fernando Macián¹,
Magda Villarroya, Juan C. Escudero,
Marta Martínez-Vicente, Erwin Knecht
and M. Eugenia Armengod²

Instituto de Investigaciones Citológicas, Fundación Valenciana de Investigaciones Biomédicas, Valencia 46010, Spain

¹Present address: Center for Blood Research and Department of Pathology, Harvard Medical School, Boston, MA 02115, USA

²Corresponding author
e-mail: armengod@ochoa.fib.es

H. Cabedo, F. Macián and M. Villarroya contributed equally to this work

The evolutionarily conserved 50K protein of *Escherichia coli*, encoded by *o454*, contains a consensus GTP-binding motif. Here we show that 50K is a GTPase that differs extensively from regulatory GTPases such as p21. Thus, 50K exhibits a very high intrinsic GTPase hydrolysis rate, rather low affinity for GTP, and extremely low affinity for GDP. Moreover, it can form self-assemblies. Strikingly, the 17 kDa GTPase domain of 50K conserves the guanine nucleotide-binding and GTPase activities of the intact 50K molecule. Therefore, the structural requirements for GTP binding and GTP hydrolysis by 50K are without precedent and justify a separate classification in the GTPase superfamily. Immunoelectron microscopy reveals that 50K is a cytoplasmic protein partially associated with the inner membrane. We prove that *o454* is allelic with *trmE*, a gene involved in the biosynthesis of the hypermodified nucleoside 5-methylaminomethyl-2-thiouridine, which is found in the wobble position of some tRNAs. Our results demonstrate that 50K is essential for viability depending on the genetic background. We propose that combination of mutations affecting the decoding process, which separately do not reveal an obvious defect in growth, can give rise to lethal phenotypes, most likely due to synergism.

Keywords: GTPase/synthetic lethality/translation/tRNA modification

Introduction

GTP-binding proteins are involved in cell proliferation, signal transduction, protein synthesis and protein targeting (for reviews, see Bourne *et al.*, 1990; Kjeldgaard *et al.*, 1996). The binding of GTP causes a conformational change in these proteins that allows interaction with a target (effector) molecule, whereas the GDP-bound form is inactive (Bourne *et al.*, 1990). The similarity within this large class of proteins is confined to three consensus sequence elements (called G-1, G-3 and G-4), all involved in binding the guanine nucleotide (for reviews, see Bourne

et al., 1991; Kjeldgaard *et al.*, 1996). A fourth sequence motif (G-2), which is highly conserved within each GTPase family but not between different families, is not involved in GTP binding but is thought to play a role in interaction with the effector molecule. A threonine residue is the only invariant of G-2 between families.

The last few years have witnessed a tremendous increase in our understanding of the structures and functions of eukaryotic GTP-binding proteins. In contrast, there is little information about similar factors in bacteria (see March, 1992; Okamoto and Ochi, 1998; Zhong and Tai, 1998 and references therein). In *Escherichia coli*, the 50 kDa protein encoded by *o454* (Figure 1) contains a GTP binding site sequence, identified by similarities to other GTP-binding proteins, but the function(s) of this protein remains to be clarified (Burland *et al.*, 1993). Homologous DNA sequences to *o454* have been found in all prokaryotic genomes of Bacteria determined so far, but not in those of Archaea. Interestingly, the bacterial 50 kDa protein is highly homologous to the putative protein encoded by *MSS1*, a nuclear gene of *Saccharomyces cerevisiae* (Decoster *et al.*, 1993), whose product seems to play a role in some aspect of mitochondrial translation (Decoster *et al.*, 1993; Colby *et al.*, 1998).

In the *E. coli* chromosome, *o454* lies in a cluster of genes involved in protein synthesis, the order of genes being *rpmH-rnpA-o548-o454* (Burland *et al.*, 1993). Transcription of the four adjacent genes is in the clockwise direction (see Figure 1). *rpmH* encodes the 5.4 kDa ribosomal protein L34 and *rnpA* encodes the 13.8 kDa protein component of RNase P, an endoribonuclease that processes tRNA. *o548* codes for a 60 kDa inner membrane protein (Hansen *et al.*, 1985; Sääf *et al.*, 1998) whose precise function has not been identified so far. This protein is homologous to that encoded by *OXA1*, a *S. cerevisiae* nuclear gene involved in a novel and general protein export machinery of the mitochondrial inner membrane (Hell *et al.*, 1998).

o454 has been assigned by Burland *et al.* (1993) to *trmE*, a gene involved in tRNA modification (Elseviers *et al.*, 1984), and to *thdF*, a gene involved in thiophene and furan oxidation (Alam and Clark, 1991). Several amber mutations in *o548* and *o454* were isolated by Wright and O'Day (cited by Hansen *et al.*, 1985) in a selection for mutations in essential genes in the *dnaA* region of the *E. coli* chromosome. In all cases, these amber mutations conferred temperature sensitivity to a strain containing a temperature-sensitive amber suppressor, suggesting that both the 60 and the 50 kDa proteins are essential for *E. coli* growth.

In this work, we characterize the biochemical activities of the 50K protein and the GTPase domain of 50K, prove that *o454*, the gene encoding for 50K, is allelic with *trmE* (renamed *mnme*; see Leung *et al.*, 1998), and demonstrate

Fig. 1. Map of the *E. coli* chromosomal *rpmH* region. Data have been taken from Burland *et al.* (1993), arbitrarily assigning co-ordinate 1 in the figure to nt 73725. Promoters are represented by arrowheads. The constitutive terminator located between *o548* and *o454* is represented by a vertical arrow. Nucleotides corresponding to the 5' and 3' ends of the open reading frames and relevant restriction sites are indicated.

that *o454* is essential for growth in specific genetic backgrounds.

Results

Purification of the 50K protein

50K was expressed as a protein fused to the C-terminal region of the glutathione *S*-transferase (GST)-encoding gene from *Schistosoma japonicum*. As the synthesis of this GST–50K chimera was controlled by an isopropyl-1-thio- β -D-galactopyranoside (IPTG)-inducible promoter, the addition of this inducer led to the overproduction of the chimera, which ran at ~80 kDa (Figure 2A, compare lanes 1 and 2). Cleavage with thrombin of the chimera, which was purified by affinity chromatography (Figure 2A, lane 3), allowed the isolation of a 50K protein containing 28 foreign amino acids at the N-terminus. This protein, hereafter designated 53K because it runs at ~53 kDa (Figure 2A, lane 5), was used to raise antibodies in rabbits. It should be noted that a 50 kDa protein, which is recognized by the anti-53K antibody, seems to be co-fractionated with the overexpressed GST–50K chimeric protein (Figure 2A, lanes 3 and 5; Figure 2B, lane C). We propose that such a protein is the chromosomally produced 50K protein and that its co-purification with the GST–50K chimeric protein results from its ability to self-associate. In fact, when applied to an S-200 gel filtration column, the purified 53K protein (and also the native 50K protein from crude extracts of *E. coli* IC3492) eluted with an apparent molecular mass ranging between 50 and 250 kDa (Figure 2B; data not shown), which supports the proposal that 50K is able to undergo polymerization.

Multimerization of 50K

To investigate further the ability of 50K to multimerize, we performed *in vivo* cross-linking experiments by using formaldehyde as the cross-linking agent. This small reactive molecule, capable of polymerization to a variety of lengths, can be used in whole cells to reflect protein–protein interactions as they occur *in vivo* (Peters and Richards, 1977). Furthermore, the cross-link can be destroyed by heating, thus liberating the unaltered individual components of a cross-linked product (Jackson and Chalkley, 1974). Figure 2C shows the results obtained when cells cross-linked with 1% formaldehyde for various

times were analysed by SDS–PAGE and immunoblotting with anti-53K antibody. A band with an apparent molecular mass of 100 kDa arose after 30 min of treatment (lanes 2, 3 and 4). The disappearance of this band upon boiling (lanes 5 and 6) demonstrates it to be the result of cross-linking by formaldehyde. An additional band running at ~150 kDa was detected immediately after the addition of formaldehyde (lanes 1–4). In this case, cross-linking could not be reversed by boiling (lanes 5 and 6).

A more direct approach to the question of self-association can be provided by *in vitro* chemical cross-linking. Extensive cross-linking in solution is taken to be indicative of specific interactions, since random collisions are expected to produce a minimal amount of cross-linking (Maman *et al.*, 1994). Dithiobis(succinimidyl propionate) (DTSP) was used as the cross-linking molecule; it reacts primarily with ϵ -amines of lysine residues, and is cleavable under reducing conditions (Lomant and Fairbanks, 1976). Therefore, the ability of DTSP to cross-link the purified 53K protein was analysed by SDS–PAGE under non-reducing conditions (Figure 2D). Analysis of untreated 53K allowed the detection, in addition to the band of 53K monomers, of a clear band that corresponded in size to 53K dimers (lane 1). This additional band disappeared when 53K was mixed before loading with the strong reducing agent 2-mercaptoethanol (ME) (Figure 2D, lane 3). These results suggest that 53K is able to form intermolecular disulfide bonds by means of its sole cysteine residue, which forms part of the tetrapeptide motif C-I-G-K at the extreme C-terminus. It should be noted that this motif shows good homology with the C-a-a-X motif (where 'a' represents an aliphatic residue and 'X' any residue) characteristic of the Ras, Ras-related and Rho proteins of eukaryotic cells, which has been shown to be essential for the membrane association of these proteins (see Bourne *et al.*, 1990). Many other GTPases contain a pair of C-terminal cysteines, at least one of which is essential for proper membrane localization and function (Bourne *et al.*, 1990).

Incubation of 53K with DTSP increased the yield of dimers (Figure 2D, compare lanes 1 and 2), whereas the subsequent addition of ME induced complete dissociation into monomers (Figure 2D, compare lanes 2 and 4). It should be stressed here that the DTSP-induced dimerization is reversed by ME because, as mentioned above, the cross-linker is cleavable under reducing conditions.

In order to prevent 53K dimerization by disulfide bonds involving the C-terminal cysteine, we mutated Cys451 to Ser. Untreated mutant 53K-C451S did not produce dimers when resolved by non-reducing SDS–PAGE (Figure 2D, lane 5), but it could be efficiently cross-linked with DTSP (Figure 2D, lane 6). This result indicates that 53K is able to form self-assemblies by non-covalent interactions. Accordingly, and taking into consideration that cysteine residues remain free in the reducing environment of the *E. coli* cytoplasm (Derman and Beckwith, 1991; Snyder and Silhavy, 1995), it is possible that the 100 kDa complexes detected after treatment of cells with formaldehyde (Figure 2C, lanes 2–4) were dimers of 50K maintained in the cytoplasm by non-covalent interactions.

Biochemical activities of the 50K protein and the G domain

The predicted amino acid sequence of 50K contains the three elements and correct spacing definitive of a consensus

Fig. 2. The purified 53K protein forms oligomers. (A) SDS-PAGE analysis of samples from different purification steps of the GST-50K fusion protein from strain MC1000/pIC684. The gel was stained with Coomassie Blue. Lane 1, crude extract from an uninduced culture; lane 2, crude extract from a culture induced for GST-50K overexpression with 0.5 mM IPTG for 3 h; lane 3, purified GST-50K; lane 4, protein remaining in the glutathione-Sepharose 4B beads (Pharmacia) after partial digestion with thrombin; lane 5, protein 53K resulting from treatment of GST-50K with thrombin. (B) Elution profile of the purified 53K protein through gel filtration. A sample (2.5 mg) of purified 53K was subjected to a Sephacryl S-200 gel filtration column. Fractions of 1 ml were collected and aliquots of the indicated fractions were analysed by Western blotting with the anti-53K antibody. The migrations of thyroglobulin (669 kDa), catalase (232 kDa) and bovine serum albumin (67 kDa) during calibration runs are indicated. Lane C shows an aliquot of the sample before gel filtration. (C) Immunoblot analysis of 50K protein in strain IC3492 cross-linked *in vivo* for the indicated times with 1% formaldehyde. The 30 and 90 min samples on the right (lanes 5 and 6) were heated at 95°C for 10 min to release cross-links. Lane 7 shows strain IC3492 without formaldehyde. Positions of mass markers and their size in kilodaltons are indicated on the left. Positions of 50K and 50K-specific complexes are indicated on the right, together with the apparent molecular weights of the complexes. (D) *In vitro* cross-linking of 53K and 53K-C451S with DTSP. Proteins 53K and 53K-C451S, untreated or cross-linked with DTSP, were analysed by non-reducing SDS-PAGE. Lanes 3 and 4 show the effect of 2-ME on the reactions. DTSP-induced dimerization of 53K-C451S (see lane 6) was also reversed by ME, as expected (data not shown). Positions of 53K and a 53K-specific complex are indicated on the right.

GTP-binding motif. To investigate whether 50K actually binds GTP, we attempted to cross-link GTP photochemically to an overexpressed protein in crude cell extracts following the method of March and Inouye (1985). IC3038 cells, which harbour a plasmid system arranged to overproduce 50K after a shift at 42°C, synthesized a 50 kDa protein at 42°C that was very efficiently cross-linked to [α - 32 P]GTP (data not shown). In another approach, purified soluble 53K protein (95% purity; Figure 2A) was cross-linked to [α - 32 P]GTP by UV irradiation (Figure 3, lane 1). The specificity of GTP cross-linking was tested in the presence of nucleotide competitors. As shown in Figure 3, GTP, GDP and dGTP could compete effectively, whereas GMP, CTP, UTP and ATP failed to abolish GTP cross-linking. Altogether, these results provide clear evidence that 50K is a GTP-binding protein.

To characterize the binding activities further, a fixed amount of purified 53K was incubated with various concentrations of the non-hydrolysable GTP analogue GTP γ S (35 S labeled), and the amounts of bound nucleotide

Fig. 3. SDS-PAGE profile of UV-cross-linking of [α - 32 P]GTP to 53K in the presence of competitors. Lane 1, complete reaction mixture without nucleotide competitor. Lanes 2–8, the following unlabelled nucleotides (50 μ M) were added to the complete reaction mixture before cross-linking: lane 2, GTP; lane 3, GDP; lane 4, GMP; lane 5, dGTP; lane 6, CTP; lane 7, UTP; lane 8, ATP. Lane 9, bovine serum albumin was added to the complete reaction mixture in place of 53K. Only the relevant part of the blot is shown.

were quantified for each reaction by filtration on nitrocellulose filters (Figure 4). From the saturation curve shown in Figure 4A, the dissociation constant, K_d , of binding of 53K to the GTP analogue was found to be ~ 280 μ M.

Fig. 4. Determination of GTP γ S dissociation constants for 53K and the G domain of 50K. Filter binding assays were performed as described in Materials and methods. (A) and (C) represent the concentration-dependent binding of GTP γ S to 53K and the G domain of 50K, respectively. (B) and (D) represent the corresponding Scatchard plots of the data displayed in (A) and (C). r = [bound GTP γ S]/[total protein]. F is the concentration (mM) of free GTP γ S.

Saturation experiments also indicated that the affinity of 53K for GDP was even lower than for GTP γ S (data not shown). This feature did not allow us to measure the K_d of 53K for GDP accurately, which we estimated to be >1 mM. Similar experiments performed with the 17 kDa GTPase domain of 50K revealed that the K_d of this domain to GTP γ S was ~ 55 μ M (Figure 4C). This means that the GTPase domain binds GTP γ S 5-fold more efficiently than the purified 53K protein, suggesting that other domains of 50K modulate the access of the nucleotide to the protein. From the Scatchard plots shown in Figure 4B and D, it can be calculated that at saturation the molar ratios of GTP γ S bound to 53K and the G domain were ~ 0.8 and 0.4 , respectively. The lower value for the G domain might be due to misfolding of this domain when synthesized separately as a GST fusion or to relaxation of control mechanisms exerted on the G domain by the N- and/or C-terminal domains.

Many proteins that contain consensus tripartite GTP-binding motifs have been demonstrated to exhibit intrinsic GTPase activities *in vitro*. To test whether 50K is capable of hydrolysing GTP, we measured by thin-layer chromatography analysis the ability of the purified 53K protein to convert [α - 32 P]GTP to [α - 32 P]GDP. In time course experiments performed essentially as described by RayChaudhuri and Park (1992), we observed that

radiolabelled GTP incubated with 53K was rapidly converted to GDP in a magnesium-dependent manner and that GDP was not hydrolysed to GMP even after 180 min of incubation (data not shown). Therefore, 53K possesses intrinsic GTPase activity *in vitro*.

To determine the enzymatic parameters of GTP hydrolysis by 53K, the substrate concentration was varied from 0 to 2 mM, and the reaction products were quantified (Figure 5A). From the Lineweaver–Burk plot (Figure 5B), the K_m for GTP hydrolysis by 53K was estimated to be 378 μ M. Independent preparations of 53K varied slightly in activity; typically, they had specific GTPase activities of 250–500 nmol of GTP/min/mg of protein, corresponding to a turnover number of up to 26 min^{-1} . Strikingly, substrate saturation experiments performed with the purified G domain of 50K (Figure 5C) revealed that it displays a GTPase activity similar to that of the entire protein. Thus, the K_m for GTP hydrolysis by the G domain was 565 μ M and the maximum hydrolysis rate was 1823 nmol of GTP/min/mg of protein, corresponding to a turnover number of 31 min^{-1} (Figure 5D).

Subcellular localization of 50K

Escherichia coli strains expressing 50K at wild-type (C600) or elevated levels (IC3038) were subjected to subcellular fractionation followed by immunoblotting to

Fig. 5. GTPase activity of 53K and the G domain of 50K. GTP saturation curves (top) and Lineweaver–Burk plots (bottom) of the GTPase activity of 53K (A and B) and the G domain of 50K (C and D).

identify 50K in the fractions. As shown in Figure 6A and B, 50K was mostly found in the soluble fraction, although at least a portion was also detected in the inner membrane fraction. It should be pointed out that the presence or activity of β -galactosidase, used as a cytoplasmic marker when appropriate (strain C600), was negligible in the inner membrane fraction (data not shown), suggesting little cross-contamination with the soluble fraction.

We further investigated the cellular distribution of 50K by immunoelectron microscopy, using affinity-purified anti-53K antiserum and secondary antibodies labelled with gold particles [immunogold staining (IGS) procedure; see Materials and methods], on ultrathin sections of strains expressing 50K at wild-type or elevated levels. Labelling was seen diffusely in the cytoplasm and also close to or actually on the inner membrane (Figure 6C, D and E). Labelling was very low or practically non-existent on the outer membrane, periplasm and in the embedding matrix. Control incubations (see Materials and methods) provided evidence of the specificity of the labelling (data not shown). Since the number of colloidal gold particles in cells expressing 50K at wild-type levels was low, we used the 50K-overexpressing strains IC3037 and IC3492 for the quantitative evaluation of the electron micrographs (Table I). The data confirmed that 50K is localized in both the cytoplasm and, to a lesser but significant extent, the inner membrane.

Fig. 6. Subcellular localization of 50K. (A and B) Western blot analysis with anti-53K antibody of cell fractions from strains C600 (A) and IC3038 (B). IC3038 cells were grown at 30°C in LBT to an absorbance at 600 nm of 0.3, induced by incubation at 42°C for 30 min, and collected after incubation for 2 h at 37°C. T, total cell lysate; S, soluble fraction; IM, inner membrane fraction; OM, outer membrane fraction. (C–E) Immunoelectron microscopy with anti-53K antibody and immunogold of MC1000 (C), IC3037 (D) and IC3492 (E) cells. Strain IC3492 was grown in LBT to an absorbance at 600 nm of 0.3, induced with 0.5 mM IPTG, and collected 60 min after induction. Note that expression of *o454* on pIC576 is mediated by its own promoter in strain IC3037 and by the *o454* and T7 promoters in the IC3492 strain induced by IPTG. The bar represents 0.1 μ m.

The *o454* gene is essential for viability

As a first step to clarify the possible role(s) of 50K in cell metabolism, we attempted to disrupt *o454* on the chromosome to create a null mutant by using a linear transformation method (Winans *et al.*, 1985). For this purpose, pIC720 was constructed as described in Materials and methods with *o454* interrupted by the insertion of a 1.3 kb *EcoRI* fragment containing the gene for kanamycin resistance (Kan^R) into the unique *MunI* site [nucleotide (nt) 3620; see Figure 1]. Plasmid pIC720 was then linearized and transformed into strain JC7623 (*recBC sbcBC*) lysogenized by λ IC718, a λ bacteriophage containing a wild-type copy of *o454* under the control of the *tac* promoter. Kan^R colonies were isolated and one of them (hereafter called IC3652) was selected for subsequent manipulations. Bacteriophage P1 was used to transduce the *o454::kan* allele (hereafter designated *o454-1::kan*) from IC3652 into JC7623 and JC7623/ λ IC718. More than 600 Kan^R transductants were obtained with strain JC7623/ λ IC718, whereas only one Kan^R transductant was obtained when JC7623 was the recipient. This rare transductant carried a wild-type copy of *o454*, as shown by long-range PCR analysis (data not shown). These results suggest that *o454* is essential for *E.coli*. To confirm this idea, the following strategy was used. The *o454-1::kan* allele was P1 transduced from IC3652 into strain V5701 harbouring pIC755. This plasmid, which carries a wild-type copy of *o454*, is temperature sensitive for replication and contains the gene for chloramphenicol resistance (Cm^R). Note that V5701 harbours a *bgl* mutation (Armengod and Blanco, 1978), which confers ability to ferment salicin (Sal^+), and that the *bgl* locus is co-transducible with *o454*. Accordingly, a Kan^R Sal^- Cm^R transductant was selected and designated IC4130. A *recA*⁻ derivative, IC4133, was constructed by transduction to Tet^R (tetracycline resistance) by a P1 bacteriophage lysate bearing a *recA13 srl::tet* allele. Southern blot analysis confirmed that the chromosomal copy of *o454* was interrupted in IC4130 and IC4133 (data not shown). Then, to investigate whether survival of the chromosomally disrupted *o454* mutants requires the complementary *o454* on the helper plasmid, appropriate dilutions of exponential cultures of IC4130 and IC4133 grown at 30°C were spread on LAT plates at 30 and 42°C. As shown in Table II, the *o454-1::kan* strains (IC4130 and IC4133) exhibited a strict growth dependence on pIC755 and, hence, temperature sensitivity. Note that only ~5% of the colonies recovered from plating the control strain (V5701/pIC755) at 42°C were Cm^R ,

which indicates that pIC755 has been lost in most of them, as expected. In contrast, all the surviving colonies recovered from plating the *o454-1::kan* strains at 42°C were Cm^R , which means that they were able to grow at the non-permissive temperature only after the integration of plasmid carrying the wild-type copy of *o454* (pIC755) into the bacterial chromosome. Integration occurred in the *recA*⁻ strain (IC4133), which is deficient in the homologous recombination process, at a much lower frequency than in the *recA*⁺ strain (IC4130), as expected. These data demonstrate that the plasmid-borne *o454* copy was absolutely required to support growth when chromosomal *o454* was non-functional. Collectively, our data demonstrate that *o454* is essential for *E.coli*, consistent with the idea that this gene has an important cellular function.

The *trmE* (*mnME*) mutant: effect of genetic background on the phenotype conferred by null *o454* mutations

trmE mutants of *E.coli* are defective in the biosynthesis of the hypermodified nucleoside 5-methylaminomethyl-2-thiouridine ($\text{mm}^5\text{s}^2\text{U}$), which is found in the wobble position of some tRNAs (Elseviers *et al.*, 1984; Krüger and Sørensen, 1998). The presence of $\text{mm}^5\text{s}^2\text{U}$ in the wobble position has been shown to be important in the recognition of such tRNAs by their synthetases and in the codon-anticodon interaction (Krüger and Sørensen, 1998; Krüger *et al.*, 1998). The *trmE* gene product was identified by transforming the minicell strain P678-54 with *trmE*⁺ and *trmE*⁻ plasmids (Elseviers *et al.*, 1984). The *trmE*⁺ plasmid expressed a 48.5 kDa protein that was absent in the mutant plasmid. Thus, it was concluded that the *trmE* gene product was the 48.5 kDa protein and that the *trmE* mutation studied was itself a deletion or nonsense mutation.

Table II. *o454* is essential for viability in the V5701 background

Strain	Survival (%) at 42°C on LAT plates ^a
V5701/pIC755 (IC4126)	100 (5% Cm^R)
V5701 <i>o454-1::kan</i> /pIC755 (IC4130)	3 (100% Cm^R)
V5701 <i>o454-1::kan recA</i> /pIC755 (IC4133)	0.01 (100% Cm^R)

^aThe percentage of survivors at 42°C that were Cm^R is shown in parentheses.

Table I. Density of labelling and distribution of gold particles in different compartments of two strains of *E.coli* immunolabelled with 50K-specific antibodies followed by immunogold

Cell compartment	Labelling density ^a		V_v (%) ^b		Relative labelling ^c	
	IC3037	IC3492	IC3037	IC3492	IC3037	IC3492
Cytosol	29.0 (60.2)	90.5 (53.1)	51.3	47.8	1.2	1.1
Inner membrane	46.8 (33.9)	147.2 (28.3)	17.2	16.0	2.0	1.8
Outer membrane + periplasm	4.9 (5.9)	34.9 (18.7)	31.5	36.2	0.2	0.5

^aLabelling densities are expressed as gold particles/ μm^2 compartment area. Labelling from controls (0.6 gold particles/ μm^2) was subtracted from the experimental values. The numbers in parentheses represent the percentage of the total number of gold particles associated with each compartment.

^bVolume density: percentage of the total cell area occupied by the various compartments defined as described in Materials and methods.

^cRelative labelling was calculated by dividing the percentage of the total number of gold particles associated with each compartment by the corresponding volume density. Relative labelling >1 indicates above average labelling.

Fig. 7. Immunoblot analysis of 50K expression in wild-type and *o454* mutant strains. Whole-cell lysates were analysed by SDS-PAGE and immunoblotting using anti-53K antibody. The position of 50K is indicated on the right. Only relevant portions of the blots are shown. Lanes 1 and 9, MC1000; lane 2, DEV16; lane 3, DEV16/pIC576; lane 4, IC3652; lane 5, temperature-resistant (T^R), Cm^R derivative of IC4364 *o454-2::kan*; lane 6, T^R Cm^S derivative of IC4364 *o454-2::kan*; lane 7, T^R Cm^R derivative of IC4364 *o454-1::kan*; lane 8, T^R Cm^S derivative of IC4364 *o454-1::kan*.

Burland *et al.* (1993) have proposed that *trmE* is allelic with *o454* from comparison of restriction maps and the complementation data of Elseviers *et al.* (1984). Since the *trmE* mutants isolated by Elseviers *et al.* (1984) are viable, whereas the null mutant of *o454* constructed here is lethal, we decided to characterize at the molecular level the *trmE* mutation carried by strain DEV16 (Elseviers *et al.*, 1984), a generous gift from Dr Hagervall. First, we studied the ability of plasmid-borne *o454* to complement *trmE* mutants. Since these mutants were isolated based on a phenotype of reduced readthrough at UAG codons, we transformed strain DEV16 (*trmE*⁻), which carries the *lacZ105* (UAG) mutation, with several plasmids containing *o454* and performed β -galactosidase assays. UAG misreading, measured as β -galactosidase activity, increased ~100-fold in the transformants (data not shown) indicating that *o454* is able to complement the *trmE* mutation. Secondly, the anti-53K antibody was used to test for the presence of 50K in the *trmE* strain. As shown in Figure 7, this strain produces no detectable 50K protein (lane 2), but introduction of a plasmid carrying *o454* restored the ability of the mutant strain to express 50K (lane 3). Finally, sequence analysis of the *trmE* allele identified a transition C→T at nt 3676, which produces an amber codon starting at this position (mutation Q192X). Since the *trmE* mutation is located 56 bp downstream from the position of the *o454-1::kan* mutation, it is possible that an essential function of 50K, different to the tRNA methylating activity, could still be performed by the *trmE*-encoded peptide but not by the *o454-1::kan*-encoded peptide. It should be pointed out, however, that the anti-53K antibody, which is able to recognize the 224-amino-acid N-terminal domain of 50K (data not shown), did not detect truncated forms of 50K in both *trmE* and *o454-1::kan* mutants, suggesting that such forms are very unstable. To analyse whether the position of the stop mutation is important to determine a lethal phenotype, we constructed a new null *o454* allele (hereafter designated *o454-2::kan*), following the above described procedure, by inserting the *kan* determinant into the *A*/III site at position 4389 (see Figure 1), which is found close to the end of *o454*. The new mutant strain was

named IC4440. The *o454-2::kan* allele was P1 transduced from IC4440 into V5701 and IC4364 (a *Sal*⁺ derivative from MC1000), both harbouring pIC755. A *Kan*^R *Sal*⁻ *Cm*^R transductant was selected from each transduction (IC4450 and IC4444) and the interruption of the chromosomal copy was corroborated by long-range PCR analysis (data not shown). Interestingly, the *o454-2::kan* allele conferred a growth dependence on pIC755 in the V5701 background (4% of survival at 42°C) but not in the MC1000 background, in which full survival was observed when cultures were plated at 42°C. All the surviving colonies recovered from plating the V5701 *o454-2::kan*/pIC755 strain at 42°C (on LAT *Kan* plates) were *Cm*^R. In contrast, only ~5% of the colonies recovered from plating the IC4364 *o454-2::kan*/pIC755 strain at that temperature were *Cm*^R, which means that pIC755 had been lost in most of them. This suggests that the 50K protein is not essential for growth of the MC1000 derivative. To prove this, whole-cell lysates prepared from *Cm*^S and *Cm*^R colonies recovered from plating the IC4364 *o454-2::kan*/pIC755 strain at 42°C were examined for the presence of 50K by Western blot analysis using the anti-53K antibody. As shown in Figure 7, lysates of the *Cm*^R colonies showed the 50K protein (produced by the *o454* wild-type copy present in the integrated plasmid) together with an additional 51 kDa polypeptide resulting from the fusion, in the *o454-2::kan* allele, of *o454* with an in-frame sequence of the *Kan*^R determinant (lane 5). In contrast, only the 51 kDa polypeptide was present in lysates of *Cm*^S (lane 6), which supports the idea that the 50K protein is not essential in the MC1000 background. Experiments performed with IC4442, a *Kan*^R *Sal*⁻ IC4364 derivative carrying the *o454-1::kan* allele, harbouring pIC755, produced similar results to those described for IC4364 *o454-2::kan*/pIC755, except that no 50K-related polypeptide was detected in lysates of the *Cm*^S colonies (Figure 7, lane 8), probably because, as mentioned above, the polypeptide encoded by the *o454-1::kan* allele is very unstable.

Additional experiments confirmed the important effect of the genetic background on the phenotype conferred by the null *o454* mutations. Thus, bacteriophage P1 was used to transduce the *o454-2::kan* allele from IC4440 into JC7623 and JC7623/λIC718. Approximately 300 *Kan*^R transformants were obtained with strain JC7623/λIC718, whereas no transductants were obtained when JC7623 was the recipient. Moreover, P1 transduction experiments showed that alleles *o454-1::kan* and *o454-2::kan* can be efficiently transduced from IC3652 and IC4440, respectively, into V5701 lysogenized by λIC718 and IC4364 but not into V5701. Finally, DEV16 derivatives in which the *trmE* allele was substituted for *o454-1::kan* or *o454-2::kan*, by P1 transduction from strains IC3652 and IC4440, respectively, were viable. Overall, these results indicate that null *o454* mutations are lethal depending on the genetic background: the 50K protein is essential in the genetic backgrounds of JC7623 and V5701, but not in those of the MC1000 and DEV16 strains.

Discussion

50K is a GTPase with peculiar biochemical properties

We have shown here that 50K is a GTPase that differs extensively from typical regulatory GTPases, such as p21.

Fig. 8. Schematic organization of functional domains in different families of GTPases. The three elements (G1, G3 and G4) of the consensus GTP-binding motif and the only invariant of G2 among GTPase families, a threonine residue, are indicated by black bars. A putative G2 motif for the 50K family, deduced from the alignment of the *E.coli* 50K protein with its homologues in Bacteria and eukaryotic organisms, is shown below the diagram of 50K. The C-terminal C-a-a-X motif (where 'a' represents an aliphatic residue and 'X' any residue) of the Ras proteins and its C-I-G-K homologue in 50K are also depicted. Arrows indicate the positions of the *MunI* and *AflIII* sites at which the Kan^R determinant was introduced. The location of the *trmE* mutation (Q192X) is indicated by a large arrowhead. The shaded area in the diagram of 50K represents the region of this protein that isolated by genetic manipulation still conserves the guanine nucleotide-binding and GTPase activities of the intact 50K molecule.

Thus, 50K shows a very high intrinsic GTPase hydrolysis rate (K_{cat} 26 min⁻¹, K_m 378 μ M) and can perform multiple cycles of GTP hydrolysis in the absence of accessory factors. Moreover, it exhibits a rather low affinity for GTP and an extremely low affinity for GDP. Finally, it is able to form self-assemblies. These biochemical properties of 50K are reminiscent of those of a newly emerging family of GTPases constituted by the Mx proteins, dynamins and yeast VPS1 (for reviews, see Staeheli *et al.*, 1993; Schmid *et al.*, 1998). Mx proteins are found in all interferon-treated vertebrate cells and some of them have been shown to possess antiviral activity. Dynamins play important roles in clathrin-mediated endocytosis and synaptic vesicle recycling. Finally, the product of the VPS1 gene is required for the sorting of soluble vacuolar proteins in the yeast *S.cerevisiae*. Common features of Mx, dynamins and VPS1 are, in addition to their high intrinsic GTP hydrolysis rates, the high molecular mass (ranging from 70 to 100 kDa) and the organization of the functional domains. In this respect, the tripartite GTP-binding consensus motif is located in the N-terminal region, whereas a small domain termed GED (for GTPase effector domain), which is present in the C-terminal region of Mx and dynamins, is required for GTPase activity (Schwemmle *et al.*, 1995; Schmid *et al.*, 1998). Apparently, GED interacts closely with the N-terminal GTPase domain to form the active centre of the enzyme. This organization differs extensively from that found in 50K (see Figure 8). In the bacterial protein, the GTPase domain resides in the C-terminal half and, when isolated by genetic manipulation and purified, still conserves the basic activities (guanine nucleotide binding and GTPase activities) of the intact 50K molecule (Figures 4 and 5), which suggests that the removal of the N-terminal region and the C-terminus should not substantially affect its tertiary structure. In addition, no built-in GTPase-activating protein domain, such as that carried by the α -subunits of the heterotrimeric G proteins (Markby *et al.*, 1993), is found in the G domain of 50K.

Therefore, the structural requirements for GTP binding and GTP hydrolysis by 50K or its G domain are without precedent. It will be of interest to crystallize the G domain of 50K and to determine its structure.

The GTPase cycle of 50K

GTPases are prime candidates for molecular switches as they can assume distinct conformations in the GTP- and GDP-bound states (Bourne *et al.*, 1991). Typically, the GTP-bound form represents the active state of regulatory GTPases, whereas the GDP-bound form represents the inactive state. Cells regulate the ratio of active and inactive monomeric GTPases by modulating the rates of GDP release and GTP hydrolysis. A GTPase-activating protein (GAP) determines the rate of conversion of the GTP-bound form to an inactive GDP-bound form, whereas a guanine nucleotide-exchange protein (GEP) catalyses the release of bound GDP, allowing GTP binding and subsequent GTP hydrolysis. The biochemical parameters of the 50K-associated GTPase, which we have determined here, allow us to describe the GTPase cycle of 50K *in vivo*, which may be essentially similar to that described for the Mx proteins (Richter *et al.*, 1995) and very different to that of the Ras-like GTPases (Bourne *et al.*, 1991; Kjeldgaard *et al.*, 1996). The concentration of GTP in prokaryotic cells has been estimated to be ~900 μ M (Neuhard and Nygaard, 1987), which is above the K_d of 50K for this nucleotide (280 μ M). The intracellular concentration of GDP is ~100 μ M (Neuhard and Nygaard, 1987) and thus far below the K_d of 50K for GDP (> 1 mM). Consequently, most of the 50K molecules may be bound to GTP under physiological conditions, but once 50K-bound GTP is hydrolysed the newly formed GDP will be released very rapidly, and the empty nucleotide-binding pocket of 50K will be ready to accommodate a new GTP molecule. The biochemical parameters of 50K predict that the GTPase cycle proceeds without auxiliary factors. Nonetheless, to prevent futile degradation of GTP, the GTPase cycle of 50K may be regulated *in vivo*. Our finding that the GTPase domain of 50K binds GTP γ S with 5-fold more affinity than the entire protein suggests that other domains of 50K modulate the access of GTP to the protein and that binding rather than hydrolysis of GTP may be a regulating step of the GTPase cycle. On the other hand, the extreme instability of the 50K-GDP complexes under physiological conditions suggests that, as proposed for Mx proteins (Richter *et al.*, 1995), the empty state rather than the GDP-bound state may represent the inactive conformation also in the case of 50K.

The evolutionarily conserved 50K protein is involved in tRNA modification

50K shows a significant similarity with proteins of *S.cerevisiae* and *Caenorhabditis elegans* as well as with several expressed sequence tags from *Arabidopsis thaliana*, mouse and human. This evolutionary conservation between distantly related species suggests that 50K and its homologues possess a fundamental cellular role. It is noteworthy that the homologue to 50K in yeast (called MSS1) has been demonstrated to be a nuclear-encoded mitochondrial protein (Decoster *et al.*, 1993). Phylogenetic trees constructed from aligned rRNAs show purple bacteria to be the closest living relatives to

mitochondria (Olsen *et al.*, 1994). Therefore, the eukaryotic '50K' gene might have evolved from a 50K gene present in the mitochondrial ancestor. Consistent with this idea, we have not been able to detect a homologue of the 50K bacterial protein in the genomes of Archaea determined so far.

Results presented here definitively prove that *o454* is allelic with *trmE*. It has been shown previously that *trmE* mutants are defective in the biosynthesis of the hypermodified nucleoside $\text{mnm}^5\text{s}^2\text{U}$, which is found in the wobble position of bacterial tRNAs specific for lysine, glutamic acid and possibly glutamine (see Krüger *et al.*, 1998 and references therein). The synthesis of $\text{mnm}^5\text{s}^2\text{U}$ is complex and requires several enzymatic reactions (Hagervall *et al.*, 1987):

The genetic symbols for genes involved in tRNA modification have recently been revised and it was suggested that *trmU* (or *asuE*) be replaced by *mnmA*, *trmE* by *mnmE*, and *trmC* by *mnmC* (Leung *et al.*, 1998). The *mnmA* gene product (Sullivan *et al.*, 1985) carries out the thiolation step. The *mnmE* gene product is responsible for the first step of the modification in the 5-position, but it is unclear how many steps precede the formation of $\text{cmnm}^5\text{s}^2\text{U}$ (Elseviers *et al.*, 1984; Hagervall *et al.*, 1987). Modifications in the 2- and 5-positions occur independently of each other (Elseviers *et al.*, 1984; Sullivan *et al.*, 1985), and several attempts at isolating a double mutant strain having a completely unmodified U34 have been unsuccessful (Krüger and Sørensen, 1998). The *mnmC* gene has two enzymatic activities. One activity demodifies $\text{cmnm}^5\text{s}^2\text{U}$ to $\text{nm}^5\text{s}^2\text{U}$ and the other catalyses the *S*-adenosyl-methionine-dependent methylation of $\text{nm}^5\text{s}^2\text{U}$ (Hagervall *et al.*, 1987). The *mnmC1* and *mnmC2* mutations abolish these two activities, respectively.

The *mnmA*, *mnmE* and *mnmC* genes are scattered on the bacterial chromosome (Björk, 1996). *mnmA* and *mnmC* are located at 25.3 and 50 min on the *E. coli* chromosome map, respectively. *mnmE* lies in the *rpmH* region (83 min) and might be autonomously regulated since it has its own promoter region, and a constitutive terminator located between *o548* and *o454* (see Figure 1) abates most transcription coming from upstream promoters (F. Macián, J.C. Escudero and M.E. Armengod, in preparation). At present, whether a regulatory device operates to coordinate expression of the genes involved in the synthesis of mnm^5s^2 remains to be established.

Null *o454* (*trmE*, *mnmE*) mutations can give rise to synthetic lethal phenotypes

Our results indicate that the null *mnmE* (*o454*) mutations are lethal depending on the genetic background. In *E. coli*, some 50 genes (~1% of the genome) are devoted to tRNA modification (Björk, 1996). Although the modified nucleosides influence the function of tRNA, no modified nucleoside has been shown to be essential for viability so far. Curiously, Persson *et al.* (1992) have found that total absence of $\text{m}^5\text{U54}$ from *E. coli* tRNA scarcely affects the growth rate, but disruption of the gene for tRNA($\text{m}^5\text{U54}$)-methyltransferase (*trmA*) is lethal. Thus, these authors have

proposed that *trmA* has two different catalytic activities: the synthesis of $\text{m}^5\text{U54}$ and another, as yet unknown, essential function. On the other hand, the slowed-growth effect that results from mutation of the *E. coli* *hisT* gene (renamed *truA*; see Leung *et al.*, 1998), which encodes pseudouridine synthase I, has been described as 'catastrophic' (Tsui *et al.*, 1991). The *hisT* gene product catalyses pseudouridylation in anticodon stems and loops, and is required for normal growth of *E. coli* because a lack of *hisT*-mediated pseudouridine tRNA modification causes a uracil requirement that interferes with cell division. Our results constitute a new example of a complex situation in which the lack of a nucleoside-modifying enzyme may be deleterious.

It is surprising that *mnmE* (*o454*) was essential in JC7623 and V5701 and dispensable in DEV16 and MC1000. Interestingly, mutations in *MSS1*, the *S. cerevisiae* gene evolutionarily related to *o454*, render the yeast cells respiratory deficient only in the presence of paromomycin-resistance (*par*^R) mutations (Decoster *et al.*, 1993), which reside in the mitochondrial 15S rRNA gene and may alter the conformational changes involved in the decoding process (Fourmy *et al.*, 1998). Double *MSS1 par*^R mutants generate spontaneous respiratory-competent revertants that are still paromomycin resistant (Decoster *et al.*, 1993). Since these mutants were shown to carry the suppressor mutations on the 15S rRNA sequence, it was concluded that the action of the *MSS1* gene product is associated with the function(s) of the small subunit of mitoribosomes (Decoster *et al.*, 1993). These results, together with those shown here, prompt us to postulate that the lack of the *o454* gene product may be tolerated depending on features of other elements involved in translation. Combination of mutations affecting the decoding process (such as certain mutations of ribosomal genes and genes encoding RNA-modifying enzymes), which separately do not reveal an obvious defect in growth, can give rise to lethal phenotypes, most likely due to synergism. This idea extends that proposed by Simos *et al.* (1996) for multifactorial lethality in yeast, claiming that combination of mutations affecting different steps in tRNA biogenesis, such as transcription, modification, splicing, 5'/3' end processing, transport and aminoacylation, can result in synthetic lethal phenotypes. Our proposal does not exclude the possibility that 50K possesses a second function, distinct from that of tRNA $\text{cmnm}^5\text{U34}$ synthesis, associated with the function of the small ribosomal subunit and essential in specific backgrounds.

Assembly and subcellular localization of 50K

Although results from gel filtration and *in vitro* cross-linking (Figure 2B and D) clearly indicate that 53K is able to form self-assemblies, the possibility that 50K may also be associated to other proteins cannot be ruled out. The formation of multienzyme complexes may be especially relevant for those tRNA modifications that, like $\text{mnm}^5\text{s}^2\text{U34}$, occur as a result of a pathway of enzymatic events where substrate channelling of the intermediate species may enhance efficiency (García and Goodenough-Lashua, 1998). On the other hand, most tRNA modifications occur at the polynucleotide level, i.e. after the primary transcript has been synthesized and concomitantly

with the action of exo- and endonucleases, which operate in the maturation process (see Lane, 1998 and references therein). Thus, the possibility that 50K forms complexes with other enzymes involved in tRNA biogenesis should also be considered. The RNA-processing enzymes RNase III, RNase E and RNase P have been reported to be associated with the inner membrane (Miczak *et al.*, 1991; Srivastava *et al.*, 1991), although further experiments are required to ascertain the real distribution of these enzymes in the different cellular compartments (Miczak *et al.*, 1991; Srivastava *et al.*, 1991; Py *et al.*, 1994). RNase III and RNase E play major roles in the processing of rRNA, whereas RNase P (whose protein component is synthesized by *rnpA*; see Figure 1) is responsible for the processing of the 5' end of all tRNAs. Our results suggest that 50K is partially associated with the inner membrane (Figure 6; Table I). The possibility that enzymes involved in the tRNA maturation process form a processome at the membrane (as postulated by Miczak *et al.*, 1991) to which tRNA-modifying enzymes, like 50K, become associated is an exciting theory that merits being tested. It might give information about the spatial organization of the stable RNA (rRNA, tRNA) biogenesis machinery.

Our *in vivo* cross-linking experiments have allowed the detection of two bands of 100 and 150 kDa, respectively, using the polyclonal anti-53K antibody (Figure 2C). The disappearance of the 100 kDa band upon boiling demonstrates it to be the result of cross-linking by formaldehyde. However, boiling could not reverse cross-links from the 150 kDa complex. Jackson and Chalkley (1974) reported that formaldehyde can introduce covalent bonds in compact forms of molecular complexes but not in extended forms. Therefore, it is possible that compact and more relaxed complexes of 50K, depicting different functional or activation states of the protein, co-exist in *E.coli* and that the boiling-resistant 150 kDa band detected after the formaldehyde treatment represents a compact complex of 50K. That the 150 kDa band is not an artefact is supported by the fact that it was not detected in strain DEV16, which lacks the 50K protein, but it was detected in DEV16 transformed with pIC576, a plasmid carrying a wild-type copy of *o454* (data not shown). Although the apparent molecular mass of this complex corresponds to that of a 50K trimer, the possibility that it results from interaction of 50K with other molecules cannot be discarded. Moreover, the rapid rate of fixation of the 150 kDa complex by formaldehyde (see Figure 2C) suggests a peripheral location of this complex in the cell.

Domain structure of 50K

The results presented here indicate that 50K is a multi-domain protein, whose structure is shown in Figure 8. Further experiments are required to understand the role of these putative domains in the function(s) of 50K. The ~224 amino-acid N-terminal domain may play a role in 50K self-assembly by non-covalent interactions (H.Cabedo, unpublished results).

The ~78 amino-acid C-terminal domain of 50K contains the tetrapeptide motif C-I-G-K at the extreme C-terminus. The C-terminal cysteine might be involved in the membrane association of 50K as it occurs with other GTPases containing C-terminal cysteines (Bourne *et al.*, 1990). However, the C-terminal cysteine of 50K might also be

involved in a putative tRNA-modifying activity of 50K, as cysteine residues have been shown to be responsible for methyltransferase activity of some tRNA-modifying enzymes (Kealey and Santi, 1991).

Finally, the GTPase domain of 50K exhibits structural features that endow it with a high endogenous GTPase activity and a very low affinity for GTP and GDP. Whether the GTPase activity of 50K plays some role in the tRNA-modifying function of this protein remains to be elucidated. Many RNA-modifying enzymes utilize some energy source, often the hydrolysis of ATP, to drive catalysis (García and Goodenough-Lashua, 1998). However, as far as we know, 50K would be the first example of an RNA-modifying enzyme using GTP.

Materials and methods

Strains, plasmids, phages, media and enzyme assays

Bacterial strains and plasmids used in this study are listed in Table III. Genetic techniques for the construction of strains and β -galactosidase assays were performed as described by Miller (1992), unless otherwise indicated. λ 718, which carries a transcriptional *tnaA::lacZ* fusion, was constructed by homologous recombination between pIC718 and λ RZ5 (see Macián *et al.*, 1994). LBT and LAT media have been described previously (Armengod *et al.*, 1988). Cells were routinely grown in LBT. Antibiotics were added as recommended (Miller, 1992).

General DNA and protein techniques

For DNA manipulations, standard procedures were followed (Sambrook *et al.*, 1989). A digoxigenin labelling and detection kit (Boehringer Mannheim) was used to probe Southern blots. DNA sequencing was performed using a 377 Automated DNA Sequencer of Applied Biosystems. Long-range PCR reactions were performed by the EXPAND PCR System (Boehringer Mannheim), according to the manufacturer's protocol.

A GST-50K protein fusion was constructed by cloning the 2619 bp *HindIII*-*NruI* fragment (Figure 1; bp 3033-5652) from pFM1 into the *EcoRI* site of pGEX-2T, previously treated with DNA polymerase I Klenow fragment to fill in the protruding ends. The resulting plasmid, pIC684, carries an in-frame fusion between GST (26 kDa) and *o454* by means of a bridge of 23 amino acids, which are encoded by the 69 triplets extending from the *HindIII* site at bp 3033 and the *o454* translational start signal at bp 3102 (see Figure 1). Positive clones were selected by restriction analysis and IPTG induction of a GST fusion protein of ~80 kDa. A fusion between the 17 kDa GTPase domain of 50K and the GST was constructed by PCR as follows. A DNA fragment extending from nt 3736 to 4232 was amplified by PCR from plasmid pFM1 using primers partially complementary to its 5' and 3' ends (5'-GCGGATCCTTGTTCGCGAAGG-3' and 5'-GCGAATTCCTAT-GCTCTGTTTGAG-3', respectively). The amplified fragment was digested at completion with *EcoRI* and partially with *BamHI*, and cloned into pGEX-2T previously cut with the same enzymes, generating plasmid pIC758. This construction, which was verified by DNA sequencing, contains an in-frame fusion between GST and the GTPase domain of 50K in such a way that GST is fused to codon 210 of 50K and four amino acids (IHRG) are added to the last codon of 50K that has been included in the construct (codon 377).

Site-directed oligonucleotide mutagenesis of the *o454* sequence included in pIC684 was performed by PCR according to the procedure described by Papworth *et al.* (1996). The mutagenic oligonucleotides used as primers in the PCR to convert Cys451 to Ser451 were 5'-GGATTTTCTCCAGCTTCAGTATTTGGTAAGTAACC-3' and 5'-GGTACTTACCAATACTGAAGCTGGAGAAAATCC-3'. The resulting plasmid was named pIC805.

Expression and purification of the GST fusion proteins were carried out essentially as reported by Smith and Johnson (1988).

SDS-PAGE and immunoblotting were performed as described (Sambrook *et al.*, 1989). Bound antibodies were visualized on immunoblots by enhanced chemiluminescence (ECL; Amersham). The anti- β -galactosidase antibody was obtained from Promega.

Production of antisera against 53K

New Zealand rabbits were inoculated with the purified 53K protein, after a pre-immune serum sample had been taken. The animals were

Table III. *Escherichia coli* strains and plasmids used in this study

Strain or plasmid	Description ^a	Reference
<i>E. coli</i> strains		
BL21(DE3)	<i>E. coli</i> B F ⁻ <i>dcm cmpT hsdS gal</i> (λDE3)	Studier <i>et al.</i> (1990)
C600	<i>thi-1 thr-1 leuB6 lacY1 tonA21 supE44 mcrA</i>	Bachmann (1996)
DEV16	F ⁻ <i>thi-1 rel-1 spoT1 lacZ105_{UAG} trmE Val^R</i>	Elseviers <i>et al.</i> (1984)
JC7623	<i>recB21 recC22 sbcB15 sbcC201 Sal⁻</i>	Bachmann (1996)
K38	HfrC (λ)	Russel and Model (1984)
MC1000	F ⁻ <i>araD139 Δ(ara-leu)7679 galUK Δ(lac)X74 rpsL thi</i>	Silhavy <i>et al.</i> (1984)
NCM631	BL21(λDE3) Δ(lac) Tn10	Govantes <i>et al.</i> (1996)
V5701	<i>bgl</i> (Sal ⁺)	Armengod and Blanco (1978)
IC3037	MC1000/pIC576	this work
IC3038	K38/pGP1-2, pIC576	this work
IC3472	NCM631/pIZ227	this work
IC3492	IC3472/pIC576	this work
IC3647	JC7623 (λIC718)	this work
IC3652	IC3647 <i>o454-1::kan</i>	this work
IC4126	V5701/pIC755	this work
IC4130	Kan ^R Sal ⁻ Cm ^R transductant of IC4126 from donor IC3652	this work
IC4133	Tet ^R <i>recA13</i> transductant of IC4130 from a donor <i>recA13 srl::Tn10</i>	this work
IC4364	Sal ⁺ transductant of MC1000 from donor V5701	this work
IC4440	IC3647 <i>o454-2::kan</i>	this work
IC4442	Kan ^R Sal ⁻ Cm ^R transductant of IC4364/pIC755 from donor IC3652	this work
IC4444	Kan ^R Sal ⁻ Cm ^R transductant of IC4364/pIC755 from donor IC4440	this work
IC4450	Kan ^R Sal ⁻ Cm ^R transductant of V5701/pIC755 from donor IC4440	this work
Plasmids		
pGP1-2	T7 RNA polymerase gene under control of the λP _L promoter	Tabor and Richardson (1985)
pLysE	pACYC184 derivative carrying the T7 lysozyme gene	Studier <i>et al.</i> (1990)
pIZ227	pLysE derivative carrying <i>lacI^q</i>	Govantes <i>et al.</i> (1996)
pT7-12	vector for overexpression of proteins under the T7 promoter	Brown <i>et al.</i> (1991)
pFM1	chromosomal DNA fragment 980–9847 inserted between the <i>HindIII</i> and <i>EcoRI</i> sites on pT7-12	this work
pFM2	chromosomal DNA fragment 980–4568 inserted between the <i>HindIII</i> and <i>EcoRI</i> sites on pT7-12	this work
pGEX-2T	parent for GST fusions	Smith and Johnson (1988)
pKK223-3	vector for overexpression of proteins under the <i>tac</i> promoter (Ptac)	Pharmacia Biotech
pUC4K	plasmid containing the Kan ^R determinant	Vieira and Messing (1982)
pMAK700	Cm ^R , temperature-sensitive replicon	Hamilton <i>et al.</i> (1989)
pIC552	parent for <i>lacZ</i> transcriptional fusions	Macfán <i>et al.</i> (1994)
pIC576	chromosomal DNA fragment 2916–4568 inserted between the <i>HindIII</i> and <i>EcoRI</i> sites on pT7-12	this work
pIC684	GST fusion of DNA fragment from 3033 to 5652	this work
pIC712	<i>SalI</i> fragment (including Ptac) from pKK223-3 inserted into <i>XhoI</i> -cut pIC552	this work
pIC714	pIC712 deleted for the Ω fragment by <i>HindIII</i> digestion	this work
pIC718	<i>HincII</i> – <i>BstXI</i> fragment 3033–5310 inserted into <i>SmaI</i> -cut pIC714	this work
pIC720	1.3 kb <i>EcoRI</i> fragment (Kan ^R determinant) from pUC4K inserted into the unique <i>MunI</i> site (nt 3620) on pFM1	this work
pIC755	<i>HindIII</i> fragment 980–7374 inserted into <i>HindIII</i> -cut pMAK700	this work
pIC758	GST fusion of DNA fragment from 3736 to 4232	this work
pIC805	pIC684 derivative containing mutation C451S in 50K	this work
pIC885	1.3 kb <i>BamHI</i> fragment (Kan ^R determinant) from pUC4K inserted into the unique <i>AflIII</i> site (nt 4389) on pFM1	this work

^aThe numbering system used to define *E. coli* chromosomal fragments present in some plasmids is that used in Figure 1.

bled and re-inoculated at the appropriate times. Serum was aliquoted and stored at –80°C in 0.02% (w/v) sodium azide. Before use, the antiserum was affinity purified on nitrocellulose-bound 53K.

Gel filtration chromatography

Purified 53K protein (2.5 mg) was first loaded onto a PD-10 desalting column (Pharmacia) equilibrated with buffer A containing 50 mM Tris–HCl pH 7.5, 1 mM 2-ME, and then applied, in a final volume of 3 ml, to a column (1.5 × 50 cm) of Sephacryl S200 (Pharmacia) equilibrated with buffer A. Protein was eluted with 150 ml of the same buffer at a flow rate of 1 ml/min. Fractions of 1.0 ml were collected, and an aliquot (20 µl) of every second column fraction was analysed by SDS–PAGE (10% polyacrylamide) followed by Western blotting with the anti-53K antibody. For calibration of the molecular mass, thyroglobulin (669 kDa), catalase (232 kDa) and bovine serum albumin (69 kDa) were used as markers.

In vivo formaldehyde cross-linking

Cross-linking was performed as previously described (Skare *et al.*, 1993) on bacteria resuspended in 0.1 M sodium phosphate buffer pH 6.8 to OD₆₆₀ = 0.5. Formaldehyde was used at a final concentration of 1% (w/w). The samples were incubated at room temperature, 1 ml volumes were then removed at various time points, and the samples centrifuged immediately to pellet the whole cells. The pellets were solubilized in 3× concentrated Laemmli sample buffer and either heated at 60°C for 10 min to maintain the formaldehyde cross-links or at 95°C for 10 min to break the chemical cross-links. Protein samples were analysed by SDS–PAGE (8% polyacrylamide) followed by immunoblotting with the anti-53K antibody.

In vitro cross-linking

Purified protein (0.3 mg/ml) was cross-linked with DTSP at 1 mM in a final volume of 50 µl containing 80 mM Tris–HCl pH 7.5, 50 mM KCl,

100 mM NaCl, 2 mM MgCl₂, 1.5 mM CaCl₂ and 5% glycerol. DTSP (Sigma) was added from a stock solution (30 mM) in *N,N*-dimethylformamide. Control reactions were performed by incubating with *N,N*-dimethylformamide without DTSP. After 10 min at room temperature, all reactions were terminated by adding lysine (final concentration 150 mM) and incubating for an additional 15 min period. Sample buffer (10 µl) was added to each reaction, whose final volume was then divided into two parts. To one of them, was added 2-ME at 100 mM and all samples were incubated for 15 min at 37°C prior to non-reducing SDS-PAGE. Proteins were visualized by staining with Coomassie Brilliant Blue.

Covalent cross-linking of [α -³²P]GTP to purified 53K protein

Purified 53K (8 µg) was irradiated with UV light for 20 min in the presence of [α -³²P]GTP as described by RayChaudhuri and Park (1992). Protein samples were precipitated with trichloroacetic acid and subjected to SDS-PAGE followed by autoradiography.

Determination of dissociation constants

The purified protein (2.5–10 µM) was incubated with increasing concentrations of [γ -³⁵S]GTP (~20 Ci/mmol) or [8-³H]GDP (~5 Ci/mmol) in 25 µl of binding buffer (50 mM Tris-HCl pH 7.5, 50 mM KCl, 1 mM dithiothreitol, 2 mM MgCl₂, 5% glycerol) at 25°C for 30 min. Aliquots were applied to a nitrocellulose filter pre-soaked with binding buffer. After washing with 3 ml of nucleotide-free binding buffer, the filter was dried and the bound radioactivity was measured by scintillation counting. The binding constants (*K*_d values) were calculated using GraphPad Prism version 3.00 for Windows (GraphPad Software, Inc., San Diego, CA; www.graphpad.com) according to the manufacturer's instructions.

GTPase assay

Substrate saturation experiments were carried out by incubating the protein (2.5–10 µM) with increasing concentrations (0–2 mM) of [α -³²P]GTP (~10 Ci/mmol) in binding buffer at 25°C. Reactions were stopped by adding an equal volume of a solution containing 20 mM EDTA and 1% SDS, then tracer amounts of unlabeled GDP and GTP were added to the mixture. Samples (2–5 µl) were placed on polyethyleneimine (PEI)-cellulose and thin-layer chromatography was carried out in 0.6 M LiCl at room temperature. The chromatogram was dried and the spots corresponding to GTP and GDP were identified with UV light, cut out and counted in a liquid scintillation counter to quantify GTP hydrolysis.

Cellular fractionations

Subcellular fractions of cells were prepared essentially as described (Forst *et al.*, 1988). Briefly, cells were collected, suspended in 20 mM sodium phosphate buffer pH 7.0, and disrupted by sonication. Cell debris were removed from the lysate by centrifugation (2000 g for 15 min). Cell envelopes were separated from the soluble fraction (containing cytoplasmic and periplasmic proteins) by ultracentrifugation (100 000 g for 30 min). Cytoplasmic membrane proteins were separated from outer membrane proteins by selective solubilization in sodium *N*-lauryl sarcosinate (30 min at room temperature) followed by ultracentrifugation (100 000 g for 30 min, twice). Fractions were tested by Western blot analysis with affinity-purified anti-53K antiserum (and anti- β -galactosidase antibody, when appropriate) for the same amount of bulk protein in each fraction.

Immunoelectron microscopy

Cells were fixed for 2 h at 4°C in a mixture of 1% glutaraldehyde and 1% paraformaldehyde, buffered with 0.1 M PIPES pH 7.3, containing 0.1% MgCl₂ and 0.05% CaCl₂, and washed overnight at 4°C in the same solution without the fixatives. Dehydration and embedding in LR White (Polysciences Ltd, Northampton, UK) were carried out following the manufacturer's instructions, using ethanol as dehydrating agent. Sections were mounted on 200-mesh nickel grids. The IGS technique (De Mey, 1983) was carried out as described (Knecht *et al.*, 1986), using 20.1 ± 2.1 nm gold particles (prepared from HAuCl₄ reduced with trisodium citrate) conjugated with anti-rabbit IgG by conventional procedures (Beesley, 1989). After the immunocytochemical procedure, grids were stained in the dark at 20°C for 15 min with 5% aqueous uranyl acetate and then for 40 s with alkaline lead citrate. Controls included incubations with pre-immune IgGs instead of the primary antibody and incubations with immunogold alone. Samples were incubated simultaneously and using the same immunogold conjugates to minimize variations due to different proportions of unconjugated IgG, different gold sizes and different antibody preparations.

Morphometric analysis was performed by determining the area of the various compartments and by counting the associated gold particles. For each measurement, >20 different electron micrographs, representing 40–60 randomly selected cells, were analysed. Micrographs were taken at random (cell areas located in the upper right corner of the supporting nickel grids) from three different grids corresponding to three different cell cultures. The lateral resolution of the procedure was calculated to be 26 nm, which corresponds to the addition of IgG_{rabbit} and IgG_{goat} sizes (~8 nm, each) and the gold particle radius (~10 nm) (Roth, 1982; Knecht *et al.*, 1986). Thus, a line was drawn on the electron micrographs following the inner membrane of the cells with a thickness corresponding to 52 nm (2 × 26 nm). This established an 'inner membrane-associated area' (Bayer *et al.*, 1987). The outer membrane plus periplasm area was considered to be that limited by the inner membrane area and a new line bordering from the outside, and at a distance of 26 nm, the contour of the outer membrane of the cell. Finally, the cytoplasm area was defined as the cell area bordered by the inner membrane area. For all these compartments, the number of gold particles was counted and areas were determined by the point-counting method using a multipurpose test system (18 × 24 cm) containing 154 test points on it as described by Weibel and Bolender (1973). Gold labelling densities are expressed as number of gold particles/µm² of each cell compartment.

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