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Era, an Essential *Escherichia coli* Small G-Protein, Binds to the 30S Ribosomal Subunit

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Era is an essential G-protein in *Escherichia coli* identified originally as a homologue protein to Ras (*E. coli* Ras-like protein). It binds to GTP/GDP and contains a low intrinsic GTPase activity. Its function remains elusive, although it has been suggested that Era is associated with the cytoplasmic membrane, cell division, energy metabolism, and cell-cycle check point. Recently, a cold-sensitive phenotype was found to be suppressed by the overexpression of 16S rRNA methyltransferase, suggesting Era association with the ribosome. Here we demonstrate that Era specifically binds to 16S rRNA and the 30S ribosomal subunit. Both GTP and GDP, but not GMP, inhibit Era binding to ribosomal component. Involvement of Era in protein synthesis is suggested by the fact that Era depletion results in the translation defect both *in vitro* and *in vivo*.

Era is an *Escherichia coli* small G-protein (1, 2). Era have considerable homology with bacterial G-proteins such as *E. coli* EF-Tu, EF-G, IF2. Homologues of Era have been identified in every bacterium sequenced to date making it an attractive target for possible antibacterial drug development. However, the finding of Era in human, mouse and antirrhinum indicated that the function of Era is not limited within bacteria (3, 4). Deletion of *era* gene is lethal to *E. coli*. Depletion of Era affects various cellular processes such as cell division, and carbon assimilation, and certain mutations in Era are associated with the cold sensitive phenotype of *E. coli* (5–8). A possible role of Era in cell cycle progression has also been proposed (3). However, despite these demonstrations from genetic experiments the function of Era still remains elusive.

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Previously, we have shown that the overexpression of *ksgA* gene for 16S rRNA transmethylase suppressed the cold-sensitive phenotype of Era (E200K) (8). Era contains an RNA-binding KH-like domain (9) at the C-terminal region (A. Lupas, personal communication; and also described in Ref. 10), suggesting a possibility that Era is an RNA binding protein. Bacterial GTP-binding proteins are known to be involved in protein synthesis (11). Our present findings that Era binds to 30S ribosomal subunit along with the observation of inhibition of ribosomal protein synthesis machinery in Era depleted cells suggest that Era function is related with protein synthesis mechanism of the cell and may thus explain its essential characteristic. It is possible that there is an obligatory interaction of cell division and initiation of protein synthesis and Era may play a central role in signaling of such interactions.

MATERIALS AND METHODS

Preparation of ribosomal fractions. Era depletion was carried out by shifting the growing *E. coli* strain CL213 (Δ*era::kan F′lacI*^{q}) (6) to 43°C for 4 h. Wild-type (JM83) and Era-depleted cells grown under identical condition were broken at 900 psi × 2 at 4°C. S100 fractions from the wild-type cells and Era-depleted cells were prepared as described in Ref. 12. Ribosomal fractions were precipitated out from the cytosolic fractions by ultracentrifugation at 350,000*g* at 4°C for 75 min. rRNA was obtained from *E. coli* ribosomes after phenol extractions.

Sucrose-density gradient fractionations. Unless otherwise stated, sucrose gradients (5–25 and 3–10%) were prepared in Beckman ultra centrifuge tubes (14 × 89 mm) in the buffer A containing 10 mM Tris–HCl (pH 7.5), 0.25 mM $MgCl_2$ and 1 mM DTT. Interaction between Era and ribosomes (or rRNAs) were carried out at 4°C for 30 min in a 100-μl reaction mixture in buffer A containing 50 pmol of Era, and 1 mg of ribosome (or 100 μg rRNA) extracted from *E. coli*. To examine the effects of guanine nucleotides, GTP (also GTPγS), GDP or GMP were included both in the gradient and in the reaction mixture at a final concentrations of 100 μM. Each reaction mixture was applied on the top of the gradient and subjected to centrifugation at 39,000 rpm in a SW41 rotor at 4°C for 3.5 h followed by fractionation into 30 to 35 fractions using a peristaltic pump. Fractions thus obtained were loaded onto a 10% SDS–PAGE, transferred onto nitrocellulose membrane and immunoblotting was performed with anti-Era antibody.

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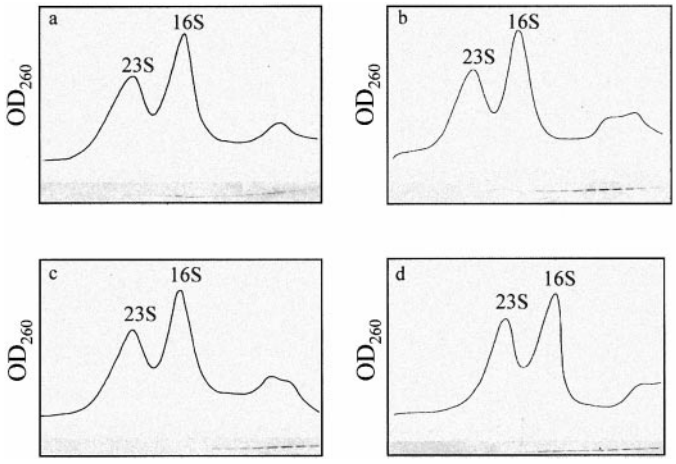

FIG. 1. Cosedimentation of Era with *E. coli* rRNA. Era and rRNA preparations were mixed in the presence of (a) none, (b) GTP, (c) GDP, and (d) GMP, and the mixture was separated into 30–35 fractions by 3–10% sucrose gradient ultracentifugation. Era in each fraction was detected by Western blot with anti-Era antibody.

In vitro translation assay. *In vitro* transcription of mRNAs of OmpF-Lpp and DHFR from the plasmids containing their respective genes were performed using SP6 RNA polymerase (Boehringer-Mannheim) and the various components of the cell-free translation systems were assembled as described previously (12). [^{35}S]Methionine-labeled translated products were loaded on to a polyacrylamide gel as described in Ref. 13. Dried gel was exposed on to X-OMAT film (Kodak).

RESULTS AND DISCUSSION

Era was incubated with E. coli rRNA at 4°C in the reaction buffer containing 0.25 mM Mg^{2+}. After 30 min of incubation, the mixture was fractionated to 30 fractions by a 3–10% sucrose density gradient ultracentrifugation (Fig. 1). Each fraction was run onto SDS–PAGE followed by Western blot analysis with anti-Era antibody shown at the bottom of each pattern. Era was specifically detected in the fractions corresponding to 16S rRNA (Fig. 1a). No Era was detected in the fractions corresponding to 23S rRNA. Unbound free Era was stayed in the upper part of the gradient.

Next we examined the effect of guanine nucleotides on Era-16S rRNA binding by adding GTP, GDP and GMP in the binding reaction. Addition of GTP and GDP in the binding reaction completely abolished Era interaction with 16S rRNA (Figs. 1b and 1c, respectively). When nonhydrolyzable analogue, GTPγS, was used instead of GTP, similar results were obtained (data not shown). However, GMP did not show any inhibitory effect on Era-rRNA interaction (Fig. 1d). Since, EF-Tu, EF-G, and IF2, three other *E. coli* G-proteins, required for protein synthesis, bind to ribosome (14), we next examined if Era binds to ribosome. Era and ribosome were mixed in the presence of 0.25 mM Mg^{2+} and after 30 min of incubation the mixture was subjected onto a 5–25% sucrose density gradient ultracentrifugation. The gradient was fractionated into 30 to 35 fractions. The presence of Era in each fraction was examined by performing SDS–PAGE followed by Western blot analysis with anti-Era antibody as above. Consistent with Era binding to 16S rRNA, Era was found to be specifically cofractionated with the small 30S subunit (Fig. 2a). It did not bind with large 50S subunit. Unbound free Era was detected in the upper fractions corresponding to lower sucrose gradient fractions and quite distinctively separated from ribosomal (30S unit) fractions. The addition of GTP and GDP inhibited this Era-ribosome interaction (Figs. 2b and 2c, respectively). The above results indicate that GTP- or GDP-bound forms of Era have reduced or inhibited binding ability to ribosomal components. It is well known that Ras proteins bind to both GTP and GDP with identical K_d values. Because the concentration of GTP in the cell is much higher (~10-fold) than that of GDP, in the presence of guanine exchange factor (GEF) Ras preferably becomes loaded with GTP to activate downstream signaling (15, 16). Similarly, it is possible that Era binding to ribosomal component is regulated by GTP, but not by GDP, *in vivo.*

Most of the G-proteins in bacteria have found to be associated with protein synthesis (11). The GTPase domain of Era has considerable homology with those of protein synthesis factors such as EF-Tu, EF-G, and IF-2 (1) and the present study demonstrates Era association with ribosomes. The above finding that Era binds to ribosome prompted us to examine the role of Era in protein synthesis, using Era-depleted cells. Era depletion was carried out using *E. coli* CL213 (Δ*era::kan F′lacI*^{q}) harboring a temperature-sensitive plasmid containing the wild-type *era* gene (6). Cells were incubated at 43°C for 4 h, where the content of Era was reduced to approximately 10% compared to that of wild-type cells, as judged by Western blot analysis. The ratio of RNA to protein in Era-depleted cells significantly increased (approximately twofold) in comparison with that of the wild-type cells. It was found that Era depletion seriously affected protein synthesis in the cell, as was observed with [^{35}S]methionine labeling of total protein synthesis after shifting the growing *E. coli* CL213 to 43°C. To determine how Era depletion affected on protein synthesis, a cell-free protein synthesis system (S100 fraction) was prepared from the Era-depleted cells and also from wild-type cells under identical condition. The mRNA for a hybrid protein, OmpF-Lpp, was used for the cell-free system (12). The cell extracts from Era-depleted cells was unable to translate *ompF-lpp* mRNA (Fig. 3A, lane 1), while the cell-free system from wild-type cells gave the 6 kDa OmpF-Lpp polypeptide product (Fig. 3A, lane 2). The translation inability of Era-depleted cell extract was confirmed with another mRNA (mRNA for DHFR, data not shown), indicating that the Era depletion causes a

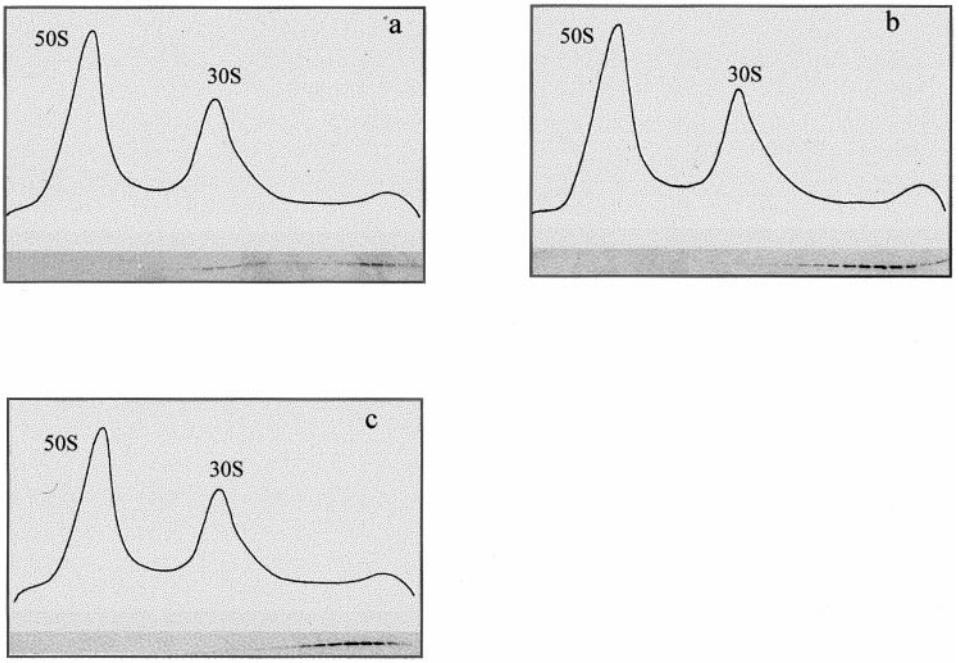

FIG. 2. Cosedimentation of Era with *E. coli* ribosomal particles. Era and ribosomes were mixed in the presence of (a) none, (b) GTP, and (c) GDP, and the mixture was separated into 30–35 fractions by 5–25% sucrose gradient ultracentifugation. Era in each fraction was detected by Western blot with anti-Era antibody.

global blockade in the protein synthesis activity and thus causes the inhibition of cell growth. It has to be noted that the addition of Era in the cell-free translation system from Era-depleted cells could not resume the protein synthesis, indicating that Era is not required as a direct factor in protein synthesis. We have also found that 70S ribosomes are abnormally accumulated in Era-depleted cells, whereas wild-type cells contain 70S ribosomes as well as 30S and 50S subunits. Previously, Nashimoto reported that a cold-sensitive mutation of Era resulted in defective processing of 16S rRNA (17). In consistent with this report, we have also found that the accumulated 70S ribosomes in Era-depleted cells contain unprocessed 16S rRNA (data not shown).

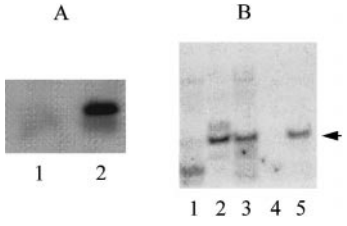

FIG. 3. *In vitro* translation assay with cell extracts prepared from wild-type cells and Era-depleted cells. The *ompF-lpp* mRNA was used as template transcript. (A) Translation assay was performed with S100 extracts from Era-depleted cells (lane 1) and wild-type cells (lane 2). (B) Translation assay was performed with reconstituted S100 system by cytosolic (Cyt) and ribosomal (Rib) fractions from Era depleted cells (CL) and wild-type (wt) cells with different combinations such as wt Cyt and wt Rib (lane 3); wt Cyt and CL Rib (lane 2); CL Cyt and CL Rib (lane 4); CL Cyt and Rib (lane 1); and CL Cyt and CL Rib supplemented with wt Cyt (lane 5) as described in the text. Arrow indicates the position of OmpF-Lpp.

To further examine if the defect in translation is caused at the level of ribosomes or at cytoplasmic soluble factor(s), we fractionated the ribosomal and postribosomal (cytosolic) fractions by ultracentrifugation. Cell-free translation was carried out with different combination of ribosomal fractions (Rib) and cytosolic fractions (Cyt) from wild-type cells and CL213 Era-depleted cells (Fig. 3B). The combination of wild type Cyt and CL213 Rib efficiently translated the added *ompF-lpp* mRNA (Fig. 3B, lane 2) as well as that of wild-type Cyt and wild-type Rib (Fig. 3B, lane 3), whereas the combination of both fractions from CL213 failed to support any translation (Fig. 3B, lane 4). The addition of CL213 Cyt to wild-type Rib could not also support the translation of the mRNA (Fig. 3B, lane 1), indicating that the Era depletion resulted in defect (depletion or inactivation) in cytosolic factors required for protein synthesis. Interestingly, the protein synthesis was resumed by the S100 fractions (mixture of Rib and Cyt of CL213) if supplemented with as low as 10% amount of the wild-type Cyt required for translation assay in lane 2 (Fig. 3B, lane 5). However, the wild-type Cyt supplement after a heat treatment could not resume protein synthesis in S100 extracts from Era-depleted cell (data not shown). This indicates that an

essential 'putative' protein factor, which is abundant in wild-type cells, is depleted or defective in Era-depleted cells. Era may be required for expression (synthesis), folding, complex formation, peptide cleavage, or modification of this essential factor.

GTPases play a key role in ribosomal protein synthesis (14, 18, 19). All the major reactions, except peptide bond formation, involve the hydrolysis of GTP to GDP. On the basis of the present finding Era seems to be another GTPase related to ribosomal protein synthesis activity, although the action of Era seemed to be quiet different from those of EF-Tu and EF-G. First, EF-Tu and EF-G bind to 50S subunit by interacting with 23S rRNA, whereas Era binds to 30S subunit by interacting with 16S rRNA. Second, both GTP and GDP inhibits Era-ribosome (and 16S rRNA) binding. EF-Tu and EF-G become "activated" upon GTP binding which facilitates their association to ribosomes. When bound GTP is hydrolyzed by their intrinsic GTPase activity, a conformational change occurs and the resulting GDP-bound form is released from ribosomes. In contrast, Era is most likely not to be directly involved in protein synthesis, as the addition of Era to the cell-free system for the Era-depleted cells did not restore protein synthesis. However, it is difficult at this point to ascertain whether Era GTPase activity plays a role in the 'indirect' involvement of Era in ribosomal mechanics. Identification and characterization of the putative protein-synthesis factor described above would be vital in this regard. Nonetheless, the data presented here support the previous assumption that the cell cycle arrest in *era* mutants may be related with aborted protein synthesis (3).

Note added in proof. While this manuscript was being prepared a report on crystallographic analysis of *E. coli* Era has been published (20). The X-ray structure of Era has depicted a two-domain structure for Era: an N-terminal Ras-like domain and a C-terminal domain containing the probable RNA-binding site.

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