Translation Elongation by a Hybrid Ribosome in Which Proteins at the GTPase Center of the Escherichia coli Ribosome Are Replaced with Rat Counterparts*

Ribosomal L10/L7/L12 protein complex and L11 bind to a highly conserved RNA region around position 1070 in domain II of 23 S rRNA and constitute a part of the GTPase-associated center in Escherichia coli ribosomes. We replaced these ribosomal proteins in vitro with the rat counterparts P0/P1/P2 complex and RL12, and tested them for ribosomal activities. The core 50 S subunit lacking the proteins on the 1070 RNA domain was prepared under gentle conditions from a mutant deficient in ribosomal protein L11. The rat proteins bound to the core 50 S subunit through their interactions with the 1070 RNA domain. The resultant hybrid ribosome was insensitive to thiostrepton and showed poly(U)-programmed polyphenylalanine synthesis dependent on the actions of both eukaryotic elongation factors 1α (eEF-1α) and 2 (eEF-2) but not of the prokaryotic equivalent factors EF-Tu and EF-G. The results from replacement of either the L10/L7/L12 complex or L11 with rat protein showed that the P0/P1/P2 complex, and not RL12, was responsible for the specificity of the eukaryotic ribosomes to eukaryotic elongation factors and for the accompanying GTPase activity. The presence of either E. coli L11 or rat RL12 considerably stimulated the polyphenylalanine synthesis by the hybrid ribosome, suggesting that L11/RL12 proteins play an important role in post-GTPase events of translation elongation.

The “GTPase center” of the ribosome is a region involved in interaction with GTP-bound translation factors, GTP hydrolysis (1), and post-GTPase events including tRNA movements on the ribosome (2, 3). Translation elongation is markedly stimulated by the interaction of this region with two elongation factors in a GTP-dependent manner. The GTPase center includes two essential RNA regions around positions 1070 and 2660 (Escherichia coli numbering) of the 23 S/28 S rRNA (4), which appear to bind to the elongation factors (5, 6). Despite the highly conserved structure of the 1070 and 2660 RNA regions, ribosomes show a kingdom-dependent accessibility for translation factors, i.e., prokaryotic ribosomes do not engage in translation elongation with the eukaryotic factors instead of the prokaryotic factors (7–9). Furthermore, there are differences in the rate of GTPase turnover between the two systems; in vitro eukaryotic eEF-2/70 S ribosome-dependent GTP hydrolysis is 10-fold slower than the prokaryotic EF-G/70 S ribosome system (10). This may reflect, in part, the elaborate regulation of eukaryotic translation. The other important component of the GTPase center is the acidic stalk protein, termed L7/L12 in prokaryotes (11–15). Four copies of this proteins bind to protein L10 and form a stable complex (16), designated here as L10/L7/L12. This protein complex and another protein, L11, are assembled on the 1070 RNA domain (17). Flexible property of L7/L12 protein in the ribosome (16, 18–20) seems to be correlated with the fast turnover of EF-G-dependent GTPase. The eukaryotic counterparts of the prokaryotic L7/L12 and L10 are P1/P2 and P0, respectively (21, 22). Although formation of the complex, termed P0/P1/P2, has been clarified (21, 23–26), its structure and function have not been characterized extensively. We previously tried replacement of the acidic stalk protein complex L10/L7/L12 in the E. coli ribosome with rat P0/P1/P2 in vitro and showed, by this replacement, that the ribosome acquired GTPase activity dependent on the eukaryotic translocase eEF-2 instead of prokaryotic EF-G (10). This activity was comparable with that of the rat 80 S ribosome. Meanwhile, other groups exchanged the 1070 RNA region within 23 S/28 S rRNA, with which the acidic stalk protein complex interacts between E. coli and yeast, and this showed no major functional effect (27, 28). These studies suggest that P0/P1/P2 protein complex on the 1070 RNA domain, but not the RNA itself, are important for the kingdom-specific function.

The E. coli ribosome in which L10/L7/L12 was replaced with rat P0/P1/P2, however, showed no significant activity of eEF-1α/eEF-2-dependent polyphenylalanine synthesis in our previous study. This may be due to damage caused during preparation of the core ribosome lacking both L10 and L7/L12, using 50% ethanol, 0.5 M NH₄Cl at 30 °C. To prepare the core ribosome employing milder conditions, we use here an L11-lacking ribosomal mutant from which L10-L7/L12 complex is easily removed at 0 °C. Both rat P0/P1/P2 complex and RL12 (rat counterpart of E. coli L11) are incorporated into the core ribosome. This hybrid ribosome has appreciable activity in polyphenylalanine synthesis dependent on the two eukaryotic elongation factors. The present results clearly show that the eukaryotic ribosomal proteins bound to the 1070 RNA domain play crucial roles in translation elongation regulated by the eukaryotic factors.

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Materials and Methods

E. coli Core Ribosomes Lacking L10-L7/L12 and L11. E. coli—AM68, lacking ribosomal protein L11 (29), was grown to late exponential stage in phosphate-buffered medium (pH 7.1) containing 2 mM sodium citrate, 0.4 mM MgSO₄, 0.5% glucose, 10 g/liter tryptophane, and 5 g/liter yeast extract and harvested. Salt-washed ribosomes were prepared as described (10). The 50 S and 30 S subunits were isolated by density gradient centrifugation using a 10–28% sucrose gradient in buffer (0.5 mM MgCl₂, 50 mM NH₄Cl, 20 mM Tris·HCl, pH 7.6, and 5 mM 2-mercaptoethanol) at 24,000 rpm and 4°C for 14 h with a Hitachi P28-S rotor. To remove the L10-L7/L12 complex, the 50 S subunits were incubated in 50% ethanol, 0.5 M NH₄Cl solution as described previously (10), except that the incubation was performed at 0°C and the solution contained 5 mM MgCl₂. The 50 S core subunit and the released L10-L7/L12 complex were recovered as described (11). The intact 70 S ribosomes were from E. coli Q13 (10).

Rat Ribosomal Proteins and Their Binding to the E. coli Core Ribosome—The rat P0/P1/P2 complex and RL12, counterparts of E. coli L10-L7/L12 and L11, respectively, were prepared as described previously (24, 30). In a typical experiment, 10 pmol of the 50 S subunit cores were incubated with 2 μg of P0/P1/P2 complex and 0.4 μg of RL12 in a solution (25 μl) containing 10 mM MgCl₂, 75 mM NH₄Cl, 20 mM Tris·HCl, pH 7.6, at 37°C for 5 min and used for various assays. In some experiments, E. coli L10-L7/L12 and L11 (30) were added instead of the rat proteins. Incorporation of these proteins was confirmed by sucrose density gradient (10) and native agarose-acrylamide composite gel (see below).

Elongation Factors—Eukaryotic eEF-1α and eEF-2 were isolated from pig liver as described by Iwasaki and Kaziro (31). E. coli EF-Tu (32) and EF-G (33) were prepared as described.

Polyphenylalanine Synthesis—The eukaryotic eEF-1α/eEF-2-dependent reaction was performed in a mixture of 100 μl containing 10 pmol of hybrid 50 S subunits, 50 pmol of 30 S subunits (optimum for the activity), 10 μg of poly(U), 80 μg of E. coli tRNA precharged with 40 pmol of [¹⁴C]phenylalanine (400 cpm/pmol), 0.2 mM GTP, 10 mM MgCl₂, 75 mM NH₄Cl, 50 mM Tris·HCl, pH 7.6, 0.2 mM dithiothreitol, 5 pmol of eEF-2, and 40 pmol of eEF-1α, which was incubated at 37°C for 2.5–15 min. The eukaryotic EF-Tu/eEF-G-dependent reaction was performed under the same conditions, except that the reaction mixture contained 5 mM MgCl₂, 120 mM NH₄Cl, 5 pmol of eEF-G, and 40 pmol of EF-Tu. The polymerized radioactivity was counted as described by Möller et al. (12).

GTPase Activity—For eEF-1α-dependent activity, the reaction mixture (30 μl) contained 10 pmol of hybrid 50 S subunits, 30 pmol of 30 S subunits, 300 pmol of [γ-³²P]GTP (400–500 cpm/pmol), 10 μg of poly(U), 20 pmol of Ph-e-tRNA, 5 mM MgCl₂, 50 mM NH₄Cl, 20 mM Tris·HCl, pH 7.5, 0.2 mM dithiothreitol, 5 pmol of eEF-1α, and 40 pmol of GTP. The polymerized radioactivity was assayed by thin-layer chromatography on silicic acid plates.

Eukaryotic Elongation Factors—The eukaryotic elongation factors were isolated from pig liver and rat liver as described by Iwasaki and Kaziro (31). The eukaryotic eEF-2 was isolated from pig liver as described by Iwasaki and Kaziro (31). The eukaryotic elongation factors were isolated from pig liver as described by Iwasaki and Kaziro (31).

Results

We have characterized rat ribosomal proteins P0/P1/P2 complex and RL12, counterparts of E. coli L10-L7/L12 and L11, respectively (24, 30). To investigate the functional significance of these rat proteins in eukaryotic translational mechanism, here we attempted to substitute the proteins for L10-L7/L12 and L11 in E. coli 50 S ribosomal subunits. The E. coli core particle lacking L10-L7/L12 and L11 assembled on the domain around 1070 of 23 S rRNA was prepared with the 50 S subunit of the L11-deficient AM68 strain (Fig. 1A, lane 2). L10-L7/L12 was easily and selectively removed from the mutant 50 S subunit (Fig. 1A, lanes 3 and 4) in 50% ethanol, 0.5 M NH₄Cl at 0°C. Binding of the rat proteins to the E. coli core particle was examined by native agarose-acrylamide composite gel electrophoresis (Fig. 1B). The mobility of the core particle (lane 2) was much higher than the intact 50 S subunit (lane 1). The addition of the rat proteins changed the gel mobility of the core particle (lane 3). This mobility shift by rat proteins was prevented by adding an excess amount of an RNA fragment containing residues 1029–1127 of E. coli 23 S rRNA to which
rat P0/H18528/P1/P2 complex (10) and RL12 (30) cross-bind (lane 4), suggesting that the rat proteins bind to the 1070 RNA domain within the E. coli 50 S core particle. The binding of rat proteins to the 1070 RNA region was also confirmed by chemical footprinting.3 The region of the gel with the shifted ribosomal band (lane 3) was cut out and tested by immunoblotting for reactivity with autoimmune serum that recognizes rat ribosomal proteins P0, P1, P2, and RL12 (35) (Fig. 1C). Fig. 1C, lane 2, clearly shows that all of the added rat proteins comigrated with the E. coli core particle. From these results, we concluded that E. coli L10/H18528/L7/L12 and L11 are replaced with rat protein counterparts on the 1070 RNA domain of 23 S rRNA in the 50 S subunit, as illustrated in Fig. 1D.

The hybrid ribosomes were tested for activity in poly(U)-dependent polyphenylalanine synthesis. Unlike intact E. coli ribosomes, hybrid ribosomes showed activity to be dependent on eukaryotic eEF-1α and eEF-2 (Fig. 2A) but not on prokaryotic EF-Tu and EF-G (Fig. 2B). Therefore, the ribosomal specificity for elongation factors was changed by replacing E. coli L10/L7/L12 and L11 on the 50 S subunit with rat counterparts. This eEF-1α/eEF-2-dependent activity was suppressed by the addition of the RNA competitor (data not shown), which prevented the hybrid formation (Fig. 1B, lane 4). The polyphenylalanine synthetic activity of the hybrid ribosome was not as high as that of the rat intact ribosome (Fig. 2C); the initial rate of polymerization by the hybrid ribosome was about one-third that of the rat 80 S ribosome. To confirm whether the polymerization activity of the hybrid ribosome depends on the actions of both eEF-1α and eEF-2, the activity was assayed without either eEF-1α or eEF-2. As shown in Fig. 2D, the polymerization activity of the hybrid ribosome was detected only when both eEF-1α and eEF-2 were present, indicating that the hybrid ribosome allow functional access to both the eukaryotic elongation factors.

To investigate individual contributions of P0-P1/P2 complex and RL12 to translation elongation by the hybrid ribosome, we performed partial replacement of either L10/L7/L12 or L11 in E. coli 50 S subunit with the respective rat counterparts. The core ribosome lacking L10/L7/L12 and L11 (Fig. 1A) was incubated with L10/L7/L12-like proteins (E. coli L10/L7/L12 or rat...
P0-P1/P2) and of L11-like proteins (E. coli L11 or rat RL12), and we tested the ribosomal functions dependent on eukaryotic elongation factors (Fig. 3). The GTPase activities dependent on eEF-2 (Fig. 3A) and eEF-1α (Fig. 3B) were markedly stimulated by addition of rat P0-P1/P2 to the core ribosome. The addition of E. coli L11 or rat RL12, together with P0-P1/P2, slightly enhanced eEF-2-dependent GTPase (Fig. 3A) but had no effect on eEF-1α-dependent GTPase (Fig. 3B). In contrast to GTPase, polyphenylalanine synthetic activity with eEF-1α and eEF-2 was stimulated to only a small extent by the addition of P0-P1/P2 complex alone to the core ribosome (Fig. 3C). This activity was, however, enhanced 3-fold by the further addition of E. coli L11 and more than 4-fold by the addition of rat RL12. Replacement of E. coli L11 alone with RL12 gave no appreciable effect on the polyphenylalanine synthesis as well as GTPase.

The hybrid ribosomes were tested for sensitivity to the antibiotic thiostrepton, which recognizes the E. coli 1070 RNA domain associated with L11 (1). The E. coli ribosomes in which L10-L7/L12 alone was replaced with rat P0-P1/P2 retained thiostrepton sensitivity, as described previously (10). Replacement of both L10-L7/L12 and L11 with P0-P1/P2 and RL12 resulted in ribosomes insensitive to the drug (Fig. 4), suggesting that rat RL12 is responsible for the thiostrepton insensitivity of the hybrid ribosome.

**DISCUSSION**

The ribosomal proteins L10-L7/L12 and L11 bind to the 1070 RNA region in domain II of 23 S rRNA, forming a mobile region that constitutes a part of the GTPase-related functional center of the E. coli ribosome. We here performed in vitro replacement of L10-L7/L12 complex and L11 in the 50 S subunit with rat counterparts P0-P1/P2 complex and RL12, respectively. By this replacement, ribosomal specificity for elongation factors is changed; the hybrid ribosome is engaged in polypeptide synthesis by the actions of two eukaryotic elongation factors, eEF-1α and eEF-2, but not of prokaryotic EF-Tu and EF-G. It has been shown since the earliest work that prokaryotic 70 S ribosomes do not engage in protein synthesis with the eukaryotic translation factors and that eukaryotic 80 S ribosomes are inactive with the prokaryotic factors (7–9). The present results strongly suggest that a limited number of ribosomal proteins assembled on the 1070 RNA domain are major components responsible for the kingdom-dependent specificity between ribosomes and GTP-bound translation factors.

Functional contributions of rat P0-P1/P2 and RL12 in the hybrid ribosome were clarified by their individual substitutions for E. coli L10-L7/L12 and L11, respectively (Fig. 3). P0-P1/P2 complex, but not RL12, contributes substantially to the specificity for the eukaryotic factors and GTPase. Not only rat RL12 but also E. coli L11, however, stimulates polyphenylalanine synthetic activity dependent on eEF-1α and eEF-2, although the stimulation level by RL12 is higher than that of E. coli L11. The availability of mutants deficient in L11-type proteins in bacteria (29, 36, 37) and yeast (38) indicates that L11-like proteins are not essential for cell viability. However, the growth rate of E. coli mutant AM68 lacking L11 was very slow; its doubling time was five times longer than strain Q13, which does have L11 (data not shown). This finding suggests that the efficiency of protein synthesis by ribosomes lacking L11 is quite low within cell, in line with the present in vitro data on poly(Phe) synthesis. RL12/L11 appears to participate in improving the efficiency of a step in post-GTPase events such as translocation of tRNAs. There is a clear difference between E. coli L11 and rat RL12. L11 but not RL12 binding to the 1070 RNA domain makes the ribosome sensitive to the antibiotic thiostrepton (Fig. 4). This is consistent with our previous binding experiment, i.e., thiostrepton stabilizes a complex between L11 and the E. coli 1070 RNA domain but not the RL12-RNA complex (30). The difference in thiostrepton sensitivity may be due to N-terminal differences in amino acid sequences between L11 and RL12 around residue 22, which is important for thio-
streptol binding (39). Despite the divergence of the sequences, a conformation important for function appears to be conserved between the two proteins.

The present results clearly show that P0-P1/P2 complex plays a crucial role in the functions of the two eukaryotic elongation factors. The involvement of P1/P2 proteins in translation elongation has been suggested previously by immunological inhibition assays (40) and partial reconstitution studies with yeast and yeast ribosomes (23, 41). An essential role of P0 for cell viability has been demonstrated in yeast (23). Cryoelectron microscopic studies of the complex containing E. coli ribosome EF-Tu and aminoacyl-tRNA (42), as well as the ribosome-EF-G complex (43, 44), have demonstrated direct contacts of these factors (GTP-binding domain) with the L7/L12 stalk and also with its base region. Considering these previous data and the present results together, eukaryotic P1/P2 stalk and P0/RL12 constituting its base region seem to bind directly to eEF1 and eEF2. This view is also supported by chemical cross-linking of eEF-2 with P2, P0 (La20), and RL12 (45) and of eEF1 with RL12 (46). Therefore, the ribosome factor specificity may be explained by the direct interaction between the ribosomal proteins and elongation factors.

In addition to interactions with the translation factors, P0-P1/P2 complex and RL12 have another important function, which is rRNA binding. Because rRNAs appear to play essential roles in translational mechanism (47), it is important to know the effect of the protein binding on rRNA. P0-P1/P2 complex and RL12 bind to overlapping regions of the 1070 (E. coli numbering) domain of 28 S rRNA and affect the RNA conformation (24). In the 1070 RNA region, there is also a site for eEF-2 binding as detected by footprinting (48). It is likely that adjustment of the 1070 RNA region by protein binding may be important for the functional interaction of eEF-2 with the RNA. This is also the case in the hybrid ribosome. Because rat P0-P1/P2 and RL12 cross-bind to the E. coli 1070 RNA domain (10, 30) and stimulate the ribosome function dependent on eEF1 and eEF2 (present study), these rat proteins appear to affect the structure and function of the 1070 RNA region within the E. coli ribosome. An important and interesting point yet to be addressed is the effect of protein binding on the 2660 RNA region (sarcin/ricin loop), another important RNA region to which elongation factors bind. Because the 1070 and 2660 RNA regions are neighbors in the 50 S subunit (4), it is also likely that bindings of rat proteins to the 1070 region may affect the interactions of elongation factors with the 2660 region.

The activity of our hybrid ribosomes in translational elongation implies that tRNA movement as well as its binding occur properly in this artificial construction. It has been shown that translocation of the A-site tRNA to the P-site is stimulated by GTP hydrolysis on EF-G (2) and possibly by the interaction of EF-G with several sites including the decoding region of the E. coli ribosome (24). In the 1070 RNA region, there is also a site to which elongation factors bind. Because the 1070 and 2660 RNA regions are neighbors in the 50 S subunit (4), it is also known the effect of the protein binding on rRNA. P0 may be important for the functional interaction of eEF-2 with the ribosome, provide a novel methodology for the researches of eukaryotic ribosomal proteins in vitro. The present replacement studies, “molecular plantation,” as it were, of mammalian proteins into the E. coli ribosome, provide a novel methodology for the researches of eukaryotic ribosomal proteins.
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