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Interaction Network of Human Aminoacyl-tRNA Synthetases and Subunits of Elongation Factor 1 Complex

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Aminoacyl-tRNA synthetases (ARSs) ligate amino acids to their cognate tRNAs. It has been suggested that mammalian ARSs are linked to the EF-1 complex for efficient channeling of aminoacyl tRNAs to ribosome. Here we systemically investigated possible interactions between human ARSs and the subunits of EF-1 (α, β, γ, and δ) using a yeast two-hybrid assay. Among the 80 tested pairs, leucyl- and histidyl-tRNA synthetases were found to make strong and specific interaction with the EF-1γ and β while glu-proly-, glutaminyl-, alanyl-, aspartyl-, lysyl-, phenylalanyl-, glycyl-, and tryptophanyl-tRNA synthetases showed moderate interactions with the different EF-1 subunits. The interactions of leucyl- and histidyl-tRNA synthetase with the EF-1 complex were confirmed by immunoprecipitation and *in vitro* pull-down experiments. Interestingly, the aminoacylation activities of these two enzymes, but not other ARSs, were stimulated by the cofactor of EF-1, GTP. These data suggest that a systematic interaction network may exist between mammalian ARSs and EF-1 subunits probably to enhance the efficiency of *in vivo* protein synthesis.
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Key Words: **aminoacyl-tRNA synthetase; elongation factor-1; protein–protein interaction; multi-tRNA synthetase complex; translation.**

Aminoacyl-tRNA synthetases (ARSs) play an essential role in translation by attaching amino acids to their cognate tRNAs. In higher eukaryotes, ARSs do not appear to exist in isolation but do appear to be associated with other cellular frameworks or translational machinery (1–5). Among the macromolecular assemblage involving ARSs, an ARS-rich complex consists of at least eight different ARS polypeptides (EPRS, IRS, LRS, MRS, QRS, KRS, RRS, and DRS) and three nonenzymatic factors such as p43, p38, and p18 (6). VRS also forms a different complex tightly associated with the subunits, $\beta\gamma\delta$, of elongation factor-1 (EF-1) (5).

Elongation factor-1 (EF-1) transfers the charged tRNA to ribosome (7, 8). Mammalian EF-1 complex is composed of α and $\beta\gamma\delta$ subunit (9). EF-1α is a G-protein responsible for transferring the charged tRNA to the A site of mRNA-ribosome consuming the bound GTP. Both of the β and δ subunits function as guanine nucleotide exchange factors (10). EF-1α is converted to an active form when GTP binds, and thus transient interaction between EF-1α and the EF-1$\beta\gamma\delta$ complex maintains a pool of EF-1$\alpha \cdot$ GTP.

In eukaryotic system, the presence of a channeling system has been suggested in the transfer of tRNA. Namely, tRNAs can be delivered from nucleus to ARSs for aminoacylation and the charged tRNAs are subsequently transferred to ribosome via EF-1 without being diffused to the cytoplasmic medium. This hypothesis is supported by several reports (11–14). VRS provides the best example for the channeling for tRNA via its association with EF-1. VRS has been purified as a component of the EF-1$\beta\gamma\delta$ complex from rabbit liver and artemia (15–17). Recently VRS has been shown to interact with the EF-1δ subunit (18), and also its activity was increased by coincubation of EF-1α and GTP with EF-1$\beta\gamma\delta \cdot$ VRS complex (19). Other ARSs such as FRS and DRS are activated by EF-1 co-incubation (20–22). These data suggest that there may be a systematic functional linkage between ARSs and the EF-1 subunits. However, most of studies regarding the relationship between ARS and the EF-1 subunits have been done individually. In this work, we explored the possible interaction network between ARSs and the EF-1 subunits in a systematic manner using all 20 human ARSs. The experimental results suggested several pu-

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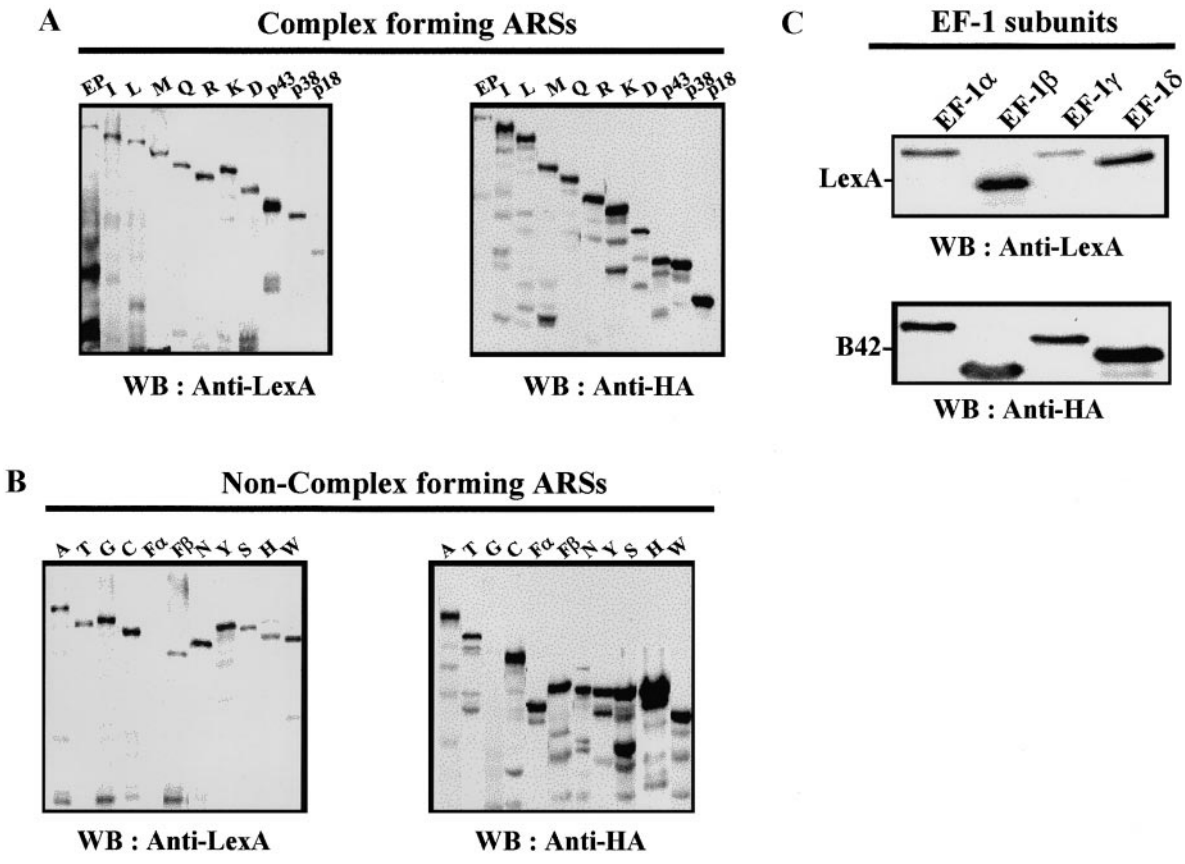

FIG. 1. The LexA and B42 fusion proteins of ARSs (A, complex forming ARSs; B, non-complex forming ARSs) and EF-1 subunits (C) were expressed in yeast. The expression of LexA- and B42-fusion proteins were detected by Western blotting using anti-LexA and anti-HA antibodies, respectively.

tative interactions between these enzymes and EF-1 complex. The functional significance for these interactions has been also investigated.

MATERIALS AND METHODS

Materials. ^{3}H-labeled histidine, leucine, alanine, threonine, and isoleucine were purchased from Amersham-Pharmacia. Antibody against EF-1α was obtained from Upstate Biotechnology and bovine liver total tRNA was purchased from Sigma. Yeast tRNA was purchased from Boehringer Mannheim. Preparation of polyclonal rabbit antibodies against LRS was previously described (23). EF-1β, and -1γ were expressed and purified as His-tagged proteins in *E. coli* and injected into mice to raise antibodies as previously described (24). Anti-HA and anti-Myc antibodies were obtained from Santa Cruz Biotechnology and Anti-LexA antibody obtained from Invitrogen. Anti-HRS antibody is a special gift from Dr. Plotz. The Accession Nos. for human ARS genes used for this study are as follows: AlaRS; D32050, DRS; J05032, EPRS; X54326, FRSα; AF042347, FRSβ; AF042346, GRS; U09510, HRS; Z11518, IRS; U04953, KRS; D32053, LRS; D84223, MRS; X94754, NRS; D84273, QRS; X76013, RRS; S80343, SRS; NM_006513, TRS; M63180, VRS; X59303, WRS; M61715, YRS; NM_003680, p43; U10117, p38; U24169 and p18; AF054186.

Construction of plasmids. The cDNAs encoding each subunit of human elongation factor-1 complex were obtained by PCR with the corresponding primers. (The sequences would be available upon request.) Human lymphocyte cDNA library (Clontech) was used as a template for cloning of cDNAs for EF-1α, and IMAGE clones 1736996 and 50603 (Research Genetics) for EF-1β and -1δ, respectively. Human fetal brain cDNA library (Clontech) was used for cloning EF-1γ. The PCR products for EF-1α, β, and δ were digested with *Eco*RI and *Xho*I, and ligated into the same sites of pEG202 (for construction of LexA-fusion proteins) or pJG4-5 (for construction of B42-fusion proteins). The EF-1γ PCR product was cut with *Sal*I and *Not*I, and ligated into the same vectors. Human ARS genes were inserted into pEG202 or pJG4-5. For expression of ARSs and EF-1 in human 293 cells, the PCR products for EF-1α, β, and δ and human ARS genes were inserted into pcDNA3 (Invitrogen) containing Myc-tag.

In vitro pull-down assay. GST-EF-1β or -1γ was expressed from pGEX4T-1 containing the corresponding gene in *E. coli* BL21 (DE3). The harvested cells were lysed by ultrasonication and the protein extracts were prepared. LRS was synthesized by *in vitro* translation in the presence of [^{35}S]methionine using pcDNA3-LRS by the TNT coupled translation kit (Promega). The GST fusion proteins bound to the glutathione Sepharose beads were incubated with the [^{35}S]methionine-labeled LRS in the binding buffer of 25 mM Hepes, pH 7.6, 20% glycerol, 1 mM DTT, 150 mM KCl and 0.3 μM phenylmethylsulfonyl fluoride (PMSF). The binding mixture was incubated overnight at 4°C with rotation and washed four times with the binding buffer containing 1% Triton X-100. After addition of the SDS sample buffer, the binding proteins were eluted by boiling and separated by SDS gel electrophoresis. The presence of LRS was determined by autoradiography.

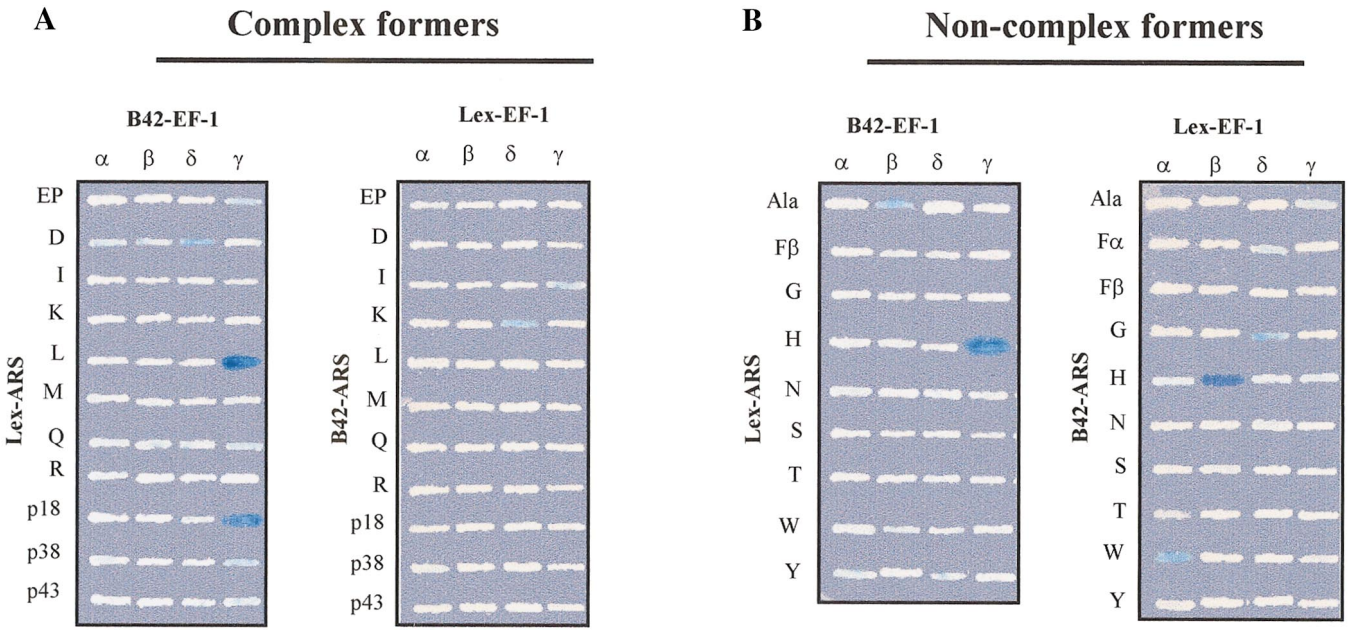

FIG. 2. Interactions of human ARSs with EF-1 subunits by yeast two-hybrid assay. (A) Interactions between complex-forming ARSs and the subunits of EF-1. The three ARS cofactors (p43, p38, and p18) were included in the assay. (B) Interactions between non-complex-forming ARSs and the subunits of EF-1. The indicated ARSs including three auxiliary proteins and the four EF-1 subunits were expressed as both LexA and B42 fusion proteins in yeast. The positive interactions were determined by the formation of blue colonies on the plates containing X-gal.

Preparation of EF-1β. Human EF-1β cDNA clone was ligated to pET28a using *Eco*R1 and *Xho*1 sites. The recombinant protein was expressed in *E. coli* BL21 (DE3) by IPTG induction. The cells were harvested and sonicated in the binding buffer (20 mM KH_2PO4, 500 mM NaCl, pH 7.8, 2 mM 2-mercaptoethanol). The lysate was centrifuged at 26,000*g* for 30 min. The recombinant protein was purified by the affinity chromatography using His-tag attached to the protein according to the manufacturer's protocol (Invitrogen).

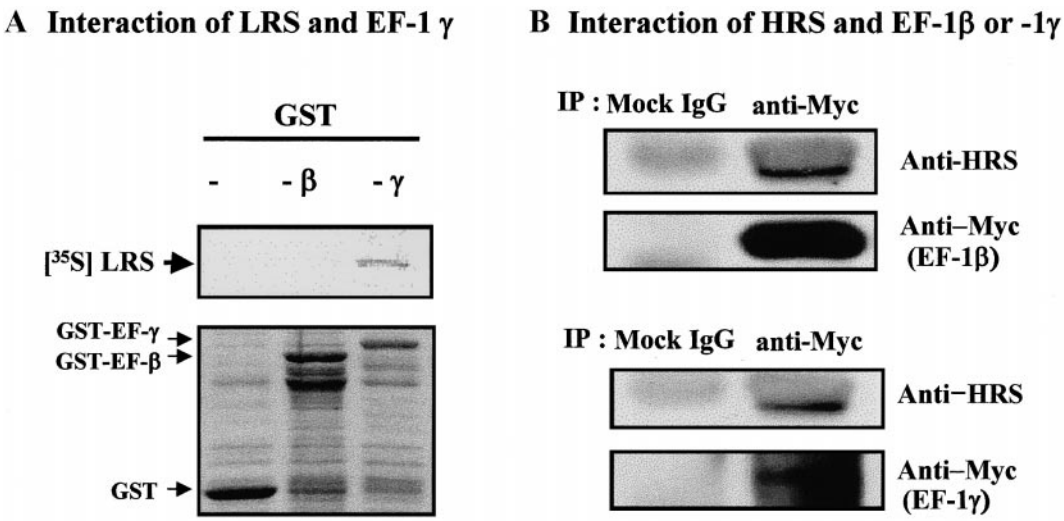

FIG. 3. *In vitro* interaction between ARSs and EF-1 subunits. (A) The LRS binds directly to the EF-1γ. The GST-EF-1β and -1γ fusion proteins expressed in *E. coli* were purified. The radiolabeled LRS was tested for the specific binding to the GST-EF-1γ, -1β or GST alone and visualized by autoradiography (upper panel). 20% of input LRS was also shown in a lane indicated by input. The Coomassie blue staining of eluted proteins from the glutathion Sepharose beads was shown in the lower panel with the proteins indicated by arrows. (B) Immunoprecipitation of HRS and EF-1β, -1γ. The pcDNA3 encoding Myc-EF1-1β or -1γ was transiently transfected into 293 cells. The Myc-tagged EF-1 subunits in 293 cells were immunoprecipitated with anti-Myc antibody, and coprecipitation of HRS was determined by a Western blot using anti-HRS antibody.

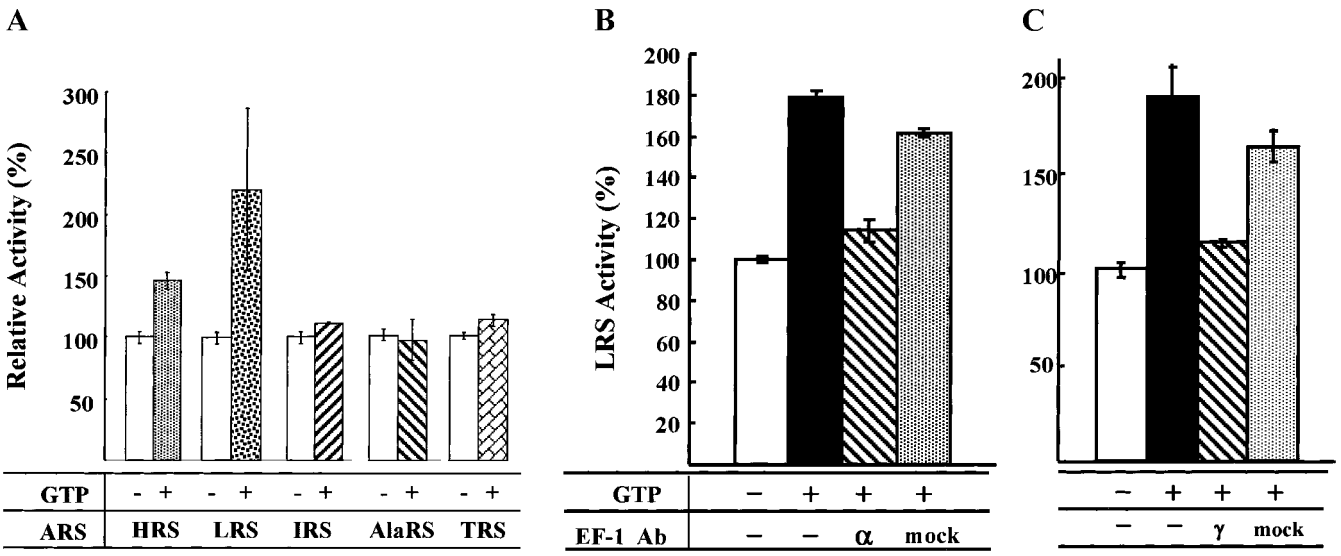

FIG. 4. The catalytic activities of HRS and LRS were stimulated by GTP (A). The activities of ARSs were measured using corresponding ^{3}H-labeled amino acid in the presence of 10 mM GTP. The catalytic activities of HRS and LRS were increased by the addition of GTP while other enzymes were not. The stimulatory effect of GTP was abolished by coincubation with the antibodies against EF-1α (B) or EF-1γ (C). Mouse anti-EF-1α (5 μg), anti-EF-1γ (10 μg), or mouse normal IgG (10 μg) was preincubated with the assay mixture on ice before the addition of tRNA.

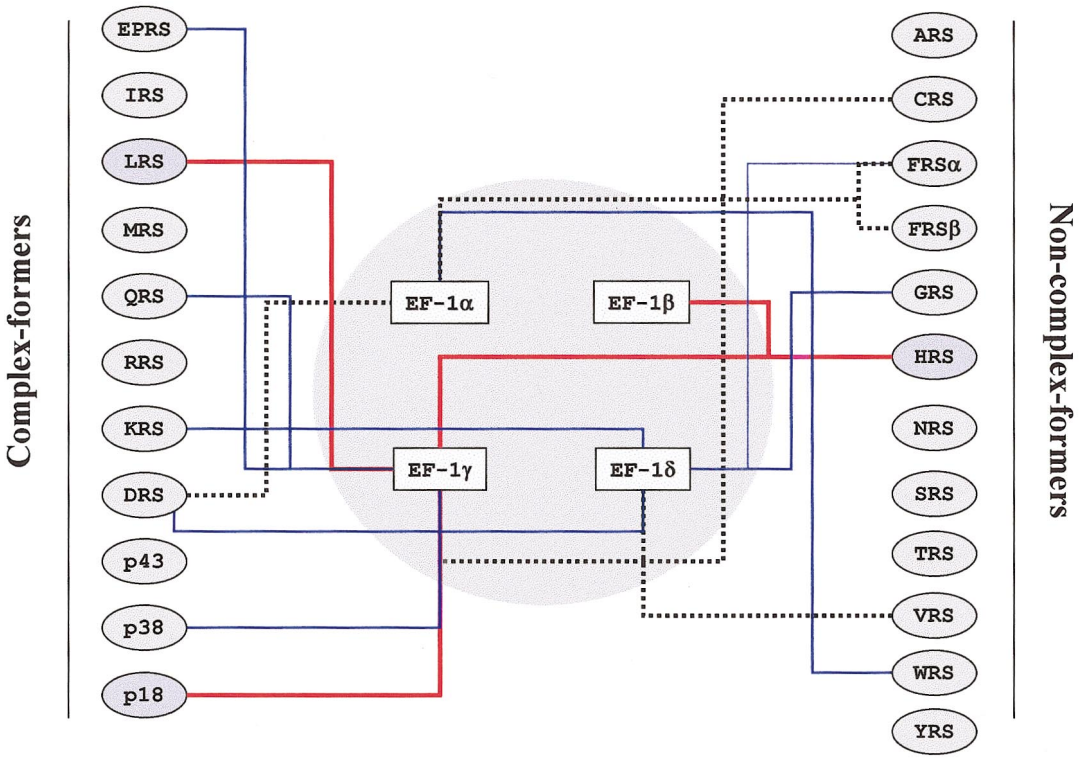

FIG. 5. Schematic diagram illustrating interaction network of ARSs and EF-1 subunits. The complex- and non-complex-forming ARSs are vertically arrayed left and right, respectively. The strong interaction of LRS, HRS and p18 with the corresponding subunits of EF-1 complex are connected with red lines. The moderate interactions of EPRS, QRS, KRS, DRS, FRSα, GRS, WRS, and p38 with EF-1 subunits are marked with blue lines. The functional or physical interactions of DRS (21, 22), VRS (15-19), CRS (28), FRS (20) with EF-1 complex are marked with dotted lines.

Yeast two-hybrid assay. The interactions of the elongation factor-1 subunits with ARSs were tested using the yeast two-hybrid system. Positive interaction was determined by the induction of β-galactosidase as described previously (25,26). For the interaction test, colonies were picked and grown on the Ura^-, His^-, Trp^-/glucose plate. Approximately equal amounts of cells were transferred to the selection plates of Ura^-, His^-, Trp^-/galactose containing X-gal and raffinose. The pairs forming blue color in a whole patched area were recorded as positive interaction.

Aminoacylation activity assay. For measuring the activities of LRS and HRS, human embryonic kidney 293 cells grown in DMEM (Dulbecco's modified Eagle medium) containing 10% FBS (fetal bovine serum) and penicillin/streptomycin were harvested in a buffer (10 mM Hepes pH 7.6, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM EGTA, 10 mM NaF, 12 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM PMSF, 5 μg/ml aprotinin). After harvesting, cells were lysed by homogenization using Dounce homogenizer. Then cell lysates were centrifuged for 10 min at 14 K rpm to remove insoluble cell debris. The protein amounts of cell lysates were measured by using a Bio-Rad kit.

The aminoacylation reaction was initiated by incubation of 0.1 μg/μl total protein of the cell lysate with bovine liver total tRNA (0.4 μg/μl) in the reaction mixture (50 μl) consisting of 0.2 mg/ml BSA, 20 mM KCl, 5 mM $MgCl_2$, 20 mM 2-mercaptoethanol, 5 mM ATP, 50 mM Hepes (pH 7.4), and 0.12 μCi/μl of the corresponding ^{3}H-labeled amino acid. 10 mM of GTP, CTP, UTP or GMP-PNP (β;γ-imidoguanosine 5′-triphosphate, Sigma Co.) was added where indicated. All of the assays were done in triplicate and repeated at least twice. Samples (15 μl) were taken at 1.5 and 3 min after incubation and spotted on 2.3-cm glass fiber filter disks (Whatman) presoaked with 5% trichloroacetic acid. The filters were dried for a minute and washed 3 times with ice-cold 5% trichloroacetic acid at 4°C. After washing, the filters were dehydrated by incubation with 95% ethanol at 4°C. The radioactivity of the filters was measured using liquid scintillation counter (Wallac 1409).

Immunoprecipitation The Myc-tagged pcDNA3-EF-1β or -1γ plasmids were transfected into 293 cells using GenePORTER (Gene Therapy System) as previously described (27). The transfected cells were lysed with a lysis buffer (20 mM Tris–Cl, pH 7.5, 150 mM NaCl, 5 mM EGTA, 10 mM NaF, 1% NP-40, 12 mM β-glycerophosphate, 3 mM DTT, 1 mM Na_3VO_4, 1 mM PMSF, 5 μg/ml aprotinin). After lysis, cell lysates were obtained by incubation for 20 min on ice and centrifugation for 10 min at 14 K rpm. The protein amounts of cell lysates were measured by using a Bio-Rad kit. The equal amount (1 mg) of proteins in the cell lysate was incubated with 5 μg anti-Myc antibody for 1 h and subsequently with protein G-agarose for additional 1 h. The bound proteins were separated by SDS–PAGE, electrotransferred into a PVDF (polyvinylidene difluoride) membrane (Millipore Co.) and detected by a Western blot with anti-HRS and anti-Myc antibodies.

RESULTS

Construction of LexA- and B42-Fusion Proteins

The interactions between human ARSs and the subunits of EF-1 complex have been investigated by yeast two hybrid assay. To know if LexA-/B42-fusion proteins with ARSs or EF-1 subunits were expressed in yeast cells, pEG202 or pJG4-5 plasmids containing the corresponding genes were transformed into yeast strain EGY 48/SH. The expression level of each LexA-fusion protein was detected by Western blots using anti-LexA antibody. For determining the expression of B42-fusion proteins, immunoblotting with anti-HA antibody was performed because B42-fusion proteins are also tagged with HA. As shown in Fig. 1, most of the fusion proteins were expressed in yeast cells except LexA-FRSα, LexA-VRS and B42-VRS fusion proteins. Therefore, VRS constructs were not used for further interaction studies. In addition, CRS was not included in this study because it is expressed as multiple isoforms and one of its isoforms was previously shown to interact with EF-1γ (28).

Interaction of ARSs with EF-1 Subunits

To determine the interactions of ARSs and the subunits of EF-1 complex systemically, we carried out extensive yeast two hybrid assay between human ARSs and the four subunits of human EF-1 complex (Fig. 2). Human ARSs were grouped into two classes depending on their ability to form a macromolecular ARS complex to see if there is any difference between these two groups in the interaction with EF-1 complex. Positive interactions were determined by the induction of the reporter genes, β-galactosidase. Thus the strength of the interaction is roughly reflected by the intensity of blue color of colony. Figure 2A shows the results of the interactions between the complex-forming ARSs and the EF-1 subunits. EF-1γ strongly interacted with LRS and p18 among the pairs between LexA-ARS and B42-EF-1 subunits (Fig. 2A, left panel). Moderate interactions of EPRS, QRS and p38 with EF-1γ and of DRS with EF-1δ were also observed. In the pairs of LexA-EF-1 and B42-ARS, KRS showed moderate interaction with EF-1δ (Fig. 2A, right panel).

We also tested the interaction of the non-complex-forming ARSs with the EF-1 subunits. The pairwise test of LexA-ARS and B42-EF-1 demonstrated the strong interaction of HRS with EF-1γ, and the moderate interaction of AlaRS with EF-1β (Figure 2B, left panel). In LexA-EF-1/B42-ARS pairs, HRS appeared to strongly interact with EF-1β. In addition, GRS and FRSα, and WRS had a moderate interaction with EF-1δ and -1α, respectively.

Among the strong and moderate interaction pairs, we have focused on LRS and HRS that showed the strong interaction with the subunits of EF-1 complex. The interaction of the two molecules was confirmed by *in vitro* pull-down assay and coimmunoprecipitation. Since LRS is one of the components of the multi-ARS complex, immunoprecipitation of LRS would bring down all of the other complex-forming ARSs and their associating factors. Thus, the direct interaction of LRS with EF-1γ was tested by *in vitro* binding assay using recombinant proteins. EF-1β and -1γ were expressed as GST fusion protein and LRS was synthesized by *in vitro* translation. The ^{35}S-labeled LRS was mixed with each of GST, GST-EF-1β and -1γ that were bound to glutathione Sepharose. The bound LRS was eluted by SDS and detected by autoradiography. LRS was specifically precipitated with GST-EF-1γ, but not with

either of GST-EF-1β or GST alone (Fig. 3A). Furthermore, we found that EF-1γ was also copurified with the multi-ARS complex from bovine liver (data not shown). For testing the interaction between HRS and EF-1β or 1γ, coimmunoprecipitation was used because HRS is not associated with the multi-ARS complex. HRS was coimmunoprecipitated with EF-1β or -1γ consistent with the interaction determined by the yeast two hybrid assay (Fig. 3B). Therefore, the interactions of LRS or HRS with the EF-1 subunits were confirmed by two different assays.

LRS and HRS Activities Are Enhanced Specifically by GTP

VRS and DRS were previously shown to be associated with the EF-1 complex. In both cases, their aminoacylation activities were stimulated about twofold by the addition of EF-1α and a cofactor of EF-1, GTP (19, 22). If LRS and HRS are associated with the subunits of EF-1 complex for the similar functional reason, their activities may be affected by the interaction with the EF-1 subunits. The aminoacylation activities of HRS, LRS, IRS, AlaRS and TRS were determined using the 293 whole cell lysates in the presence or absence of GTP. LRS and HRS showed about 1.5- to 2.3-fold increase by the addition of GTP while other enzymes did not (Fig. 4A). However, the LRS and HRS activities were not stimulated by the addition of CTP, UTP or the nonhydrolyzable GTP analogue, GMP-PNP (data not shown), indicating that the enhancement of the LRS and HRS activities requires the hydrolysis of GTP. Thus, the stimulatory effect of GTP on the catalytic activities of LRS and HRS is related to the interaction of these enzymes with EF-1 complex. This possibility was further supported by the inhibitory effect of anti-EF-1α and γ antibodies on the GTP-dependent increase of LRS activity (Figs. 4B and 4C).

DISCUSSION

It is thought that the translational components would form a supramolecular network *in vivo* for efficient protein synthesis. Since cells contain 20 different ARSs in charge of 20 natural amino acids, it would be reasonable to believe that their catalytic activities and locations would be controlled in some coordinated manner. Although stable macromolecular complexes consisting of eight ARSs (EP, I, L, M, Q, R, K, D) and a few cofactors (p43, p38, and p18), or the VRS · EF-1 complex partly support this notion (6, 15, 16, 29), it has not been studied whether there is a systematic functional or physical network between ARSs and EF-1 complex. Here, we employed genetic analysis to determine the interactions between all twenty ARSs and the subunits of EF-1 complex because this method is sensitive enough to detect weak or transient interactions.

The EF-1 complex is involved in the delivery of the charged tRNAs to ribosome. EF-1α transfers aminoacyl-tRNA to the A site of ribosome at the expense of GTP for further proceeding in protein synthesis and EF-1β/γ exchanges the bound GDP with GTP in EF-1α (10). Thus, EF-1 complex is responsible for the vectoral transfer of charged tRNAs from ARSs to ribosome during protein synthesis. If tRNAs are transferred among these translational components by active mechanisms without diffusion through cytoplasm, it would be better for these proteins to be physically linked to generate a delivery channel of tRNA. This possibility prompted us to investigate the possible interactions between each of human ARSs and the EF-1 subunits (Fig. 2). The interactions revealed in this work were summarized in Fig. 5 along with others previously reported. From the interaction studies between ARSs and EF-1 subunits, we tried to address a few questions. First, we were curious whether the interaction of ARSs with EF-1 complex is related to their propensity to form a macromolecular ARS complex. For this reason, we separated ARSs to the complex-forming and non-complex forming enzymes, and compared these two groups in their EF-1 interaction properties. As seen in Fig. 5, the complex-forming ARSs are not clearly distinguished from non-complex formers at least in the interaction with the EF-1 subunits because the strongly or moderately interacting enzymes are present in both of the groups. Second, the catalytic activities of ARSs may be affected by their interaction with the EF-1 subunits. As expected, the activities of LRS and HRS strongly interacting with the EF-1 complex subunits are increased by GTP that is required for the function of EF-1. The stimulation of HRS by EF-1 was also previously reported (20). DRS, FRS previously shown to be regulated by EF-1α showed moderate interactions with the EF-1δ in this work. Therefore, the interaction between ARSs and EF-1 complex appears to reflect their functional linkage although our assay system is not sensitive enough to monitor the activity change of ARSs that interact with EF-1 complex with moderate affinity. Third, not all ARS members were shown to interact with the EF-1 subunits in the two hybrid assay. However, the negative results in this assay do not necessarily reflect no interaction of the proteins. Thus, we do not exclude the possibility that other ARSs still make interactions with EF-1 complex. We expect that functional linkage of more ARSs and EF-1 would be revealed if more sensitive assay system could be applied. Alternatively, they may be linked via either other cellular framework or adaptor molecules (30). In this sense, one of the ARS cofactor, p18, showing the strong interaction with EF-1γ may play such a role. Lastly, many ARSs interact with the $\beta\gamma\delta$ subunits of EF-1 complex although EF-1α is the factor carrying the charged tRNAs between ARSs and ribosome. Perhaps, the additional function of these subunits is to make a

complex with ARSs to help the recruitment of EF-1α for efficient delivery of the charged tRNAs.

In summary, we have identified several new interactions between ARSs and EF-1 subunits that have not been previously reported (Fig. 5). Among them, the catalytic activities of two ARSs (LRS and HRS) were increased by GTP that is a cofactor of EF-1 complex. The detailed working mechanism for this stimulation effect is under investigation.

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