Binding Mode of the First Aminoacyl-tRNA in Translation Initiation Mediated by Plautia stali Intestine Virus Internal Ribosome Entry Site

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Eukaryotic ribosomes directly bind to the intergenic region-internal ribosome entry site (IGR-IRES) of Plautia stali intestine virus (PSIV) and initiate translation without either initiation factors or initiator Met-tRNA. We have investigated the mode of binding of the first aminoacyl-tRNA in translation initiation mediated by the IGR-IRES. Binding ability of aminoacyl-tRNA to the first codon within the IGR-IRES/80 S ribosome complex was very low in the presence of eukaryotic elongation factor 1A (eEF1A) alone but markedly enhanced by the translocation of eEF2. Moreover, eEF2-dependent GTPase activity of the IRES/80 S ribosome complex was 3-fold higher than that of the free 80 S ribosome. This activation was suppressed by addition of the antibiotics pactamycin and hygromycin B, which are inhibitors of translocation. The results suggest that translocation by the action of eEF2 is essential for stable tRNA binding to the first codon of the PSIV-IRES in the ribosome. Chemical probing analysis showed that IRES binding causes a conformational change in helix 18 of 18 S rRNA at the A site such that IRES destabilizes the conserved pseudoknot within the helix. This conformational change caused by the PSIV-IRES may be responsible for the activation of eEF2 action and stimulation of the first tRNA binding to the P site without initiation factors.

Translation initiation in eukaryotes requires participation of multiple initiation factors and generally also involves the 5′-cap structure of mRNAs. Translation of some viral mRNAs, however, is initiated by an alternative mechanism mediated by an RNA known as the internal ribosome entry site or IRES (reviewed in Ref. 1). Plautia stali intestine virus (PSIV) and cricket paralysis virus (CrPV) are members of the dicistroviruses that have a structurally conserved intergenic region (IGR)-IRES (2–4). The IGR-IRES elements of dicistroviruses consist of ~200 nt, which constitute a secondary structure containing three domains (1 to 3) and three pseudoknots (PK I, PK II, PK III) (Fig. 1A). The IGR-IRES facilitates translation initiation independently of initiation factors initiator Met-tRNA and AUG initiation codon in a wide spectrum of eukaryotes (5–7).

The initiation mechanism of IGR-IRES-mediated translation has been deduced from in vitro experimental evidence.Domains 1 and 2 including PK I and PK III of the IGR-IRES bind tightly to a site on the 40 S ribosomal subunit (8) presumably near the E site (9), whereas domain 3 including PK I appears to be placed in the P site (3, 4). Binding competition experiments with eukaryotic initiation factor 2 (10) and with anticodon stem-loop of tRNA (8), toeprinting assays (11, 12), hydroxyl radical probing using eukaryotic initiation factor 1 mutants (10), and current cryo-EM data (13) suggest that a base-paired triplet within PK I mimics the base-paired AUG codon/Met-tRNA anticodon that forms at the P site of the decoding center in general translation initiation. In the first elongation cycle, aminoacyl-tRNA should be brought to the A site with eEF1A and base-paired with the first decoded triplet at the 3′-border of PK I and then translocated to the P site by the action of eEF2. This process is quite mysterious, because the first aminoacyl-tRNA binding to the A site and its translocation occurs in the absence of decylated tRNA in the P site. Furthermore, in usual translation the translocation is an event involving peptidyl-tRNA, not aminoacyl-tRNA (14). Although this process has been demonstrated by toeprinting assays in a system reconstituted with eEF1A, eEF2, ribosomes, aminoacyl-tRNA, and CrPV IRES (11, 12), it remains to be clarified how the IRES binding affects each activity of eEF1A or eEF2 on the ribosome.

The cryo-electron microscopic structure of the IGR-IRES-ribosome complex shows that IRES induces conformational changes in the 40 S subunit, which consists of a rotation of the head relative to the body as well as the relative positions of helices 18 and 34 (9). Unexpectedly, binding of the IGR-IRES to the 80 S ribosome largely changes conformational ordering of the P protein stalk located in the GTPase-associated center of the 60 S subunit (9). It is highly likely that these IRES-dependent conformational changes of the ribosome make...
the unusual first elongation cycle possible. We here report the effect of binding of the PSIV IGR-IRES to the ribosome on each activity of isolated eEF1A and eEF2 in the first elongation cycle of the IGR-IRES-mediated translation. We show that aminoacyl-tRNA for the first codon is very unstable at the A site in the presence of eEF1A alone, but stabilized at the P site only after translocation by the actions of both eEF1A and eEF2. The ribosomal eEF2-dependent GTPase activity increases 3-fold upon IRES binding, suggesting that IRES activates the ribosomal GTPase-associated center. In addition, IRES markedly enhances chemical modification of bases in helix 18 of 18 S rRNA located at the A site. These effects of IRES binding on ribosomal structure and function seem to be closely related to the unique process of IGR-IRES-mediated translation initiation.

**MATERIALS AND METHODS**

**Plasmids**—The region encompassing nucleotides 5961–6300 of the PSIV sequence that includes domains 1 to 3 (see Fig. 1A) and a part of the coding sequence for capsid protein (designated here as Domain 1-2-3) was amplified by PCR using the dicistronic plasmid pT7CAT-5375 (5) as a template. The amplified fragment was inserted between the HindIII and EcoRI sites of pT7Blue (Novagen) and cloned. The resultant plasmid pT7-IRE5961–6300 was used as template to introduce mutations into pseudoknots I to III (mut-PK I to mut-PK III, see Fig. 1A) by primer-directed PCR mutagenesis. A region covering nucleotides 6005–6149 that includes domain 1 and 2 (designated as Domain 1-2) was also amplified and cloned.

**Ribosomes, Elongation Factors, and Aminoacyl-tRNA**—High salt-washed 80 S ribosomes and 40 S subunits were prepared from cysts of the brine shrimp *Artemia salina* as described previously (16, 17). The isolated ribosomes showed no contaminating eEF1A and eEF2 activity. Eukaryotic elongation factors eEF1A and eEF2 were isolated from pig liver as described (16, 17). The isolated ribosomes showed no contaminating eEF1A and eEF2 activity. Eukaryotic elongation factors eEF1A and eEF2 were isolated from pig liver as described (16, 18, 19). Each isolated sample appeared as a single band in SDS-polyacrylamide gel electrophoresis. *Escherichia coli* total tRNA purchased from Roche Applied Science was charged with [14C]glutamine or [14C]phenylalanine (both from GE Healthcare) by addition of MgCl2 to 3 mM, the primer-annealed IRES was incubated with 6 pmol *A. salina* 80 S ribosomes in 30 l of solution containing 1 mM MgCl2, 100 mM KCl, 0.2 mM dithiothreitol, and 50 mM Tris-HCl, pH 7.6. 

**RNA Synthesis and Renaturation**—The plasmids described above were linearized with EcoRI and used as templates for transcription with T7 RNA polymerase. RNAs were synthesized and purified as described previously (22). The isolated RNA fragments were heated at 70 °C for 3 min and then cooled on ice in a solution containing 1–10 mM MgCl2, 100 mM KCl, 0.2 mM dithiothreitol, and 50 mM Tris-HCl, pH 7.6.

**Acrylamide-Agarose Composite Gel Electrophoresis**—The 40 S ribosomal subunits (10 pmol) were mixed with 40 pmol of the IGR-IRESs (Domain 1-2-3 or its mutants) in 10 l of solution containing 1 mM MgCl2, 50 mM NH4Cl, 25 mM Tris-HCl, pH 8.0, and incubated at 37 °C for 5 min to form the complex. The complexes were analyzed by electrophoresis on acrylamide-agarose composite gels composed of 3% (w/v) acrylamide (acrylamide/bisacrylamide, 19:1) and 0.5% (w/v) agarose as described previously (23). Gels were stained with 0.2% Azur B.

**Aminoacyl-tRNA Binding**—The 80 S ribosomes (20 pmol) were mixed with 80 pmol of the IGR-IRESs (Domain 1-2-3 or its mutants) in 25 l of solution containing 1 mM MgCl2, 100 mM KCl, 0.2 mM dithiothreitol, 50 mM Tris-HCl, pH 7.6, and incubated at 37 °C for 5 min to form the complex. In the experiments to test the effect of antibiotics, 80 S ribosomes were preincubated with 1 mM pactamycin or hygromycin B. The reaction mixture was mixed with another solution (25 l) containing 5 mM MgCl2, 100 mM KCl, 0.2 mM dithiothreitol, 50 mM Tris-HCl, pH 7.6, 60 pmol [14C]Gln-tRNA (400cpm/pmol), 120 pmol eEF1A, and 3 mM GTP or guanyl-5′-yl imidophosphate (GMPPNP) that had been preincubated at 37 °C for 5 min. The resultant reaction mixture containing IGR-IRES-80 S ribosome complexes [14C]Gln-tRNA and eEF1A was incubated at 37 °C for 10 min and then filtered through a nitrocellulose membrane (type HA, 0.45-mm pore size; Millipore). The membrane was washed with 3 ml of ice-cold buffer containing 5 mM MgCl2, 100 mM NH4Cl, 20 mM Tris-HCl, pH 7.6. Radioactivity retained on the filter was counted using a liquid scintillation counter (Aloka). In the experiment to test the effect of the addition of eEF2 on the aminoacyl-tRNA binding, the same incubation and filtration experiments were performed after addition of 20 pmol eEF2 to the reaction mixture containing the IGR-IRES-80 S ribosome complexes, [14C]Gln-tRNA, and eEF1A as described above.

**Toeprinting Assay**—The toeprinting assay was carried out basically as described by Takay et al. (24). In brief, 1.5 pmol IGR-IRES (Domain 1-2-3) was annealed to 10 pmol primer, which was complementary to nucleotides 6281–6300 of the coding region of PSIV, in 100 mM KCl and 50 mM Tris-HCl, pH 7.6 (10 l), by heating to 60 °C for 3 min and placing on ice. After addition of MgCl2 to 3 mM, the primer-annealed IRES was incubated with 6 pmol *A. salina* 80 S ribosomes in 30 l of binding buffer (3 mM MgCl2, 100 mM KCl, 0.2 mM dithiothreitol, 50 mM Tris-HCl, pH 7.6) at 37 °C for 5 min. An aliquot (10 l) of the IRES-80 S complex (1 pmol) was incubated with 2 pmol Gln-tRNA, 4 pmol eEF1A, 4 pmol eEF2, 1.5 mM GTP at 37 °C for 10 min. After translocation, aliquots (5 l each) of the reactions were extended by addition of 3 units of avian myeloblastosis virus reverse transcriptase and four dNTPs mixed with [α-32P]dCTP at 37 °C for 40 min. The products were resolved on a 6% sequencing gel.

**eEF2-dependent GTPase**—The IGR-IRES-80 S ribosome complexes (5 pmol) were incubated with 10 pmol eEF2 and 3 nmol [γ-32P]GTP (40 cpm/pmol) in a solution (20 l) containing 1 mM MgCl2, 50 mM KCl, 0.2 mM dithiothreitol, and 50 mM Tris-HCl, pH 7.6, at 37 °C for 10 min in the presence or absence of 1 mM pactamycin or hygromycin B. The amount of inorganic [γ-32P]phosphate liberated was determined as described previously (25).

**eEF2-GDP Binding Assay**—The IGR-IRES-80 S ribosome complexes (17 pmol) were incubated with 80 pmol [3H]GDP (750 cpm/pmol) and 30 pmol eEF2 in a solution (20 l) containing 10 mM MgCl2, 10 mM KCl, 0.2 mM dithiothreitol, 40 mM Tris-HCl, pH 8.0, at 37 °C for 5 min. The reaction was stopped by addition of 1 ml of ice-cold buffer containing 20 mM Tris-HCl, pH 8.0, 10 mM MgCl2. The mixture was filtered through a
Effects of IGR-IRES Binding on Ribosome Function

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FIGURE 1. Mutants of the PSIV IGR-IRES used in the present study and their ability to bind to the ribosome. A, base substitutions at three pseudoknot regions, PK I, PK II, and PK III, that disrupt the base pairing are shown on the secondary structure of the IGR-IRES RNA. Highly conserved nucleotides in CrP-like viruses are capitalized. Dots and asterisks represent base pairs in stems and pseudoknots, respectively. B, binding of the IGR-IRES RNA including nucleotides 5961–6300 (Domain 1-2-3) and its mutants to 40 S ribosomal subunits. 40 S subunit alone (lane 1), 40 S subunits + Domain 1-2-3 (lane 2), 40 S subunits + mut-PK I (lane 3), 40 S subunits + mut-PK II (lane 4), 40 S subunits + mut-PK III (lane 5), and 40 S subunits + Domain 1-2 containing nucleotides 6005–6149 (lane 6) were resolved by agarose-acrylamide composite gel electrophoresis as described under "Materials and Methods." The gel was stained with Azur B.

RESULTS

Effect of IGR-IRES Binding to the Ribosome on Each Action of Isolated eEF2 and eEF1A—In this study, we used PSIV IGR-IRES containing domains 1, 2, 3 and 108 nucleotides of coding sequence (Domain 1-2-3) and its various mutants (Fig. 1A), including mut-PK I, mut-PK II, and mut-PK III, which disrupted PK I, PK II, and PK III, respectively, as well as Domain 1-2 deficient in domain 3 and coding region. The ability of these IGR-IRES mutants to bind to the 40 S subunits was examined by acrylamide-agarose composite gel electrophoresis (Fig. 1B). The electrophoretic mobility of the 40 S-IRES complex was higher than that of free 40 S subunits in this gel system, because the gel mobility depends more on the negative charge of IRES RNA bound to the 40 S subunits than on the size of complexes. The band of free 40 S subunits (lane 1) was clearly shifted by addition of Domain 1-2-3 (lane 2), suggesting tight binding of the IRES RNA to the 40 S subunit. Mut-PK I (lane 3) and mut-PK II (lane 4) also strongly bound to the 40 S subunit, whereas the binding ability of mut-PK III (lane 5) was significantly lower than the other mutants. Domain 1-2 showed slightly reduced ability to bind to the 40 S subunit (lane 6).

Elongation factors eEF1A and eEF2 were isolated from pig liver according to Iwasaki and Kaziro (16) as described previously (18, 19). The purified factors were active in ribosome-coupled GTPase, [14C]Phe-tRNA binding, and polyphenylalanine synthesis dependent on poly(U) using ribosomes isolated from brine shrimp A. salina (data not shown) and were used in subsequent experiments.

It was of great interest to us to test the effect of IGR-IRES binding on ribosomal eEF2-dependent GTPase activity, because IGR-IRES changes the conformation of the ribosomal P protein complex (9), which is responsible for the GTPase activity (28). The effect of additions of IGR-IRES and its mutants on ribosomal-uncoupled GTPase activity dependent on eEF2 was therefore assayed (Fig. 2A). The GTPase activity of brine shrimp ribosomes was stimulated 3-fold by addition of the wild type IGR-IRES (Domain 1-2-3). A similar level of stimulation was observed by addition of mut-PK I, whereas only partial stimulation could be observed upon addition of the Domain 1-2 mutant. No stimulation of activity was detected by addition of mut-PK II and mut-PK III. A possible explanation for the IGR-IRES-dependent stimulation of GTPase turnover is that IRES binding may enhance eEF2-GDP release from the ribosome after GTP hydrolysis. To test this possibility, eEF2-[3H]GDP binding to the ribosome was checked by filter binding assays in low salt conditions in the presence or absence of IRES (Fig. 2B). As expected, addition of Domain 1-2-3 to the ribosome markedly reduced ribosomal ability to bind to eEF2-[3H]GDP. Addition of Domain 1-2 gave a partial effect.

The activity of eEF1A was assayed by aminoacyl-tRNA binding to the ribosomal A site by a membrane filtration method (19). When A. salina 80 S ribosomes (20 pmol) preincubated with poly(U) and decacylated tRNA were mixed with excess amount of [14C]Phe-tRNA, eEF1A, and GMPNP, 11.5 pmol [14C]Phe-tRNA bound to the ribosomes. Using the same method, we tested eEF1A-dependent binding of [14C]Gln-tRNA to the 80 S ribosome in the presence of IGR-IRES, whose first codon is CAA, which codes for glutamine. As shown in Fig. 3A, [14C]Gln-tRNA failed to bind to the ribosome-IRES complex in the presence of either GTP or GMP-PNP. However, addition of eEF2 to the reaction mixture caused the amount of [14C]Gln-tRNA binding to be significantly increased, although eEF2 alone gave no stimulation effect. It is inferable from these results that [14C]Gln-tRNA is stabilized only at the P site after translocation through the action of eEF2. When mut-PK I and mut PK II were used instead of Domain 1-2-3, no binding of the aminoacyl-tRNA was detected even in the presence of both eEF1A and eEF2 (data not shown).
To confirm the translocation in our reconstitution system, we performed toeprinting analysis. After an oligonucleotide primer was annealed to a downstream coding sequence of the IGR-IRES, it was bound to the 80 S ribosome. By addition of reverse transcriptase the primer was extended until the extension was blocked by the bound ribosome. The extension was arrested at the 14–16-nt downstream regions of the cytosine base at the 5′-side of the CUU sequence, which is a part of PK I and presumably placed at the P site considering previous biochemical and cryo data (8, 10–13) (Fig. 3B, lane 2). Addition of Gln-tRNA, together with eEF1A and eEF2, to the IGR-IRES-80 S ribosome complex gave other arrested signals at the 17–19 downstream regions (lane 3), indicating that Gln-tRNA and mRNA are translocated from the A site to the P site in the presence of both elongation factors.

Inhibitors of Translocation Suppress Binding of Aminoacyl-tRNA to the First Codon of IGR-IRES—It is known that the antibiotics pactamycin and hygromycin B bind to the highly conserved tRNA regions (29, 30) and inhibit translocation of the A site tRNA to the P site (31, 32). Because these antibiotics did not block ribosome binding to the IGR-IRES (data not shown), the effects of these antibiotics on IGR-IRES-induced ribosome function were examined (Fig. 4). Although binding of the IGR-IRES to 80 S ribosomes enhanced eEF2-dependent GTPase activity 3-fold as shown in Fig. 2A, this enhancement was considerably suppressed by pactamycin and partially suppressed by hygromycin B (Fig. 4A). Binding of Gln-tRNA to the IGR-IRES-80 S ribosome complex, which depends on both eEF1A and eEF2, decreased to 30% of control upon addition of pactamycin and to 50% of control upon adding hygromycin B (Fig. 4B). These results suggest that binding of aminoacyl-tRNA for the first codon within the IGR-IRES-80 S ribosome would be blocked by these antibiotics.
A ribosome complex is correlated with activated eEF2-dependent GTPase and translocation.

Conformational Change of 18 S rRNA at the A Site Induced by PSIV IGR-IRES—To examine conformational changes of rRNAs responsible for the IGR-IRES-induced characteristics of the ribosome reported here, we performed chemical probing experiments. *A. salina* 80 S ribosomes were treated with the chemical reagents DMS and CMCT in the presence or absence of IGR-IRES (Domain 1-2-3) and the chemical modifications in 18 S rRNA monitored by primer extension. The IRES binding gave no base protection to full-length 18 S rRNA (data not shown) but enhanced the modifications of some bases of a single stem-loop region, helix 18 (Fig. 5). Domain 1-2-3 enhanced DMS modification of G-505, G-506, and C-507 (*E. coli* numbering) and slightly enhanced the modification of A-532 (lane 2 shown).
compared with lane 1). Domain 1-2-3 also enhanced CMCT modification of G-505 and gave slight enhancement of modification of G-506 and C-507 (compare lane 8 with lane 7). It is interesting that G-505, G-506, and C-507 are the bases involved in the functionally important pseudoknot structure in helix 18 (33, 34) (Fig. 5B).

These enhanced chemical modifications were compared with the effects of the various mutants of Domain 1-2-3 used in eEF2-dependent GTPase assays (Fig. 2A). The binding of mut-PK I was also found to enhance the modification of bases G-505, G-506, C-507, and A-532 (Fig. 5, lanes 3 and 9). The extent of enhancement is comparable with that by wild type IRES (Domain 1-2-3, lanes 2 and 8). However, mut-PK II (lanes 4 and 10) and mut-PK III (lanes 5 and 11) hardly enhanced modification. Domain 1-2 gave enhancement for all the bases (lanes 6 and 12), but the extent of enhancement was smaller than that by Domain 1-2-3 (lanes 2 and 8). The enhancement of chemical modification seems to be correlated with the activation of eEF2-dependent GTPase shown in Fig. 2A.

This PSIV IGR-IRES-dependent enhancement of chemical modification in helix 18 was also observed when 80 S ribosomes from rat liver and silkworm were used instead of A. salina ribosomes. However, it was not observed when the IGR-IRESs of the other dicistoviruses Himetobi P virus and Taura syndrome virus were used instead of PSIV IGR-IRES.

**DISCUSSION**

Binding of the aminoacyl-tRNA to the first codon down-stream of the IGR-IRES in dicistoviruses is unusual, because the process occurs without tRNA in the P site. Here we have described the molecular mode of the binding of the first Gln-tRNA to PSIV IGR-IRES. By investigating the effect of the IRES binding on individual actions of eEF1A and eEF2, we have demonstrated that the first Gln-tRNA cannot bind efficiently through the action of eEF1A alone, but only bind efficiently upon addition of both eEF1A and eEF2 (Fig. 3A). Because eEF2 alone does not function in Gln-tRNA binding at all, eEF1A should play an essential role in temporal binding of Gln-tRNA to the ribosomal A site and then eEF2 efficiently translocating it to the P site. The translocation in our reconstituted assay system using PSIV IGR-IRES was demonstrated by toeprinting assay (Fig. 3B), as shown in previous reports with CrPV IGR-IRES (11, 12), and by inhibition studies using pactamycin and hygromycin B (Fig. 4B), which bind to helices 23/24 and helix 44, respectively, (29, 30) and which both inhibit translocation (31, 32). We also observed that Gln-tRNA bound by the actions of eEF1A and eEF2 protected a base, A-532, that is located in the P site (27). These lines of evidence clearly indicate that the aminoacyl-tRNA for the first codon of the IGR-IRES stably binds only to the P site after translocation from the A site.

The presence of deacylated tRNA at the P site is required for translocation in usual translation, but not in the translation initiation mediated by IGR-IRES. It has been assumed that a part of the IGR-IRES including PK I mimics tRNA at the P site (3, 4). This may be one of the essential factors to make the translocation possible. Another essential part of the mecha-nism that we infer from our results is that there is a change in the mode of action of the translocase eEF2 upon binding of IGR-IRES. In the present study, we show that eEF2-dependent GTPase is enhanced 3-fold by binding of PSIV IGR-IRES to 80 S ribosomes. This enhancement of the GTPase seems to be due to IGR-IRES-induced changes of ribosomal structural features that are involved in the interaction between the ribosome and eEF2. This view is supported by our finding of destabilization of the 80 S ribosome-eEF2-GDP complex upon IRES binding (Fig. 2B) and also by previous cryo-EM data showing that the CrPV-IRES binding changes the conformation of the P protein stalk (9). Such a rearrangement of eEF2 binding site in the ribosome is likely correlated with the unusual translocation mediated by the IGR-IRES.

We have also shown that binding of IGR-IRES enhances chemical modification of bases within helix 18 of 18 S rRNA, although it did not protect any bases. Among the enhanced bases, G-505, G-506, and C-507 are known to be involved in formation of a pseudoknot structure by base pairing with G-526, C-525, and G-524, respectively (34) (Fig. 5B). Enhancement of the chemical modification of these bases indicates a conformational change in helix 18 of 18 S rRNA. It is highly likely that the base pairs G-505:C-526, G-506:C-525, and C-507:G-524 are destabilized and the pseudoknot structure is, at least partially, disrupted by IRES binding.

The IGR-IRES-dependent conformational change in helix 18 inferred from our work is supported by the cryo-EM evidence that shows that the relative locations of helices 18 and 34 in the 80 S ribosome are changed upon binding of CrPV (9). Because helix 18, together with ribosomal protein S12, lies in the A site and presumably participates in correct codon-anticodon base pairings in bacterial ribosomes (39), the conformational change in helix 18 would be expected to significantly affect ribosome function. This is shown to be the case as IGR-IRES markedly reduces eEF1A-dependent aminoacyl-tRNA binding to the A site in the presence of either GTP or GMPNP (Fig. 3A) and also reduces stability of the complex between 80 S ribosome and eEF2-GDP (Fig. 2B) as described above. These results are consistent with the fact that mutations that disrupt the pseudoknot of helix 18 in E. coli cells lead to lethality (34). We suggest from these facts that the conformational change of helix 18 including the pseudoknot structure is one of the important events in translation initiation by PSIV IGR-IRES. However, such a conformational change of the pseudoknot structure in helix 18 seems not to be essential for translation initiation of the other dicistoviruses Himetobi P virus and Taura syndrome virus, because bindings of IGR-IRES of the latter two viruses to the 80 S ribosome did not enhance the base modifications in helix 18. The structural and binding features unique to PSIV IGR-IRES may be responsible for induction of the unique conformational change of helix 18. Further extensive studies are required to explain the discrepancy.

Interestingly, one mutant of IRES in which PKI is disrupted (mut-PKI) still stimulates GTPase to a level comparable with wild type whereas another mutant in which PKII is disrupted (mut-PKII) gives no enhancement of GTPase activity, despite the retention of ability to bind strongly to the ribosome (Fig. 1B). Another mutant, Domain 1-2 lacking domain 3 of IRES, 3 H. Yamamoto and T. Uchiimi, unpublished data.
shows a partial effect of the GTPase enhancement. Because PKII seems to participate in the correct folding of RNA that leads domain 3 toward the P site of the decoding center within the ribosome, the orientation of domain 3 as well as its presence within the ribosome is responsible for the GTPase activation. The enhancement of translocase-dependent GTPase is also observed when tRNA exists in a P/E hybrid state on the E. coli ribosome (35–38). Because cryo-EM observation indicates that the CrPV IRES binds in the intersubunit space overlapping binding sites for E, P, and A site tRNAs, it may be possible that a part of the IRES structure including domain 3 mimics the P/E state tRNA. If this is the case, the GTPase activation by IRES can be explained by a mode similar to the E. coli system in which the P/E hybrid tRNA activates the ribosomal GTPase activity, although the detailed mechanism remains to be elucidated.

Nierhaus (15) and his colleagues discovered that tRNA binding to the E site lowers the affinity of the A site for aminoacyl-tRNA in an allosteric manner, although the molecular basis of how the allosteric signal from the E site reaches the A site within the ribosome is not yet clear. We infer that tight binding of tRNA in an allosteric manner, although the molecular basis of how the allosteric signal from the E site reaches the A site lowers the affinity of the A site for aminoacyl-tRNA, may be a useful tool for studying the allosteric signaling mechanism, because the binding of Domain 1-2 lacking domain 3 still gives some effect on the GTPase-associated center, because the binding of tRNA in an allosteric manner, although the molecular basis of how the allosteric signal from the E site reaches the A site lowers the affinity of the A site for aminoacyl-tRNA, may be a useful tool for studying the allosteric signaling mechanism.

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