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COMMUNICATION

AIMP3/p18 Controls Translational Initiation by Mediating the Delivery of Charged Initiator tRNA to Initiation Complex

Taehee Kang[1]†, Nam Hoon Kwon[1]†, Jin Young Lee[1], Min Chul Park[1], Eunji Kang[1], Hyo Hyun Kim[1], Taek Jin Kang[2] and Sunghoon Kim[1,3]*

[1]*Medicinal Bioconvergence Research Center, Seoul National University, Seoul 151–742, Korea*
[2]*Department of Chemical and Biochemical Engineering, Dongguk University-Seoul, Seoul 100–715, Korea*
[3]*WCU Department of Molecular Medicine and Biopharmaceutical Sciences, Graduate School of Convergence Science and Technology, Seoul National University, Suwon 443–270, Korea*

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Aminoacyl-tRNA synthetase-interacting multifunctional proteins (AIMPs) are nonenzymatic scaffolding proteins that comprise multisynthetase complex (MSC) with nine aminoacyl-tRNA synthetases in higher eukaryotes. Among the three AIMPs, AIMP3/p18 is strongly anchored to methionyl-tRNA synthetase (MRS) in the MSC. MRS attaches methionine (Met) to initiator tRNA ($tRNA_i^{Met}$) and plays an important role in translation initiation. It is known that AIMP3 is dispatched to nucleus or nuclear membrane to induce DNA damage response or senescence; however, the role of AIMP3 in translation as a component of MSC and the meaning of its interaction with MRS are still unclear. Herein, we observed that AIMP3 specifically interacted with Met-$tRNA_i^{Met}$ *in vitro*, while it showed little or reduced interaction with unacylated or lysine-charged $tRNA_i^{Met}$. In addition, AIMP3 discriminates Met-$tRNA_i^{Met}$ from Met-charged elongator tRNA based on filter-binding assay. Pull-down assay revealed that AIMP3 and MRS had noncompetitive interaction with eukaryotic initiation factor 2 (eIF2) γ subunit (eIF2γ), which is in charge of binding with Met-$tRNA_i^{Met}$ for the delivery of Met-$tRNA_i^{Met}$ to ribosome. AIMP3 recruited active eIF2γ to the MRS–AIMP3 complex, and the level of Met-$tRNA_i^{Met}$ bound to eIF2 complex was reduced by AIMP3 knockdown resulting in reduced protein synthesis. All these results suggested the novel function of AIMP3 as a critical mediator of Met-$tRNA_i^{Met}$ transfer from MRS to eIF2 complex for the accurate and efficient translation initiation.

*Corresponding author. Medicinal Bioconvergence Research Center, Seoul National University, Seoul 151-742, Korea. E-mail address: sungkim@snu.ac.kr.

† T.K. and N.H.K. contributed equally to this work.

Abbreviations used: MRS, methionyl-tRNA synthetase; eIF2γ, eIF2 γ subunit; ARS, aminoacyl-tRNA synthetase; MSC, multisynthetase complex; AIMP, ARS-interacting multifunctional protein; eEF, eukaryotic elongation factor; eIF2, eukaryotic initiation factor 2; TC, ternary complex; MEF, mouse embryonic fibroblast; WT, wild type; GST, glutathione *S*-transferase.

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Introduction

Aminoacyl-tRNA synthetases (ARSs), which conjugate amino acids to their cognate tRNAs, form a multisynthetase complex (MSC) in higher eukaryotes.[1] This complex consists of nine ARSs and three nonenzymatic cofactors called ARS-interacting multifunctional proteins (AIMPs), namely, AIMP1/p43, AIMP2/p38, and AIMP3/p18.[2] AIMPs are involved in scaffolding the MSC structure and controlling the stability of neighboring ARSs.[3] In addition, AIMPs dissociate from MSC in response to various stimuli and are involved in diverse biological functions and signaling pathways, such as immune responses, angiogenesis, wound healing, glucose homeostasis, cell proliferation, and apoptosis.[4–11] Among them, AIMP3 is known to be a potent tumor suppressor via activation of p53 under UV damage or oncogenic stress,[12,13] and elevated levels of AIMP3 lead to cellular senescence via degradation of mature lamin A.[14]

While our understanding of the functions of AIMPs outside MSC has advanced considerably, their roles in the MSC as translation components remain unclear. Arc1p, a yeast orthologue of human AIMP1/p43, is known to increase the aminoacylation activities of methionyl-tRNA synthetase (MRS) and glutamyl-tRNA synthetase by recruiting tRNAs.[15,16] AIMP1 also binds to tRNA and enhances the aminoacylation activity of arginyl-tRNA synthetase.[17] AIMP3 has sequence similarity to valyl-tRNA synthetase and is also known as eukaryotic elongation factor (eEF) 1 epsilon 1 based on sequence similarity to eEFs.[18] These results imply that AIMPs can play an active role in the translation process as members of MSC, although little is known about their functions in protein synthesis.

AIMP3 shows strong interaction with MRS in the MSC, and knockdown of AIMP3 affects the stability of MRS protein.[3] Recently, the importance of MRS in global translational regulation was suggested.[19] Under UV irradiation, MRS was phosphorylated by GCN2 (*g*eneral *c*ontrol *n*onrepressed 2), and this modification reduced the catalytic activity of MRS, resulting in decreased levels of methionine (Met)-charged initiator tRNA (Met-tRNA$_i^{Met}$). For translation initiation, Met-tRNA$_i^{Met}$ should bind to eukaryotic initiation factor 2 (eIF2) in a GTP-dependent manner to form the ternary complex (TC), eIF2–GTP–Met-tRNA$_i^{Met}$, which delivers Met-tRNA$_i^{Met}$ to the 40S ribosomal subunit.[20] That is why MRS phosphorylation and insufficient TC formation can reduce global protein synthesis.

Because AIMP3 specifically binds to MRS in the MSC and AIMP3 has sequence similarity to eEFs, we hypothesized that AIMP3 may have a function in translation regarding MRS activation or Met-tRNA$_i^{Met}$ delivery to other translation factors. Here, we report that AIMP3 interacts with Met-tRNA$_i^{Met}$ and the eIF2 complex and plays an important role in connecting aminoacylation to translation. These results suggest that AIMP3 may be a critical mediator of the accurate and efficient delivery of the Met-tRNA$_i^{Met}$ to eIF2 and a regulator of global translation initiation.

AIMP3 specifically binds to Met-tRNA$_i^{Met}$

In yeast, Arc1p, a cofactor of MRS and glutamyl-tRNA synthetase, enhances aminoacylation activity via tRNA recruitment.[15,16] To test whether AIMP3 can function similarly, we examined the binding affinity of AIMP3 to free or charged tRNA$_i^{Met}$ using a filter-binding assay. The MRS and eIF2 γ subunit (eIF2γ) were used as controls for binding with tRNA$_i^{Met}$ and Met-tRNA$_i^{Met}$, respectively. In this assay, purified AIMP3 did not exhibit interaction with *in vitro* transcribed radioactive tRNA$_i^{Met}$ (Fig. 1a). In contrast, AIMP3 exhibited explicit interaction with Met-tRNA$_i^{Met}$ in a dose-dependent manner (Fig. 1b and c). Because AIMP3 only bound to Met-tRNA$_i^{Met}$, we hypothesized that AIMP3 might recognize Met attached to the acceptor stem of tRNA$_i^{Met}$. To test this, we analyzed the interaction between AIMP3 and radioactive Met, revealing that AIMP3 has binding affinity to Met as expected (Fig. 1d). Next, we prepared Met-tRNA$_i^{Met}$ and Met-tRNA$_e^{Met}$ to investigate the difference between tRNA^{Met} isoforms with regard to recognition by AIMP3, and we also charged these tRNAs with the nonnatural amino acid acetylated lysine (acK) to compare its binding affinity to AIMP3 with that of Met. It is interesting that the association between AIMP3 and Met-tRNA$_i^{Met}$ was more obvious than that of any other types of tRNAs tested (Fig. 1e). It shows that AIMP3 has binding preference for tRNA$_i^{Met}$ over tRNA$_e^{Met}$ only when the two are charged with Met (Fig. 1f). We introduced mutations in tRNA$_i^{Met}$ to examine whether AIMP3 recognizes discriminating base pair in the acceptor stem of tRNA$_i^{Met}$. As expected, MT1 tRNA$_i^{Met}$, which had A1:U72 substitution for G1:C72 as in tRNA$_e^{Met}$, showed reduced binding affinity to AIMP3 unlike wild-type (WT) tRNA$_i^{Met}$ (Fig. 1g). There was little difference in the binding affinity between MT1 and MT2 tRNAs. Considering that the latter had additional base-pair substitution, this suggests that the discriminating base pair A1:U72 is the most important one for the recognition by AIMP3. Collectively, these results imply that AIMP3 recognizes both Met and tRNA$_i^{Met}$, but Met is prerequisite for the interaction of AIMP3 with tRNA$_i^{Met}$. Although AIMP3 knockdown affected the stability of MRS, resulting in reduced

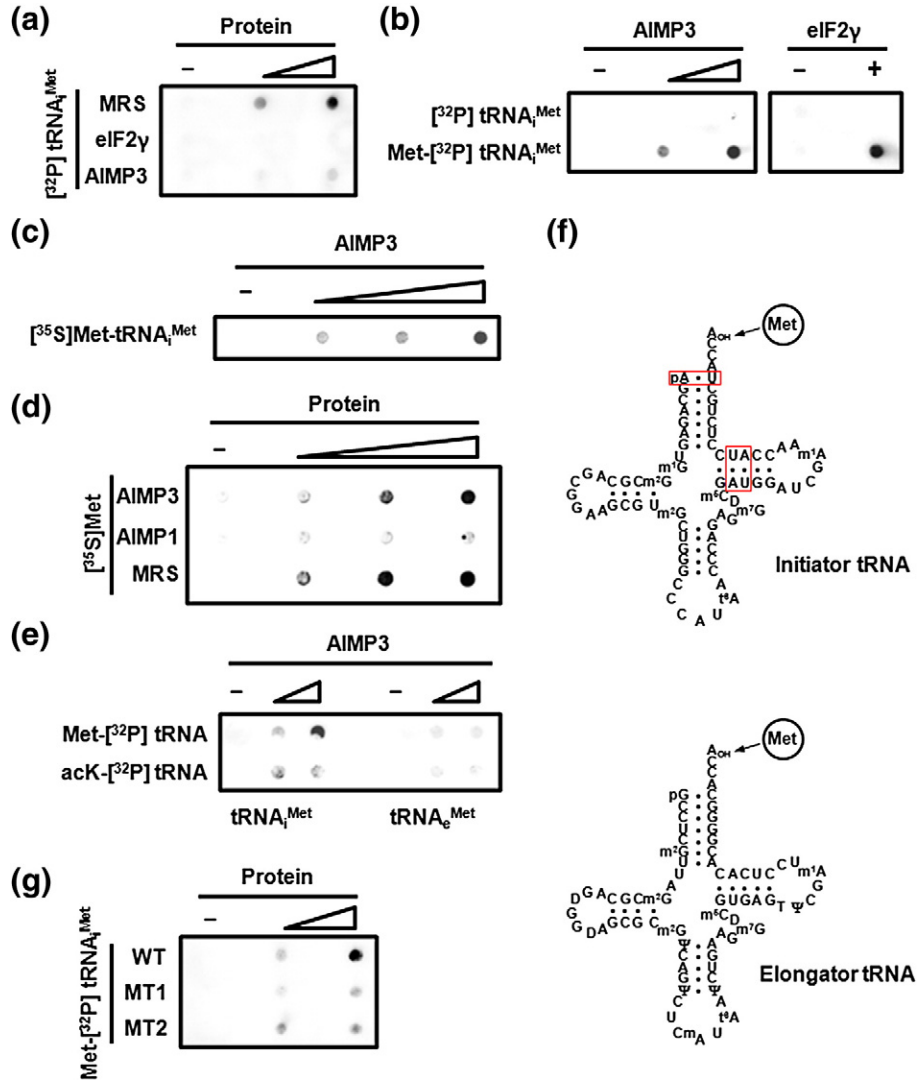

Fig. 1. Specific interaction between AIMP3 and Met-$tRNA_i^{Met}$. (a) Radioactively labeled free [^{32}P]$tRNA_i^{Met}$ was incubated with His-tagged MRS, eIF2γ, and AIMP3. Signals from tRNA bound to proteins were detected. (b) Unacylated $tRNA_i^{Met}$ and charged Met-[^{32}P]$tRNA_i^{Met}$ were subjected to a filter-binding assay with AIMP3 and eIF2γ. (c) [^{35}S]Met was ligated to $tRNA_i^{Met}$ by incubation with purified MRS and used for the binding assay after purification to investigate the interaction between Met-$tRNA_i^{Met}$ and AIMP3. (d) Interaction between AIMP3 and Met was determined by the filter-binding assay. MRS and AIMP1 were used as positive and negative controls, respectively. (e) Radioactively labeled $tRNA_i^{Met}$ and $tRNA_e^{Met}$ were charged with Met and acK using MRS and dFx (*d*initro-*fle*xizyme; a ribozyme-based tRNA-acylating catalyst),[21] respectively, and their interaction with AIMP3 was analyzed. acK, a nonnatural amino acid, was used as a substitute for comparison with Met. (f) The secondary structure of human $tRNA_i^{Met}$ and $tRNA_e^{Met}$. Red-boxed nucleotides indicate discriminating base pairs of $tRNA_i^{Met}$, whose mutations make $tRNA_i^{Met}$ work as $tRNA_e^{Met}$.[22] Met is attached to 3′ OH group via an ester linkage by MRS. (g) Mutations are introduced in $tRNA_i^{Met}$ for *in vitro* transcription of MT1 and MT2, whose A1:U72 and A1:U72/G2:C71 base pairs were substituted for G1:C72 and G1:C72/C2:G71, respectively. Charged WT, MT1, and MT2 [^{32}P]$tRNA_i^{Met}$ were subjected to a filter-binding assay with AIMP3. Protein concentrations used throughout this figure were serially increased by 2-fold with the highest concentration of 0.5 μM, 4 μM, 25 μM, and 14 μM for MRS, eIF2γ, AIMP3, and AIMP1, respectively.

aminoacylation activity of MRS in *AIMP3*$^{+/-}$ mouse embryonic fibroblast (MEF) cells, AIMP3 does not seem to give direct effect on the catalytic reaction *in vitro* (Fig. S1).

MRS and AIMP3 interact with eIF2γ

Because AIMP3 did not affect the catalytic activity of MRS, AIMP3 was assumed to mediate

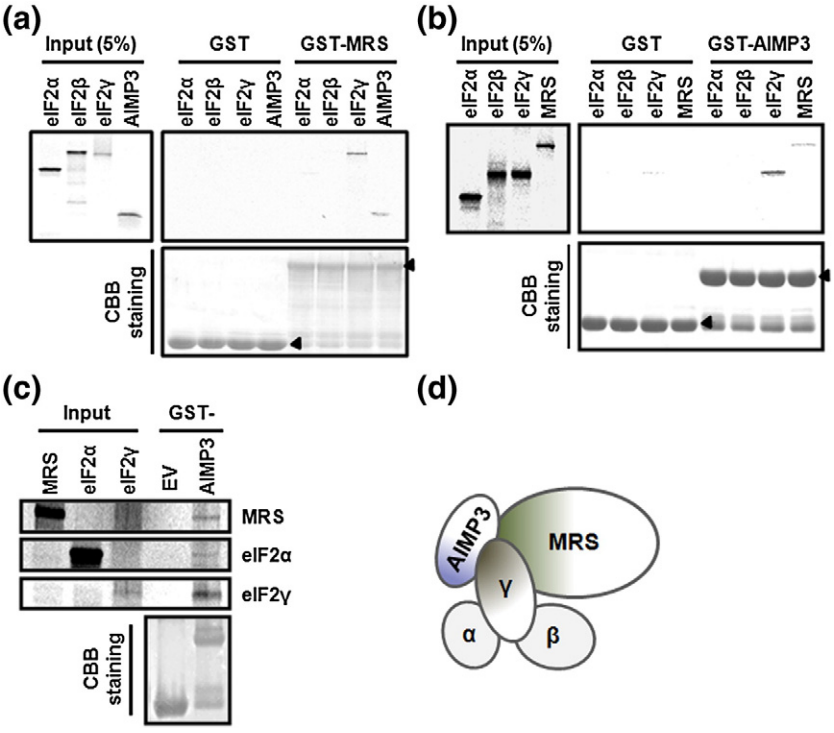

Fig. 2. Interaction of AIMP3 and MRS with eIF2γ. (a and b) Radioactively labeled eIF2 subunits (eIF2α, eIF2β, and eIF2γ) were incubated with GST-MRS or GST-AIMP3. The bound eIF2 subunits were detected by autoradiography. Each protein, stained with Coomassie Brilliant Blue (CBB), is indicated by an arrow. (c) Radioactively labeled eIF2α, eIF2γ, and MRS were mixed with GST-AIMP3. Bound proteins were detected by autoradiography. eIF2α, a binding partner of eIF2γ, was also included to investigate the possibility of eIF2 complex association with the MRS–AIMP3 complex. (d) Interactions between MRS, AIMP3, and the eIF2 subunits (α, β, and γ) are schematically represented based on domain mapping (Fig. S3). The N-termini of these proteins are indicated by dense coloration.

Met-tRNA$_i^{Met}$ delivery to downstream proteins. Met-tRNA$_i^{Met}$ should be transferred to the eIF2 complex for the formation of the TC; therefore, we analyzed the interaction between MRS, AIMP3, eIF2α subunit, eIF2β subunit, and eIF2γ subunit. The glutathione *S*-transferase (GST) pull-down assays revealed that MRS and AIMP3 commonly interacted with eIF2γ among the eIF2 subunits (Fig. 2a and b), which is in charge of binding with Met-tRNA$_i^{Met}$ and GTP.[23] Lysyl-tRNA synthetase did not show apparent interaction with eIF2 subunits unlike MRS and AIMP3 (Fig. S2). MRS and eIF2γ were pulled down together in association with AIMP3 (Fig. 2c), suggesting that this tripartite interaction is not mutually exclusive. We identified the binding domains of MRS and AIMP3 that were hypothesized to interact with eIF2γ, using deletion mutants, and confirmed that MRS and AIMP3 used different domains for each binding partner, resulting in noncompetitive association with eIF2γ (Fig. 2d and Fig. S3). This domain mapping suggests that the GST homology domains of MRS and AIMP3 are important for binding to eIF2γ and that the GTP-binding domain of eIF2γ is crucial for binding to MRS and AIMP3.

AIMP3 recruits active eIF2γ to the MRS–AIMP3 complex

Because the binding domains of each protein were not overlapping, the interactions between MRS, AIMP3, and eIF2γ may be regulated by one protein working as an adapter. To examine whether AIMP3 can mediate interaction between MRS and eIF2γ, we knocked down AIMP3 in HeLa cells using RNA interference and analyzed the change in interaction using an IP (*i*mmuno*p*recipitation) assay. As expected, decreased interaction of eIF2γ with MRS was observed in AIMP3 knockdown cells (Fig. 3a). This result suggests that the interaction between MRS and eIF2γ is dependent on the existence of AIMP3. To investigate the possibility that AIMP3 can recruit active eIF2γ, we substituted Asn190 for Asp (N190D) in the NKXD consensus sequence of eIF2γ, which is known to be an important site for GTP binding.[24] The N190D mutant mimics inactive eIF2γ, and it exhibited reduced binding to AIMP3 in comparison with WT eIF2γ in the IP assay (Fig. 3b). These results demonstrated that AIMP3 was critical for binding between MRS and eIF2γ, as well as for the recruitment of active eIF2γ to the MRS–AIMP3 complex.

AIMP3 is important for formation of the TC

Because AIMP3 interacted with Met-tRNA$_i^{Met}$ and eIF2γ, we examined whether AIMP3 could play a role in the formation of the TC via delivery of Met-tRNA$_i^{Met}$. HeLa cells were transfected with specific siRNAs (*s*mall *i*nterfering *RNA*s) for knockdown of MRS, AIMP3, eIF2α, and eIF2γ, and TC was immunoprecipitated with eIF2β-specific antibody.

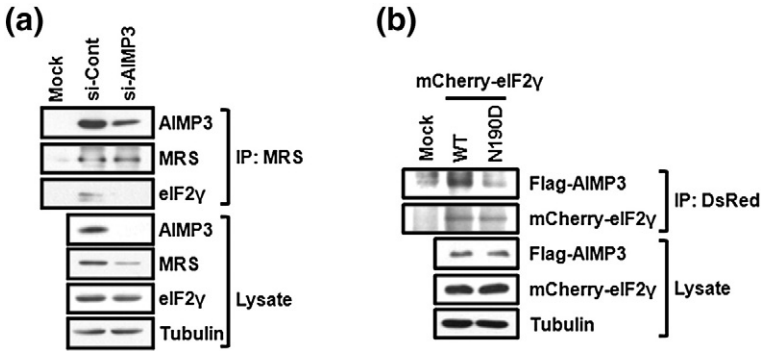

Fig. 3. The effect of AIMP3 on the interaction between MRS and eIF2γ. (a) HeLa cells were transfected with si-control and si-AIMP3. MRS was immunoprecipitated, and the bound proteins were analyzed using Western blotting. As MRS levels in the cells treated with si-AIMP3 were reduced, minimum amounts of anti-MRS antibody (0.5 μg/500 μl lysate) were used for the IP assays to equalize the amounts of captured MRS between the samples. (b) We transfected 293T cells with mCherry-tagged eIF2γ (WT or N190D mutant) and Flag-AIMP3 simultaneously, and the mCherry-eIF2γ proteins were immunoprecipitated using anti-DsRed antibody for the analysis of bound Flag-AIMP3.

The amount of $tRNA_i^{Met}$ bound to eIF2 complex was analyzed, revealing that downregulation of AIMP3 decreased $tRNA_i^{Met}$ in the eIF2 complex to the same level as seen after eIF2γ knockdown (Fig. 4a). The influence of MRS or eIF2α knockdown on the amount of $tRNA_i^{Met}$ was not as obvious as that of AIMP3 knockdown.

Next, we carried out a gel-filtration assay to confirm the effect of AIMP3 knockdown on the colocalization of eIF2 complex with ribosome. As expected, reduced levels of AIMP3 decreased the amounts of eIF2 subunits in the ribosomal fraction in comparison with MRS knockdown (Fig. S4), suggesting that levels of AIMP3 affected TC formation, which should be detected in the ribosomal fraction.[25] Collectively, these results indicated that AIMP3 played a crucial role in TC formation that was linked to protein synthesis.

To investigate the effect of AIMP3 on global translation, we calculated protein synthesis in the AIMP3 MEF and HeLa cells using a Met incorporation assay. The $AIMP3^{+/-}$ MEF cells exhibited about a 40% reduction in translation as compared with the $AIMP3^{+/+}$ MEF cells (Fig. 4b), and similar results were obtained with si-AIMP3-transfected HeLa cells (Fig. 4c). Global translation was also reduced by approximately 20% by knockdown of MRS; however, the knockdown effect of AIMP3 on protein synthesis was stronger. These results demonstrated the importance of Met-$tRNA_i^{Met}$ delivery to eIF2 via AIMP3 in global translation, even when Met-$tRNA_i^{Met}$ seemed abundant enough to negate a delay in TC formation.

Conclusions

Recently, another group reported a model that explains how the TC binds the 40S ribosomal subunit by identifying the binding motifs of eIF2γ and 40S ribosome.[26] However, it is unclear how Met-$tRNA_i^{Met}$ moves to eIF2γ from MRS in the early stage of translation initiation. We demonstrated here that AIMP3, a binding partner of MRS in the MSC, worked as a mediator of Met-$tRNA_i^{Met}$ delivery from MRS to eIF2. It is interesting that AIMP3 exhibits high affinity for Met-$tRNA_i^{Met}$ but not for Met-$tRNA_e^{Met}$, and it affected TC formation, which is critical for translation initiation.

MRS acylates $tRNA_i^{Met}$ and $tRNA_e^{Met}$, and among the acylated tRNAs, only Met-$tRNA_i^{Met}$ was recognized by AIMP3, suggesting that AIMP3 is probably involved in the translation initiation but not in elongation step. The GTP-bound form of eIF2γ can also interact with Met-$tRNA_i^{Met}$ through the recognition of Met moiety.[23] This suggests to us that tRNA sequences and Met are important for binding to AIMP3 and eIF2γ. While $tRNA_i^{Met}$ and $tRNA_e^{Met}$ share the same anticodon sequences, there are several differences between them (Fig. 1f). One of the most distinctive features of $tRNA_i^{Met}$ relates to the acceptor stem, where the discriminating base pair is located. $tRNA_i^{Met}$ has an A1:U72 base pair in its acceptor stem, whereas $tRNA_e^{Met}$ has an G1:C72 base pair.[23,27] Because of this difference, eIF2γ can discriminate Met-$tRNA_i^{Met}$ from Met-$tRNA_e^{Met}$. AIMP3 and eIF2γ may recognize Met-$tRNA_i^{Met}$ in a same way, although more study is required to fully understand the Met-$tRNA_i^{Met}$ delivery mechanism.

It seems that MRS also interacts with eIF2γ. An alternative way is the direct delivery of Met-$tRNA_i^{Met}$ from MRS to eIF2γ without the involvement of AIMP3. However, AIMP3 involvement can inhibit diffusion of Met-$tRNA_i^{Met}$ by recruiting active eIF2γ to the MRS–AIMP3 complex, thereby increases the efficiency and accuracy of TC formation. AIMP3 involvement in Met-$tRNA_i^{Met}$ transfer may be meaningful, considering the complicated regulatory mechanism of translation in higher eukaryotes. Generally, translation is regulated by phosphorylation of eIF2α. This phosphorylation prevents formation of the TC and inhibits further rounds of translation initiation. In addition, a recent study has suggested another

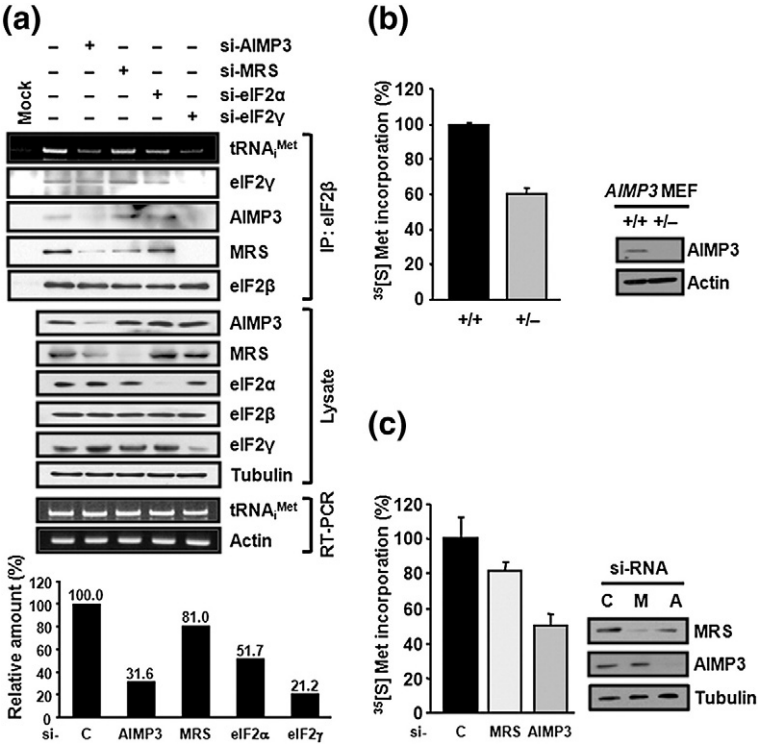

Fig. 4. The effect of AIMP3 on TC formation and global translation. (a) TC was immunoprecipitated with anti-eIF2β antibody from the siRNA-transfected HeLa cells. The bound RNA was purified using Trizol, and the amounts of bound $tRNA_i^{Met}$ were analyzed using reverse transcription PCR. Actin was used as a loading control. The proteins were purified from the Trizol-treated protein fractions and analyzed by immunoblotting. The relative amounts of bound $tRNA_i^{Met}$ from the samples treated by each siRNA were presented in the bottom panel. (b and c) Protein synthesis in the *AIMP3*$^{+/+}$ and *AIMP3*$^{+/-}$ MEFs (b) and siRNA-transfected HeLa cells (c) was analyzed using a Met incorporation assay. Mean±SD of triplicate experiments are shown. The differences between the samples were significant ($P<0.05$, one-way ANOVA).

mechanism of translation control under UV stress.[19] According to Kwon *et al.*, MRS phosphorylation occurred simultaneously with eIF2α phosphorylation and inhibited translation by reducing Met-$tRNA_i^{Met}$ production. The MRS phosphorylation site is critical for tRNA binding; however, this residue is not conserved in lower eukaryotes, such as yeast. AIMP3 only exists in higher eukaryotes, but its various functions are essential for the maintenance of life in higher eukaryotes.[12–14] These studies suggest that translational regulation mediated by MRS and AIMP3 may be an adaptation by higher eukaryotes, which developed more accurate and multistep translational regulation during evolution. There is still a possibility that AIMP3 can affect global translation via unknown pathways. In the present study, however, we elucidated the role of AIMP3 as a subunit of MSC. Furthermore, we determined that AIMP3 can capture the charged initiator tRNA and deliver it to eIF2 by recruiting active eIF2γ. We also determined that AIMP3 enables precise and efficient formation of the TC and affects global translation.

Acknowledgements

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Supplementary Data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jmb.2012.07.020

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