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# An mRNA-specific $tRNA_i$ carrier eIF2A plays a pivotal role in cell proliferation under stress conditions: stress-resistant translation of c-Src mRNA is mediated by eIF2A

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## ABSTRACT

**c-Src, a non-receptor protein tyrosine kinase, activates NF-κB and STAT3, which in turn triggers the transcription of anti-apoptosis- and cell cycle-related genes. c-Src protein regulates cell proliferation, cell motility and programmed cell death. And the elevated level of activated c-Src protein is related with solid tumor generation. Translation of c-Src mRNA is directed by an IRES element which mediates persistent translation under stress conditions when translation of most mRNAs is inhibited by a phosphorylation of the alpha subunit of eIF2 carrying the initiator tRNA ($tRNA_i$) to 40S ribosomal subunit under normal conditions. The molecular basis of the stress-resistant translation of c-Src mRNA remained to be elucidated. Here, we report that eIF2A, an alternative $tRNA_i$ carrier, is responsible for the stress-resistant translation of c-Src mRNA. eIF2A facilitates $tRNA_i$ loading onto the 40S ribosomal subunit in a c-Src mRNA-dependent manner. And a direct interaction between eIF2A and a stem-loop structure (SL I) in the c-Src IRES is required for the c-Src IRES-dependent translation under stress conditions but not under normal conditions. Finally, we showed that the eIF2A-dependent translation of c-Src mRNA plays a pivotal role in cell proliferation under stress conditions.**

## INTRODUCTION

c-Src, a notable nonreceptor protein tyrosine kinase, regulates apoptosis, cell proliferation, cell motility, and tumor growth (1–4). Activation of the c-Src pathway has been observed in ∼50% of all tumors. That is, overproduction and/or activation of c-Src protein is related with development of tumors (5–9). For example, elevated levels of c-Src proteins are often observed in colon and breast tumors (6,10). Furthermore, inhibition of c-Src expression was shown to reduce cell survival, proliferation, migration, and tumor invasion (11,12). This indicates that the accurate regulation of c-Src expression is crucial for maintaining healthy status of organisms.

It is noteworthy that a high level of c-Src protein exists in cancer cells where translation of many mRNAs is repressed by the inactivation of eIF2, a carrier of initiator tRNA ($tRNA_i$), through activation of stress signals (13). The molecular basis of stress-resistant translation of c-Src mRNA has not been uncovered except that translation of c-Src mRNA is mediated by an internal ribosome entry site (IRES) element residing in a part of 5′ untranslated region (5′UTR) and a part of the open reading frame (ORF) encoding the N-terminal 11 amino acids of c-Src gene, and that the IRES element is responsible for the stress-resistant translation (13).

Regulation of gene expression at the translation step, which is the final stage of genetic information flow, is highly responsive to temporal and spatial physiological states (14). For instance, translation of most mRNAs is repressed under stress conditions resulting in reduction of the metabolism of stressed cells. This stress response is mainly mediated by the phosphorylation of α subunit of eIF2 (eIF2α), which carries the $tRNA_i$ to the 40S ribosomal subunit (15). When the eIF2α subunit is phosphorylated by distinct kinases, which are activated by various stresses [Protein Kinase R (PKR) by viral infection, GCN2 by amino acid starvation, PKR-like ER kinase (PERK) by ER stress, and heme-regulated inhibitor (HRI) by oxidative stress], the ternary complex of eIF2, which is composed of GTP, $tRNA_i$, and eIF2, cannot be regenerated since the GTP exchange fac-

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tor for eIF2 named eIF2B is sequestered to the phosphorylated eIF2 (16–20). As a consequence of the eIF2α phosphorylation, general translation is compromised by the limited supply of translation competent ternary complex of eIF2. Nevertheless, translation of certain mRNAs encoding proteins executing special roles under stress conditions or of those required for recovery from stress response is sustained or even increased under the stress conditions. For instance, translation of X-linked inhibitor of apoptosis protein (XIAP), which is a member of the inhibitor of apoptosis (IAP) family of proteins repressing apoptotic cell death by activating degradation of caspase 3, 7 and 9 through ubiquitin-mediated protein degradation, is sustained under stress conditions (21,22). Interestingly, the translation of XIAP mRNA is also mediated by an IRES element similarly to c-Src mRNA (23). The translation of PITSLRE mRNA, which encodes isoforms of a Ser/Thr kinase, is another interesting example of eIF2α phosphorylation-resistant translation. An isoform of PITSLRE protein with molecular weight of 110 kDa ($p110^{PITSLRE}$) is translated in a cap-dependent manner throughout the cell cycle. On the contrary, a PITSLRE isoform with molecular weight of 58 kDa ($p58^{PITSLRE}$) is translated only at the G2/M phase, when eIF2α is phosphorylated (24) via an IRES element residing in the coding region of $p110^{PITSLRE}$ (25,26).

As described above, several mRNAs containing IRES elements are known to be translated even under stress conditions, whereas it has not been understood how the $tRNA_i$ is delivered to the 40S ribosomes engaged in the translation of stress-resistant mRNAs when the function of eIF2 is compromised. A translation factor eIF5B, which contains domains homologous to prokaryotic IF2, was suggested to play a role in the stress-resistant translation of an mRNA (23). However, it is not clear how eIF5B, which does not have $tRNA_i$-binding activity, delivers $tRNA_i$ to the 40S ribosomal subunit. Recently, an alternative $tRNA_i$-binding protein named eIF2A was reported to load $tRNA_i$ onto the 40S ribosomal subunit in an mRNA-specific manner (27,28). Originally, eIF2A was reported as a cellular protein interacting with $tRNA_i$ in an AUG-dependent manner similarly to the prokaryotic $tRNA_i$ carrier IF2 (29,30). Proteins homologous to human eIF2A exist in all eukaryotic cells from yeasts to mammals. However, eIF2A does not have sequence similarity with either prokaryotic $tRNA_i$ carrier IF2 or eukaryotic $tRNA_i$ carrier eIF2 (30). Instead, the crystal structure of yeast eIF2A revealed a WD domain with 9-bladed β-propeller fold containing a well-conserved positively charged top face that was predicted to participate in translation initiation (31).

Although eIF2A was suggested to function as a translation initiation factor as it facilitates loading $tRNA_i$ onto the 40S ribosomal subunit via its $tRNA_i$-binding activity, a target mRNA utilizing eIF2A or physiological role of the protein in translation initiation was unknown. Recently, some viral mRNAs were shown to utilize eIF2A for translation initiation when the function of eIF2 is compromised by the PKR activity (28). The first report on an mRNA utilizing eIF2A in translation initiation was the 26S mRNA of Sindbis virus (28). Translation of 26S mRNA of Sindbis virus was shown to be refractory to phosphorylation of eIF2α by PKR, and eIF2A was shown to be required for the translation under stress conditions using eIF2A-knockdown experiments. Moreover, the authors showed that a stem–loop structure (DLP) downstream of the authentic start codon is required for the stress-resistant translation of the mRNA (28). However, the authors did not provide an explanation of how the 26S mRNA is selectively translated by eIF2A under stress conditions. More recently, we reported that hepatitis c viral (HCV) mRNA, whose translation is mediated by an IRES element refractory to phosphorylation of eIF2α, utilizes eIF2A for its translation under stress conditions (27). It was evident that eIF2A has mRNA-binding activity as well as $tRNA_i$-binding activity. We also showed that a stem–loop (SL) structure of HCV IRES (designated as SL IIId) is the responsible site for the specific interaction with eIF2A and that the SL IIId–eIF2A interaction is required for translation of HCV mRNA under stress conditions (27). In other words, a differential binding of eIF2A to a specific mRNA is the molecular basis of selective translation of eIF2A-dependent mRNAs. Very recently, some peptide precursors loaded on MHC class I (32) and one of the PTENα isoforms targeting mitochondria (33) were shown to be translated from start codon CUGs. Moreover, it was reported that translation of the main ORF of BiP mRNA starting from AUG codon and translation of an upstream ORF (uORF) starting from UUG codon at 190 nucleotide upstream of the AUG continue under stress conditions (34). The authors suggested that translation of these polypeptide under stress conditions requires eIF2A since knockdown of eIF2A abrogated the production of these polypeptides under stress conditions. Surprisingly, the polypeptides translated from the CUG and UUG start codons begin with leucine instead of methionine. However, the molecular basis of the eIF2A-dependent translation of these polypeptides remains elusive because eIF2A does not interact with leucyl-tRNA directly (27). Furthermore, a protein–RNA interaction between eIF2A and a cellular mRNA has never been demonstrated (32–34). Therefore, it is not clear whether eIF2A functions directly or indirectly for the translation of the eIF2A-dependent cellular mRNAs using the CUG and UUG start codons.

In the present study, we revealed that eIF2A functions as a $tRNA_i$ carrier for translation of c-Src mRNA under stress conditions. We showed that eIF2A specifically interacts with the stem-loop I (SL I) of c-Src IRES and that mutations in the loop region of SL I impair the specific interaction and the stress-resistant translation of c-Src mRNA indicating the interaction between eIF2A and SL I is required for the translation of c-Src mRNA under stress conditions. Knockdown of eIF2A by a siRNA abrogated the c-Src IRES activity and lowered the level of endogenous c-Src protein under stress conditions. Surprisingly, the knockdown of eIF2A almost completely inhibited proliferation of cells under stress but not under normal conditions. Similarly, knockdown of c-Src by a siRNA also almost completely inhibited proliferation of cells under stress but not under normal conditions. The data suggest that eIF2A, an alternative $tRNA_i$ carrier, plays a key role in cell proliferation under stress conditions by facilitating translation of a specific mRNA(s) including c-Src.

## MATERIALS AND METHODS

### Cell culture

Huh-7 cells and HEK293T cells were cultivated in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with 10% fetal bovine serum (JRH) and 1% penicillin/streptomycin. Cells were maintained at 5% $CO_2$ and 37°C.

### In vitro transcription and pull-down experiments with biotinylated RNAs

Reporter RNAs were generated with T7 RNA polymerase using Not I-treated reporter DNAs containing viral IRESes and Nsi I- and T4 DNA polymerase-treated reporter DNAs containing cellular IRESes. Biotinylated RNAs used in RNA pull-down experiments were synthesized by incubating T7 RNA polymerase in the presence of biotinylated UTP as previously described (35). Template DNAs were digested with following restriction enzymes: Sal I to mono-cistronic reporter DNAs containing wild type or mutant forms of c-Src IRES, Not I to DNAs containing truncated forms of c-Src IRES, BamH I to plasmids pXIAP IRES-FLuc and pPITSLRE IRES-Fluc, EcoR I to mono luciferase reporter DNA containing EMCV IRES (36), and Acc I to mono luciferase reporter DNA containing HCV IRES (27). RNA pull-down experiments were performed with either purified 6xHis-tagged eIF2A protein or lysates of Huh-7 cells ectopically expressing Flag-tagged eIF2A protein as described elsewhere (27). Purified recombinant proteins (30 pmol) or lysates of cells (2000 μg) ectopically producing Flag-eIF2A were incubated with 15 pmol of biotinylated RNAs. In the case of eEF1A1, 200 μM of GDP was added to the reaction mixture as described previously (37).

### Transfection of RNAs and measurement of luciferase activity

SMART pool siRNA against eIF2A was purchased from Dharmacon™. A siRNA against c-Src (5′-TGTTCGGAGGCTTCAACTCCT-3′) and negative control siRNA were purchased from Bioneer. siRNAs were transfected into cells by using Oligofectamine (Invitrogen) according to the manufacturer's protocol. At 48 h after siRNA transfection, dual luciferase mRNAs were transfected into cells by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Di-cistronic mRNAs (100 ng each shown in Figure 1A) or two mono-cistronic mRNA mixtures (50 ng of *Renilla* luciferase mRNA and 100 ng of firefly luciferase mRNA shown in Figure 1D) were used in transfection of cells. Tunicamycin was added to the transfection media to provoke stress response during the transfection. Transfection efficiency of RNAs, which was measured by quantitative RT-PCR, was not affected by the tunicamycin treatment (data not shown). At 2 h after mRNA transfection, cells were harvested and lysed. Firefly luciferase and *Renilla* luciferase activities in the lysates were measured by using a dual luciferase assay system as described previously (38).

### 3-(4,5-Dimethylthiazol-2yl)-2, 5-diphenyltetrazolium bromide (MTT) assay

Huh-7 cells ($1.5 \times 10^3$ cells per well) were seeded on 96-well plates and cultivated for 24 h. Cells were transfected with control siRNA, c-Src siRNA or eIF2A siRNA with lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After additional 24 h of incubation, cells were treated with tunicamycin (5 μg/ml) for 24 h. MTT (5 mg/ml) was treated to the cells which are incubated with tunicamycin for 0 or 24 h. After 3 h incubation with MTT, media was removed carefully and MTT formazan was dissolved by adding DMSO. The absorbance of sample solutions was measured by using a victor 3 (PerkinElmer) at 590 nm.

### Sucrose gradient analysis

Huh-7 cells were transfected with a siRNA against eIF2A or a negative control siRNA. At 48 h after transfection, these cells were incubated with tunicamycin for 2 h and washed with PBS. The cells were further incubated in PBS containing 100 μg/ml cycloheximide for 30 min on the ice. The cells were lysed in polysome lysis buffer [300 mM KCl, 5 mM $MgCl_2$, 10 mM HEPES (pH 7.4), 0.5% NP-40, 5 mM DTT and 100 μg/ml cycloheximide]. Cell lysates were loaded on 5–45% sucrose gradients in polysome buffer [300 mM KCl, 5 mM $MgCl_2$ and 10 mM HEPES (pH 7.4)] and centrifuged at 32 000 rpm in a SW41Ti rotor at 4°C for 3 h. The gradients were collected using a Brandel gradient density fractionator and analyzed by an Econo UV monitor (Bio-Rad). Fractions (1 ml each) were spiked with 100 ng of firefly luciferase (*FLuc*) RNA for ensuring the technical consistency of RNA isolation. RNAs were isolated from these fractions, and reverse transcription and real-time RT-PCR were performed as described in the Supplementary Materials and Methods section.

### Filter-binding assay

The reaction mixtures (25 μl each) containing indicated components (Figure 6) in binding buffer [20 mM Tris–HCl (pH 7.4), 100 mM KCl, 5 mM $Mg(CH_3CO_2)_2$, and 1 mM DTT] was incubated for 20 min at 25°C. The amounts of components used in the experiments are as follows: 0.5 pmol of c-Src IRES or EMCV IRES RNA, 3 pmol of eIF2A, 2.5 pmol of 40S ribosomal subunit, and 1 pmol of [$^{32}$P] $tRNA_i$ (2000 c.p.m./pmol). After the incubation, 175 μl of dilution buffer [20 mM Tris–HCl (pH 7.4), 100 mM KCl, 5 mM $Mg(CH_3CO_2)_2$, 1 mM DL-methionine] was added to the mixture, and the samples were filtered through a MAHVN45 membrane filter (Millipore, 0.45 μm HVPP membrane which has low binding affinity to proteins and nucleic acids) under negative pressure. The membrane was washed five times with 200 μl of washing buffer [20 mM Tris–HCl (pH 7.4), 100 mM KCl, 5 mM $Mg(CH_3CO_2)_2$, 0.4% NP-40 and 1 mM DTT]. [$^{32}$P] $tRNA_i$ associated with filter was detected through a FLA-5100 (Fujifilm).

## RESULTS

### Resistance against stress-mediated translational inhibition varies among IRES elements

There have been reports suggesting that translation of some cellular mRNAs containing IRES elements are refractory to stress-dependent translational repression mediated by the phosphorylation of eIF2α (26). In order to investigate the underlining mechanism of the stress-resistant translation of those mRNAs, we generated di-cistronic mRNAs containing a *Renilla* luciferase (*RLuc*) gene at the first cistron, which is translated in a cap-dependent manner, and a firefly luciferase (*FLuc*) gene at the second cistron, which is translated by an IRES-dependent manner (Figure 1A). As reported previously, cap-dependent translation was inhibited by tunicamycin, which induces phosphorylation of eIF2α through generation of ER stress (Figure 1C), but HCV IRES-dependent translation was refractory to the ER stress (Figure 1B, lane 2) (27). Among other IRES elements tested in this study (EMCV, c-Src, PITSLRE, and XIAP), c-Src IRES-dependent translation was strongly refractory to ER stress at the same level as HCV IRES (Figure 1B, lane 6). EMCV, PITSLRE, and XIAP IRESes were partially refractory to ER stress-mediated translational repression (Figure 1B, lanes 4, 8 and 10, respectively). Translation of a negative control sequence (inverted XIAP IRES) was strongly affected by ER stress similarly to cap-dependent translation (Figure 1B, lanes 11 and 12). The eIF2α subunit was strongly phosphorylated by the ER stress (Figure 1C and F).

To exclude the possibility that ribosomes which fail to dissociate from the reporter mRNA after translational termination of the first ORF might contribute to the translation of second ORF of di-cistronic mRNAs, we constructed mono-cistronic mRNAs containing ApppG cap and various IRES elements (Figure 1D) and investigated the effects

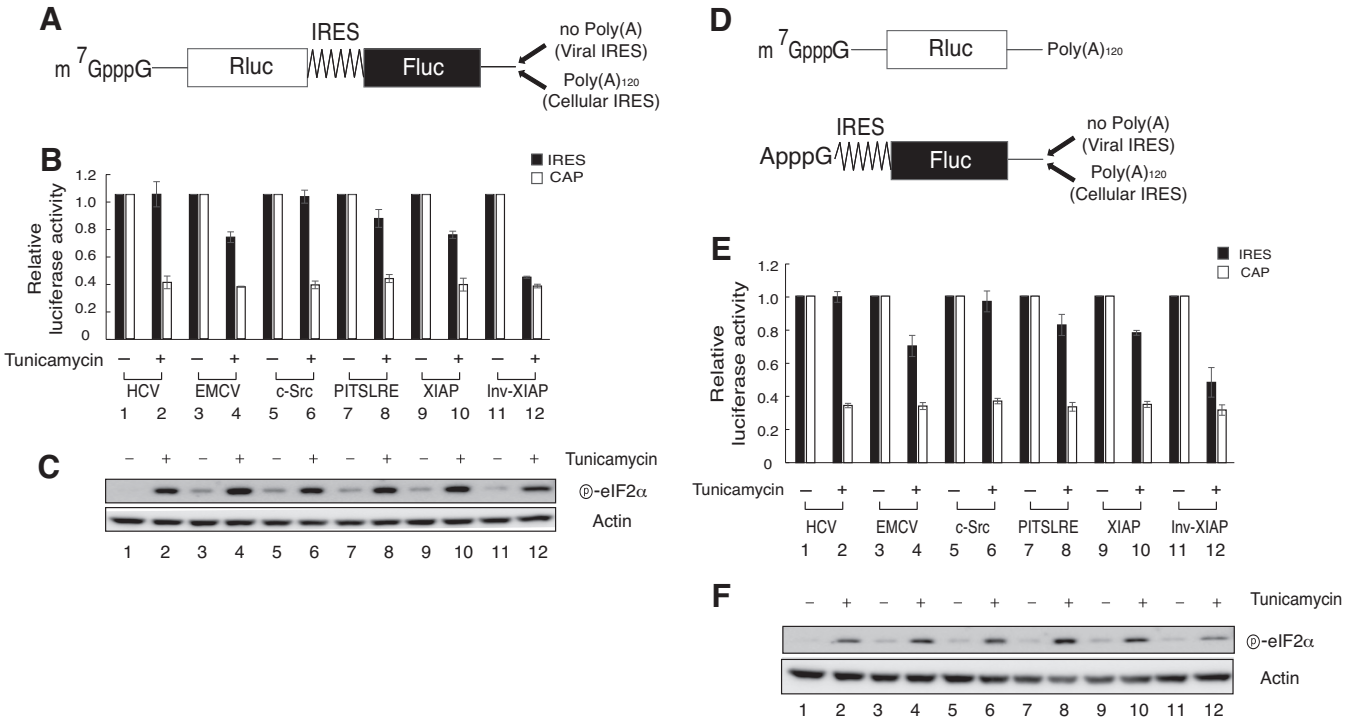


**Figure 1.** Effects of stresses on cap-dependent translation and various IRES-dependent translation. (**A**) Schematic diagram of di-cistronic mRNAs. The mRNAs contain 7-methyl guanosine cap structure at the 5′ end, *Renilla* luciferase gene at the first cistron, firefly luciferase gene at the second cistron, and various IRES elements at the inter-cistronic region. Poly(A) tails with 120 nucleotides of A's [$(A)_{120}$] were added to the mRNAs containing cellular IRESes (c-Src, PITSLRE, XIAP, and Inv-XIAP) since poly(A) tail was shown to be required for cellular IRES activities (23,52). Poly(A) tail was not added to the mRNAs containing viral IRESes (HCV and EMCV). Detailed procedures for constructing plasmids encoding reporter RNAs are described in the section of 'Supplemental materials and methods'. (**B**) Huh-7 cells were transfected with di-cistronic RNAs. Tunicamycin or DMSO was treated to cells immediately after the transfection to provoke ER stress. After the incubation for 2 h, luciferase activities in the cell lysates were measured. The experiments were performed three times. Luciferase activities were normalized to those in DMSO-treated cells which were set to 1. Solid and open columns represent *Renilla* and firefly luciferase activities, respectively. Columns and bars represent mean and standard deviation values, respectively. (**C**) The amounts of proteins (phosphorylated eIF2α subunit and actin) were monitored by Western blotting by using the indicated antibodies. Cell lysates described in panel (B) were used in this analysis. (**D**) Schematic diagram of mono-cistronic mRNAs. The mono-cistronic *Renilla* luciferase mRNA contains 7-methyl guanosine cap structure at the 5′ end followed by *Renilla* luciferase gene and $(A)_{120}$ tail. The firefly luciferase mRNAs contain ApppG cap structure at the 5′ end and various IRES elements in the 5′UTR followed by firefly luciferase gene. $(A)_{120}$ tails were added to the mono-cistronic mRNAs containing cellular IRESes but not to mono-cistronic mRNAs containing viral IRESes. (**E**) Huh-7 cells were transfected with mono-cistronic reporter RNAs. Tunicamycin or DMSO was treated to cells immediately after the transfection to provoke ER stress. After the incubation for 2 h, luciferase activities in the cell lysates were measured. The experiments were performed three times. Luciferase activities were normalized to those in DMSO-treated cells which were set to 1. Solid and open columns represent *Renilla* and firefly luciferase activities, respectively. Columns and bars represent mean and standard deviation values, respectively. (**F**) The amounts of proteins (phosphorylated eIF2α subunit and actin) were monitored by Western blotting by using the indicated antibodies. Cell lysates described in panel (E) were used in this analysis.

of ER stress on translation of these mRNAs (Figure 1E). In mono-cistronic mRNAs, translation of *RLuc* gene is directed by the m7pppG cap, and translation of *FLuc* gene is directed by various IRES elements. The ER stress induced by tunicamycin inhibited cap-dependent translation of mono-cistronic mRNA (Figure 1E). On the other hand, the effects of ER stress on translation directed by IRES elements varied. HCV and c-Src IRESes were strongly refractory to ER stress (Figure 1E, lanes 2 and 6). EMCV, PITSLRE and XIAP IRESes were partially refractory to ER stress (Figure 1E, lanes 4, 8 and 10, respectively). These effects of ER stress on translation directed by IRES elements in mono-cistronic mRNAs were the same as the effects observed from di-cistronic mRNAs. These results indicate that the IRES-dependent translation in di-cistronic mRNAs are not affected by the translation of the first gene and that c-Src IRES-dependent translation is strongly resistant to ER stress.

### eIF2A is required for translation of c-Src mRNA under stress conditions

We monitored protein–RNA interactions between eIF2A and various IRES elements by using RNA pull-down assays to investigate whether eIF2A participates in the stress-resistant translation directed by these IRES elements because a protein–RNA interaction between eIF2A and HCV IRES was shown to be required for the eIF2A-dependent translation of HCV mRNA under stress conditions. The RNA pull-down experiments were performed with cell lysates ectopically expressing Flag-tagged eIF2A (Flag-eIF2A) and biotinylated RNAs corresponding to various IRES elements. RNA-bound proteins were visualized by western blotting with an antibody against Flag peptides (Figure 2A). The eIF2A interacted with the c-Src IRES and the HCV IRES (a positive control) (Figure 2A). On the contrary, eIF2A did not interact or very weakly interacted with the EMCV, PITSLRE, and XIAP IRESes (Figure 2A). None of the IRES elements tested associated with a negative control protein eEF1A1, but a positive control RNA ($tRNA_e$-$^{Met}$) associated with eEF1A1 in the presence of GDP (Supplementary Figure S1A). Therefore, the interactions of eIF2A with HCV and c-Src IRESes are specific ones. Taken together, the data shown in Figure 1B, 1E and 2A, we speculated that eIF2A may function as a $tRNA_i$ carrier in the c-Src IRES-dependent translation under stress conditions similarly to its function in the HCV IRES-dependent translation. In the present study, therefore, we focused on the mechanism of stress-resistant translation of c-Src mRNA that showed the strongest resistance against stress-mediated translational repression and the strongest binding to eIF2A among cellular IRESes tested.

We investigated the effect of a siRNA against eIF2A on cap- and IRES-dependent translations. The knockdown efficiency of a siRNA against eIF2A and the phosphorylation of eIF2α subunit by tunicamycin treatment were monitored by Western blotting (Figure 2C). The knockdown of eIF2A did not affect cap-dependent translation under normal or stress conditions (Figure 2B, compare open columns on lane 1 with 3, and those on lane 2 with 4, respectively). Both HCV and c-Src IRES-dependent translations were not

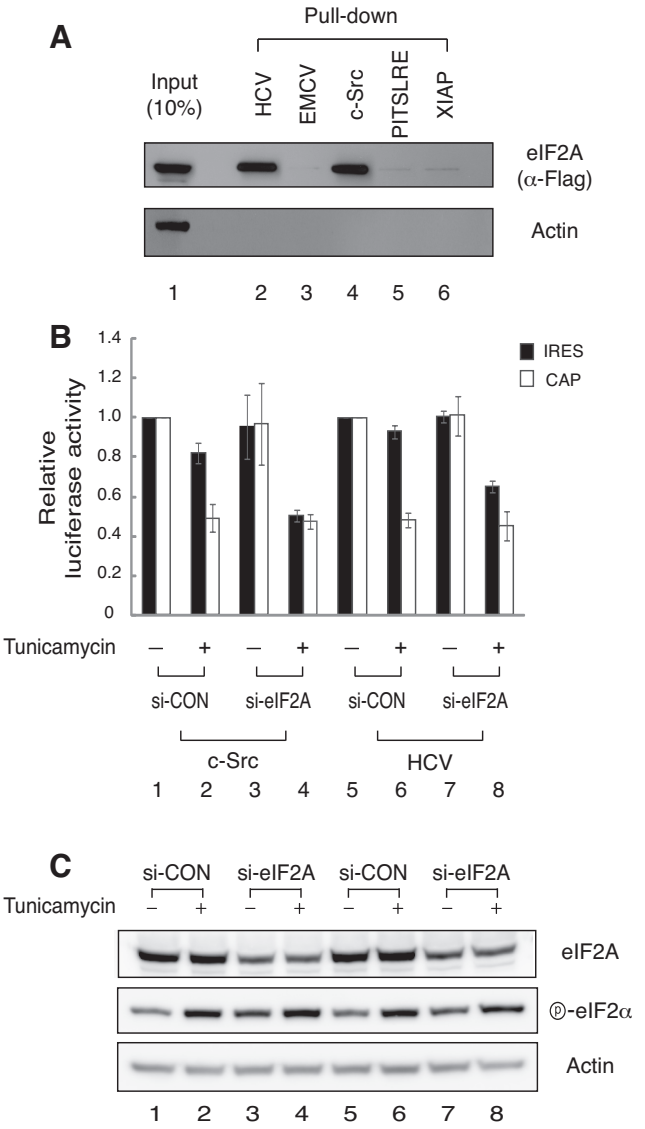


**Figure 2.** eIF2A is required for stress-resistant translation of c-Src mRNA. (**A**) Interaction between eIF2A and various IRES elements. RNA pull-down experiments were performed with biotinylated RNAs corresponding to the various IRES elements and lysates of cells ectopically expressing Flag-tagged eIF2A (Flag-eIF2A). (**B**) Effects of a control siRNA (lanes 1, 2, 5 and 6) or a siRNA against eIF2A (lanes 3, 4, 7 and 8) on cap-dependent and IRES-dependent translation. Huh-7 cells were transfected with a siRNA against eIF2A or a negative control siRNA. Cells were transfected with reporter RNAs. Tunicamycin or DMSO was treated to cells immediately after transfection to provoke ER stress. After the incubation for 2 h, cells were harvested and lysed. Luciferase activities in the cell lysates were measured. The experiments were performed three times. Luciferase activities were normalized to those in DMSO-treated cells which were set to 1. Solid and open columns represent *Renilla* and firefly luciferase activities, respectively. Columns and bars represent mean and standard deviation values, respectively. (**C**) The amounts of proteins (eIF2A, phosphorylated eIF2α subunit, and actin) were monitored by Western blotting by using the indicated antibodies. Cell lysates described in panel (B) were used in this analysis.

affected by the knockdown of eIF2A under normal conditions (Figure 2B, solid columns on lanes 7 and 3, respectively). On the contrary, HCV IRES-dependent and c-Src IRES-dependent translations were greatly reduced by the knockdown of eIF2A under stress conditions (Figure 2B, solid columns on lanes 8 and 4, respectively). The results strongly indicate that eIF2A plays a pivotal role in the stress-resistant translation of c-Src and HCV mRNAs under stress conditions but not under normal conditions.

### eIF2A interacts with the stem-loop I (SL I)

Allam and Ali discovered the c-Src IRES and named the domains as shown in Figure 3A according to the degree of structural conservation (13). The authors revealed that the Domain II is required for the activity of c-Src IRES and that the stem–loop IV (SL IV) and domain III are dispensable for IRES activity (13). Interestingly, the SL I corresponding to the coding region encoding N-terminal 11 amino acids, which is highly conserved in mammalian c-Src mRNAs, was shown to be essential for the IRES activity of c-Src mRNA. In order to determine the eIF2A-binding site in c-Src IRES, we performed RNA pull-down experiments using biotinylated RNAs corresponding to various regions of c-Src IRES. Biotinylated RNAs corresponding to domains I [nucleotides (nt) 1–52 and nt 346–381], II (nt 197–345), and III (nt 53–196) were synthesized by *in vitro* transcription and pull-down experiments were performed as described in the Materials and Methods section. The full-length IRES and the domain I interacted with eIF2A whereas domains II and III did not (Figure 3D). This indicates that domain I is responsible for the interaction with eIF2A. We further divided the domain I into SL IV (nt 1–35) and SL I+b (nt 36–53 plus nt 345–381). The SL I+b interacted with eIF2A (Figure 3E and Figure S1B) whereas SL IV did not (Supplementary Figure S1B). Through further truncations of SL I+b, we found that the SL I (nt 351–375) is sufficient for the interaction with eIF2A (Figure 3E). The importance of SL I in the interaction with eIF2A was further confirmed by pull-down experiments with mutant RNAs containing nucleotide deletions or substitutions in the loop regions of SL IV and SL I. Nucleotide deletions or substitutions in the loop region of SL IV of c-Src IRES RNAs composed of domains I–III did not affect the binding with eIF2A (lane 3 in Figure 3F and 3G). On the contrary, nucleotide deletions or substitutions in the loop region of SL I of c-Src IRES RNAs greatly impaired the binding with eIF2A (lane 4 in Figure 3F and G). In order to rule out the possibility of indirect interaction of eIF2A with SL I through a putative protein assisting the interaction between eIF2A and SL I, we performed RNA pull-down experiments with purified eIF2A proteins and the wild type and the mutant c-Src IRESes with nucleotide deletions or substitutions in SL IV or SL I (Figure 3H and I). The binding patterns of purified eIF2A proteins with the wild type and mutant c-Src IRES RNAs were the same as those of eIF2A in the cell extracts (compare panels 3F and 3G with panels 3H and 3I, respectively). Altogether, the data indicate that eIF2A by itself interacts directly with the SL I of c-Src IRES and that the loop region of SL I plays a pivotal role in the interaction with eIF2A.

### The interaction between eIF2A and c-Src IRES is required for stress-resistant translation of c-Src mRNA

We found that the loop region of SL I is critical for the interaction between eIF2A and c-Src IRES through the RNA pull-down experiments with wild type and mutant RNAs corresponding to c-Src IRES (Figure 3F–I). In order to investigate whether the interaction between eIF2A and c-Src IRES is required for the stress-resistant translation of c-Src mRNA, we examined the effects of mutations in the loop region of SL I on translation of wild type and mutant mRNAs under normal and stress conditions. IRES-dependent translation of a SL I mutant with a deletion in the loop region, which impairs the interaction between eIF2A and c-Src IRES, was greatly impaired under stress than normal conditions (compare the solid column on lane 6 with the solid column on lane 5 in Figure 4A). On the contrary, IRES-dependent translation of wild type and that of a SL IV mutant with a deletion in the loop region, which does not impair the interaction between eIF2A and c-Src IRES, were not affected by ER stress (compare the solid column on lane 1 with the solid column on lane 2 in Figure 4A, and compare the solid column on lane 3 with the solid column on lane 4 in Figure 4A, respectively).

Similarly, IRES-dependent translation of a SL I mutant with nucleotide substitutions in the loop region, which impairs the interaction between eIF2A and c-Src IRES, was greatly impaired under stress than normal conditions (compare the solid column on lane 6 with the solid column on lane 5 in Figure 4C). On the contrary, IRES-dependent translation of the wild type and a SL IV mutant with nucleotide substitutions in the loop region, which does not impair the interaction between eIF2A and c-Src IRES, were not affected by ER stress (compare the solid column on lane 1 with the solid column on lane 2 in Figure 4C, and compare the solid column on lane 3 with the solid column on lane 4 in Figure 4C, respectively). ER stress response by tunicamycin treatment was monitored by western blotting of phospho-eIF2α (Figure 4B and D). Altogether, these results indicate that the interaction between eIF2A and the SL I of c-Src IRES is required for translation of c-Src mRNA under stress conditions but not under normal conditions.

### Sucrose gradient analysis shows that eIF2A is required for translation of c-Src mRNA under stress conditions

In order to confirm that eIF2A plays a pivotal role in translation of c-Src mRNA under stress conditions, we monitored the associations of c-Src mRNA and a control GAPDH mRNA with polysome or non-polysomes with or without depletion of eIF2A under normal and stress conditions through sucrose gradient analyses (Figure 5). Phosphorylation of eIF2α induced by tunicamycin treatment attenuated the general translation as shown by the diminished polysome peaks and the increased non-polysome peaks (compare the dotted lines with solid lines in Figure 5A). This ribosome profile pattern is confirmed by the quantitative analysis of a typical stress-sensitive GAPDH mRNA (Figure 5B and C). The ratio of GAPDH mRNA in polysome fraction to that in non-polysome fraction was ∼1.5 under normal conditions (open columns in Figure

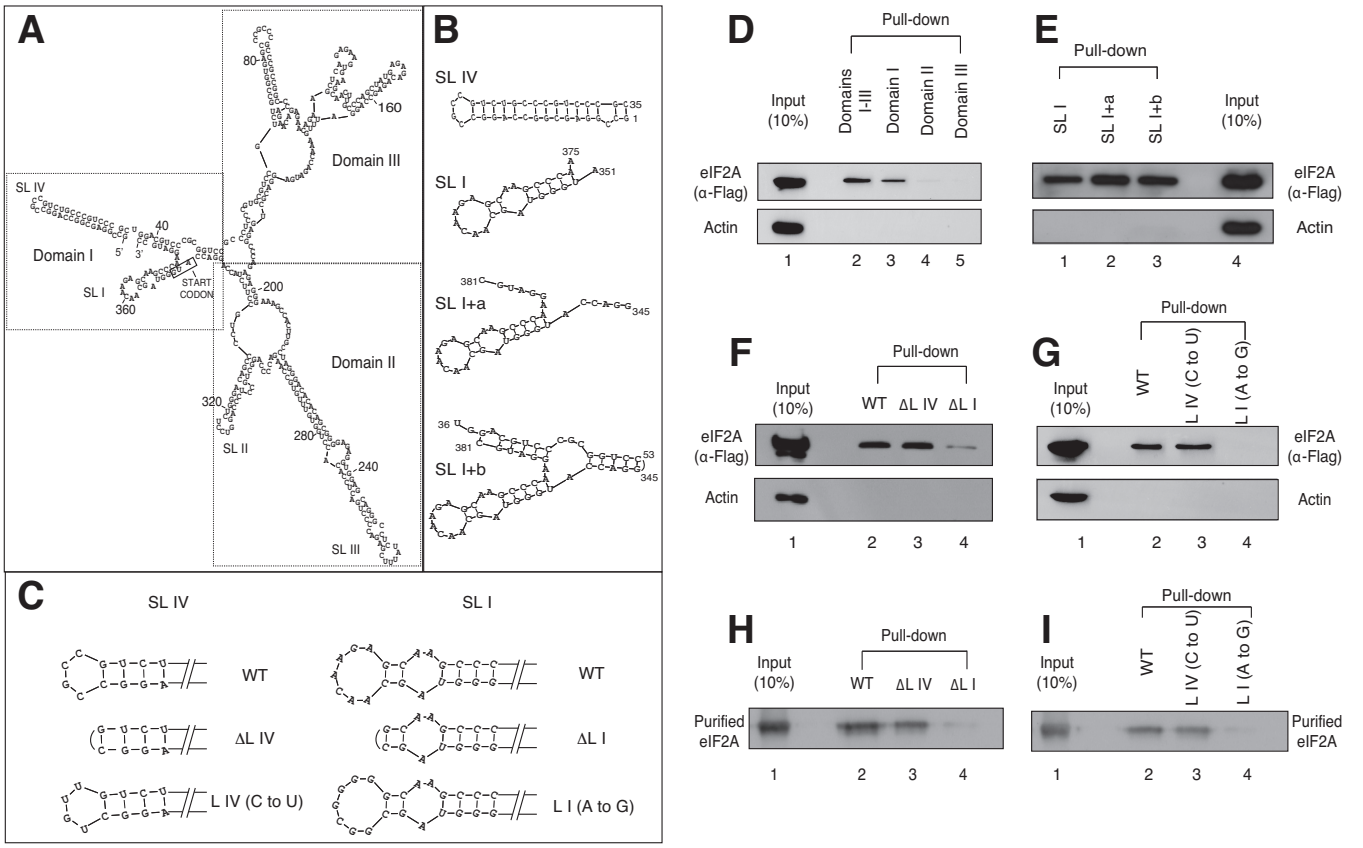


**Figure 3.** Determination of eIF2A binding site in the c-Src IRES. (**A**) The secondary structure of c-Src IRES was predicted by M-fold program, and the names of domains were depicted as Allam and Ali suggested (13). The initiation codon is depicted in a box. Each domain used in RNA pull-down experiments (panel D) is indicated by dashed lines. (**B**) Nucleotide sequences and predicted secondary structures of SL IV and SL I RNAs used in RNA pull-down experiments (panel E) are depicted. (**C**) Nucleotide sequences and predicted secondary structures of mutated RNAs used in RNA pull-down experiments (panels F and G) are depicted. All of the RNAs contain domains I to III with mutations in SL IV or in SL I. ΔLoop IV (ΔL IV) and ΔLoop I (ΔL I) contain deletions in the loop region of SL IV or SL I, respectively. Loop IV from C to U [L IV (C to U)] and Loop I from A to U [L I (A to G)] contain substitutions from Cs to Us in loop IV or substitutions from As to Gs in loop I, respectively. Only the tips of SL IV or SL I of wild type (WT) or mutant RNAs are depicted. (D–G) RNA pull-down experiments were performed with indicated biotinylated RNAs and lysates of cells ectopically expressing Flag-eIF2A. RNA-bound proteins were precipitated with streptavidin conjugated agarose resins, and RNA-bound proteins were visualized by Western blotting with an antibody against Flag peptide. (**D**) RNA pull-down experiments were performed with the lysates of cells ectopically expressing Flag-eIF2A and the RNAs corresponding to the full-length c-Src IRES (lane 2) or to individual domains (lanes 3–5). (**E**) RNA pull-down experiments were performed with the lysates of cells ectopically expressing Flag-eIF2A and the RNAs corresponding to SL I, SL I+a, and SL I+b. (**F**) RNA pull-down experiments were performed with the lysates of cells ectopically expressing Flag-eIF2A and the RNAs corresponding to WT c-Src IRES and mutant IRESes with a deletion in the loop I or IV. (**G**) RNA pull-down experiments were performed with the lysates of cells ectopically expressing Flag-eIF2A and the RNAs corresponding to WT c-Src IRES and mutant IRESes with nucleotide substitutions in the loop I or IV. (**H** and **I**) RNA pull-down experiments were performed with indicated biotinylated RNAs and purified eIF2A protein. RNA-bound proteins were precipitated with streptavidin conjugated agarose resins, and RNA-bound proteins were visualized by Western blotting with an antibody against eIF2A. (H) RNA pull-down experiments were performed with purified eIF2A proteins and the RNAs corresponding to WT c-Src IRES and mutant IRESes with a deletion in the loop I or IV. (I) RNA pull-down experiments were performed with purified eIF2A proteins and the RNAs corresponding to WT c-Src IRES and mutant IRESes with nucleotide substitutions in the loop I or IV.

5C). On the other hand, the ratio was dropped ∼3-fold under stress conditions (solid columns in Figure 5C). Depletion of eIF2A did not affect the distribution of GAPDH mRNA in the ribosome profile (compare the left panel with the right panel in Figure 5C). The results indicate that translation of GAPDH mRNA is greatly inhibited by ER stress and that eIF2A does not participate in translation of GAPDH mRNA.

On the contrary, the distribution of c-Src mRNA was only weakly affected by ER stress (left panels in Figure 5D and E). The ratio of c-Src mRNA in polysome fraction to that in non-polysome fraction was ∼2.0 under normal and stress conditions (left panel in Figure 5E). On the other hand, the ratio was dropped ∼3-fold under stress conditions when eIF2A was depleted (right panel in Figure 5E). This indicates that translation of c-Src mRNA is refractory to ER stress and that eIF2A is essential for the stress-resistant translation of c-Src mRNA. Knockdown efficiency of eIF2A and phosphorylation state of eIF2α were monitored by Western blotting (Figure 5F). The level of c-Src mRNA was not affected by the siRNA and tunicamycin treatments (Figure 5G).

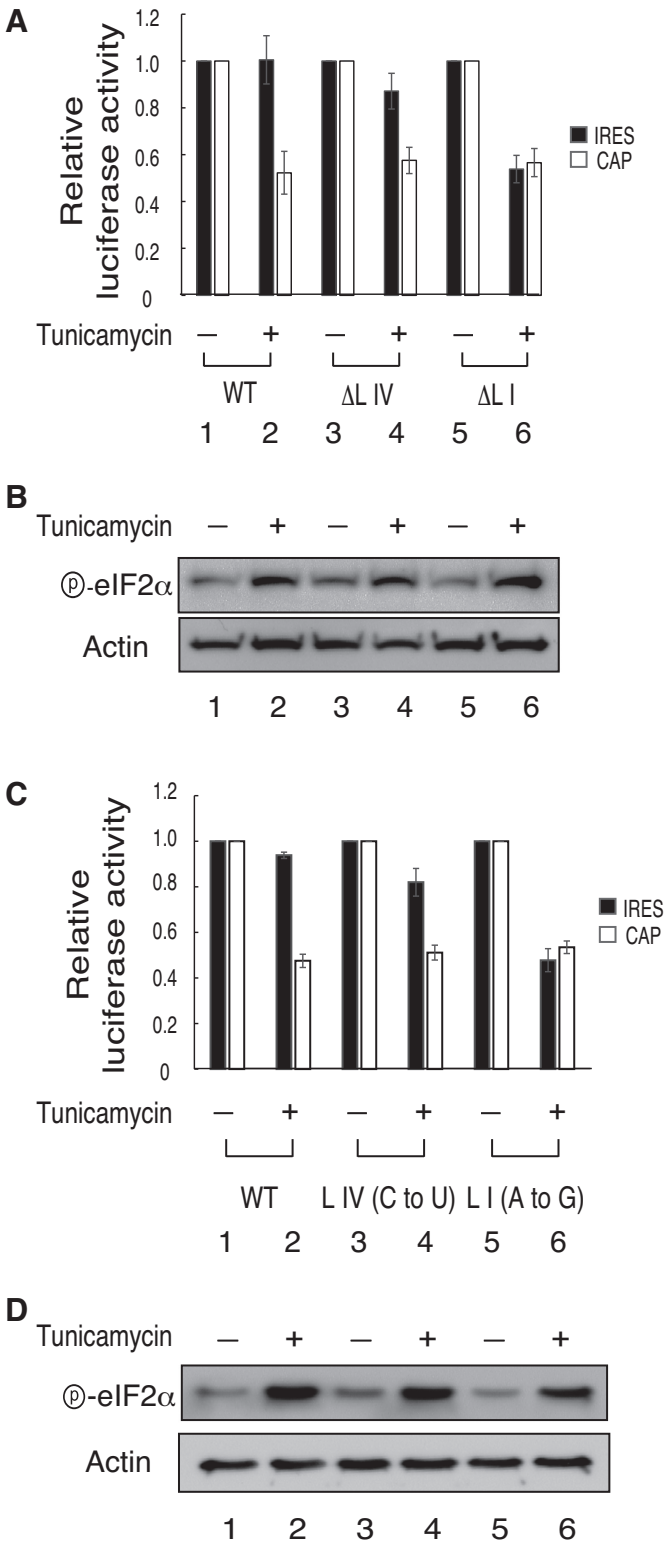


**Figure 4.** Effects of mutations in SL I on c-Src IRES-dependent translation under normal and stress conditions. (**A**) Effects of deletion mutations in loop regions of SL I and SL IV on c-Src IRES-dependent translation. Di-cistronic mRNAs were transfected into Huh-7 cells followed by the treatment of DMSO or tunicamycin. After the incubation for 2 h, luciferase activities in the cell lysates were measured. The experiments were performed three times. Luciferase activities were normalized to those in mock-treated cells which were set to 1. Solid and open columns represent *Renilla* and firefly luciferase activities, respectively. Columns and bars represent mean and standard deviation values, respectively. (**B**) The amounts of proteins (phosphorylated eIF2α subunit and actin) were visualized by Western blotting by using the indicated antibodies. Cell lysates described in panel (A) were used in this analysis. (**C**) Effects of nucleotide substitutions in the loop regions of SL I and SL IV on c-Src IRES-dependent translation. Huh-7 cells were transfected with reporter RNAs followed by the treatment of DMSO or tunicamycin. After the incubation for 2 h, luciferase activities in the cell lysates were measured. The experiments were performed three times. Luciferase activities were normalized to those in mock-treated cells which were set to 1. Solid and open columns represent *Renilla* and firefly luciferase activities, respectively. Columns and bars represent mean and standard deviation values, respectively. (**D**) The amounts of proteins (phosphorylated eIF2α subunit and actin) were monitored by Western blotting by using the indicated antibodies. Cell lysates described in panel (C) were used in this analysis.

### eIF2A facilitates the recruitment of tRNA$_i$ onto the 40S ribosomal subunit in a c-Src IRES-dependent manner

We investigated whether eIF2A and c-Src IRES can facilitate the recruitment of tRNA$_i$ onto the 40S ribosomal subunit by using a filter-binding assay similarly to the method described by Kim *et al.* (27). Filter-binding assays were performed with reaction mixtures containing the components depicted in the table of Figure 6 as described in Materials and Methods. The filter used in this assay has big pore sizes and low binding affinity with proteins and nucleic acids. Therefore, [$^{32}$P]-labeled tRNA$_i$, which is not associated with 40S ribosomal subunit, passes through the filter under negative pressure, but [$^{32}$P]-labeled tRNA$_i$, which is associated with 40S ribosomal subunit, remains on the filter after washing step. In our results, [$^{32}$P]-labeled tRNA$_i$ did not associate with the 40S ribosomal subunit in the absence of either eIF2A or an RNA corresponding to c-Src IRES (compare lanes 2 and 3 with negative control lanes 1 and 5 in Figure 6). The association of [$^{32}$P]-labeled tRNA$_i$ with the 40S ribosomal subunit was increased more than 5-fold in the presence of both c-Src IRES RNA and eIF2A. Replacement of c-Src IRES with EMCV IRES resulted in no augmentation of association of tRNA$_i$ with the 40S ribosomal subunit (compare lane 3 with 1 in Figure 6). The results indicate that eIF2A facilitates the recruitment of tRNAi onto the 40S ribosome in the presence of c-Src mRNA, and that eIF2A can function as an alternative tRNA$_i$ carrier in the presence of a specific mRNA such as c-Src mRNA.

### eIF2A-mediated translation of c-Src mRNA is required for cell growth under stress conditions

In order to confirm the requirement of eIF2A in translation of endogenous c-Src mRNA under stress conditions, we monitored the amount of endogenous c-Src and activated c-Src (phosphorylated in Y419) proteins with or without depletion of eIF2A (Figure 7A–C). Considering that the half-life of c-Src protein is 5–6 h in cells (39), we incubated Huh-7 cells with tunicamycin for 0, 6 and 12 h. The amount of c-Src protein was not changed by the tunicamycin treatment when eIF2A existed in cells (compare lanes 2 and 3 with 1 in Figure 7A and B). On the contrary, the amount of total c-Src protein was reduced more than 50% under stress conditions when eIF2A was depleted (compare lanes 5 and

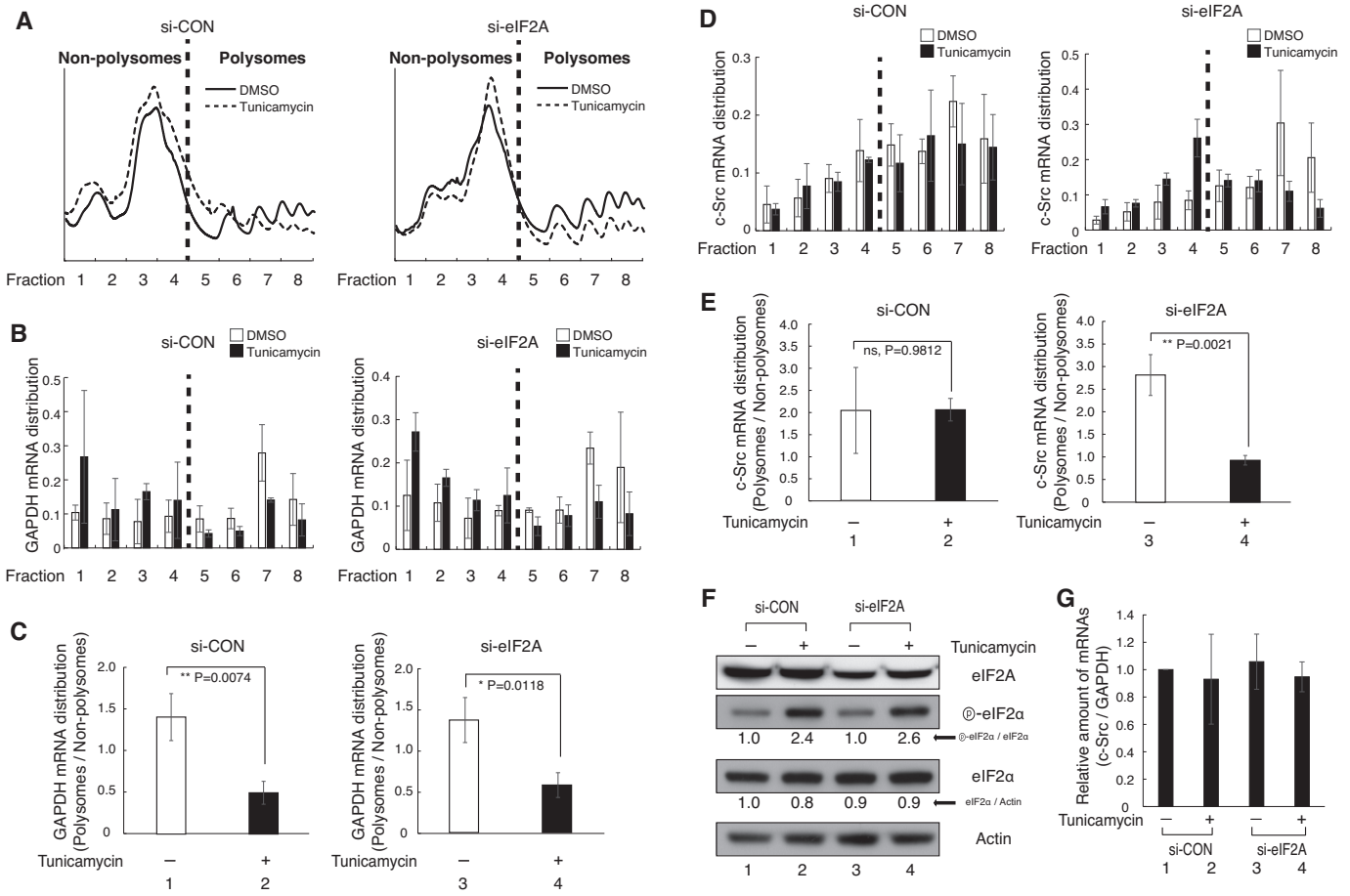


**Figure 5.** Sucrose gradient analyses of c-Src mRNA with or without depletion of eIF2A. Huh-7 cells were transfected with either a negative control siRNA or a siRNA against eIF2A under normal or stress conditions, and the distributions of eIF2A and GAPDH mRNAs were monitored by real time RT-PCR. (**A**) Sucrose gradient analysis was performed with Huh-7 cell extracts with (right panel) or without (left panel) depletion of eIF2A. Dotted lines and solid lines represent the ribosome profiles of tunicamycin-treated and mock-treated cells, respectively. Representative ribosome profiles among three independent analyses are depicted. (**B** and **D**) Distributions of GAPDH and c-Src mRNAs in the ribosome profiles. The amounts of GAPDH and c-Src mRNAs in the fractions (1-8 in panel A) of the sucrose gradients were measured by real time RT-PCR. Fluc RNAs of known concentration were spiked into each fraction and use it in normalization of RT-PCR data. The total amount of a specific mRNA was set to 1 in the graphs. Solid and open columns represent the relative mRNA level in individual fractions with or without tunicamycin treatment, respectively. Columns and bars represent mean and standard deviation values, respectively. (**C** and **E**) Ratios of mRNAs associated with polysomes (fractions 5–8) and non-polysomes (fractions 1–4). The sum of mean values of specific mRNAs [GAPDH in panel (B) and c-Src in panel (D)] in fractions 5–8 was divided by that in fractions 1–4, and the relative values are depicted in panels (C and E). Open and solid columns represent the relative values obtained from cells cultivated under normal and stress conditions, respectively. Columns and bars represent mean and standard deviation values, respectively. (**F**) The amounts of proteins (eIF2A, phosphorylated eIF2α subunit, eIF2α subunit and actin) in the cells used in the sucrose gradient analyses were monitored by Western blotting with the indicated antibodies. The amount of each protein was compared with that in DMSO- and control siRNA-treated cells which were set to 1. (**G**) Amounts of mRNAs in cell lysates used in the sucrose gradient analyses were monitored by real time RT-PCR, and the amount of c-Src mRNA was normalized with that of GAPDH. The relative level of c-Src mRNA was compared that that in DMSO- and control siRNA-treated cells which were set to 1. The experiments were performed three times. Columns and bars represent mean and standard deviation values, respectively.

6 with 4 in Figure 7A and B). Similarly to the level of total c-Src, the level of active c-Src, which has shorter half-life than inactive c-Src (39), remained the same under stress conditions when eIF2A existed in cells (compare lanes 2 and 3 with 1 in Figure 7A and C). The level of active c-Src was reduced up to 80% under stress conditions upon treatment of tunicamycin for 12 h when eIF2A was depleted (compare lane 6 with 4 in Figure 7A and C). The amount of c-Src mRNA was not changed by the treatments of tunicamycin and/or a siRNA against eIF2A (Figure 7D). The results strongly suggest that eIF2A plays a pivotal role in the translation of c-Src mRNA under stress conditions but not under normal conditions.

Finally, we investigated a potential role of eIF2A in cell proliferation under stress conditions since c-Src protein plays a key role in cell proliferation under stress conditions (40). The role of c-Src in proliferation of cells under normal and stress conditions was monitored by depleting c-Src protein with a siRNA against c-Src. MTT activity in cells was measured as an indication of cell growth (11). Depletion of c-Src did not affect cell growth under normal conditions (compare open column on lane 3 with 1 in Figure 8A and Supplementary Figure S2A). Under stress conditions, on the contrary, cell growth was reduced ∼40% (compare open column lane 2 with 1 in Figure 8A and Supplementary Figure S2A). Surprisingly, knockdown of c-Src in-

| | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| 40S ribosomal subunit | + | + | + | + | − |
| EMCV IRES | − | − | + | − | − |
| c-Src IRES | − | + | − | + | + |
| eIF2A | − | − | + | + | + |
| [$^{32}$P] tRNA$_i$ | + | + | + | + | + |

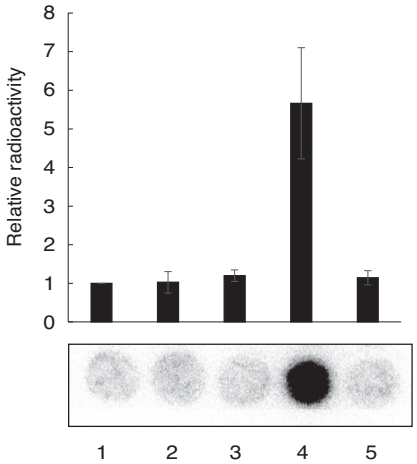


**Figure 6.** eIF2A and c-Src IRES cooperatively facilitate the recruitment of tRNA$_i$ onto the 40S ribosomal subunit. The binding of [$^{32}$P]-labeled tRNA$_i$ to 40S ribosomal subunit was monitored by a filter-binding assay as Kim *et al*. described (27). c-Src IRES RNA, EMCV IRES RNA, and [$^{32}$P]-labeled tRNA$_i$ were synthesized by *in vitro* transcription with T7 RNA polymerase. Recombinant His-eIF2A proteins were purified from *E. coli*, and 40S ribosomal subunits were purified from HeLa S3 cells. Mixtures containing components depicted in the table were incubated and analyzed by filter binding assay. The experiments were performed three times. Relative radio activities were normalized to those in lane 1 which was set to 1. Columns and bars represent mean and standard deviation values, respectively.

hibited cell growth more than 90% under stress conditions (compare open column on lane 4 with 3 in Figure 8A and Supplementary Figure S2A). The results indicate that c-Src is required for cell growth under stress conditions. The levels of c-Src, active c-Src, eIF2A, eIF2α and phosphorylated eIF2α proteins were monitored by Western blotting (Figure 8C).

Similarly, knockdown of eIF2A weakly affected cell growth under normal conditions (compare open column on lane 3 with 1 in Figure 8B and Supplementary Figure S2B). Under stress conditions, cell growth was reduced ∼40% (compare lane 2 with 1 in Figure 8B and Supplementary Figure S2B). Depletion of eIF2A inhibited cell growth >90% under stress conditions (compare lane 4 with 3 in Figure 8B and Supplementary Figure S2B). It is worth to note that the level of phospho-eIF2α at 24 h after tunicamycin treatment was almost the same as that of mock treated condition (Figure 8C and D). This indicates the stress response is nullified by this time as reported by Ghosh *et al*. (41). Nonetheless, the amount of c-Src protein was reduced in eIF2A-depleted cells after the treatment of tunicamycin for 24 h. Considering that eIF2A is required for the translation of c-Src mRNA under stress conditions, the inhibition of cell growth by the depletion of eIF2A under stress conditions is likely due to the blockage of translation of c-Src mRNA. This suggests that constitutive translation of c-Src mRNA mediated by eIF2A is essential for cell growth under stress conditions.

## DISCUSSION

Owing to the poor vascularization inside fast growing tumor mass, the tumor cells are exposed to various stresses such as poor supply of oxygen (hypoxia) and limited supplies of nutrients including glucose and amino acids. The stresses in tumor cells trigger activation of eIF2α kinases resulting in translational repression of most mRNAs (42–44). For example, hypoxia and unfolded protein response (UPR) in tumor cells activate PERK leading to phosphorylation of eIF2α (43,45). Due to the activated stress responses, only limited tumor cells, which successfully overcome the inhibitory stress responses, can outgrow in a tumor mass (46). c-Src protein, a tyrosine kinase, is known to play pivotal roles in overcoming inhibitory stress responses. For instance, c-Src induces tumor growth (47) through the activation of NF-κB, a transcription factor inducing anti-apoptotic gene expression, which results in survival of cells in hypoxia (48). Moreover, depletion of c-Src inhibits cell proliferation and cell cycle progression through reduced phosphorylation of ERK1/2, Akt and GSK3β (49).

Interestingly, expression of c-Src is sustained even when translation of most mRNAs are inhibited by the phosphorylation of eIF2α in tumors (5). Allam and Ali showed that the IRES element in c-Src mRNA is responsible for the stress-resistant translation of c-Src mRNA (13). However, the molecular basis of the stress-resistant translation of c-Src mRNA remained to be elucidated.

Here, we report that eIF2A, a stress-insensitive tRNA$_i$ carrier, mediates the c-Src IRES-dependent translation under stress conditions through a specific interaction with c-Src mRNA (Figures 1–3). A specific interaction between eIF2A and the SL I in c-Src mRNA was required for the stress-resistant translation of c-Src mRNA (Figure 4). In other words, a differential binding of eIF2A with a specific mRNA (c-Src) is the underlying mechanism of the persistent translation of c-Src mRNA under stress conditions. We also confirmed the requirement of eIF2A in translation of c-Src mRNA under stress conditions using sucrose gradient analyses (Figure 5). Moreover, we demonstrated that eIF2A facilitates the recruitment of tRNA$_i$ onto the 40S ribosomal subunit in a c-Src mRNA-dependent manner using a filter-binding assay of [$^{32}$P]-labeled tRNA$_i$ (Figure 6). Lastly, we discovered that the eIF2A-mediated translation of c-Src mRNA plays a key role in the cell proliferation under stress conditions (Figure 8). Depletion of eIF2A reduced the amount of c-Src protein under stress conditions, but not under normal conditions. Surprisingly, the level of active c-Src protein was reduced up to 80% after treatment of tunicamycin for 12 h when eIF2A was depleted (Figure 7A and C). On the other hand, the level of total c-Src protein was reduced ∼50% by this time by the same treatment (Figure 7A and B). The discrepancy is most likely attributed to the difference of stability of active c-Src and inactive c-Src proteins in cells. The half-life of total c-Src protein and

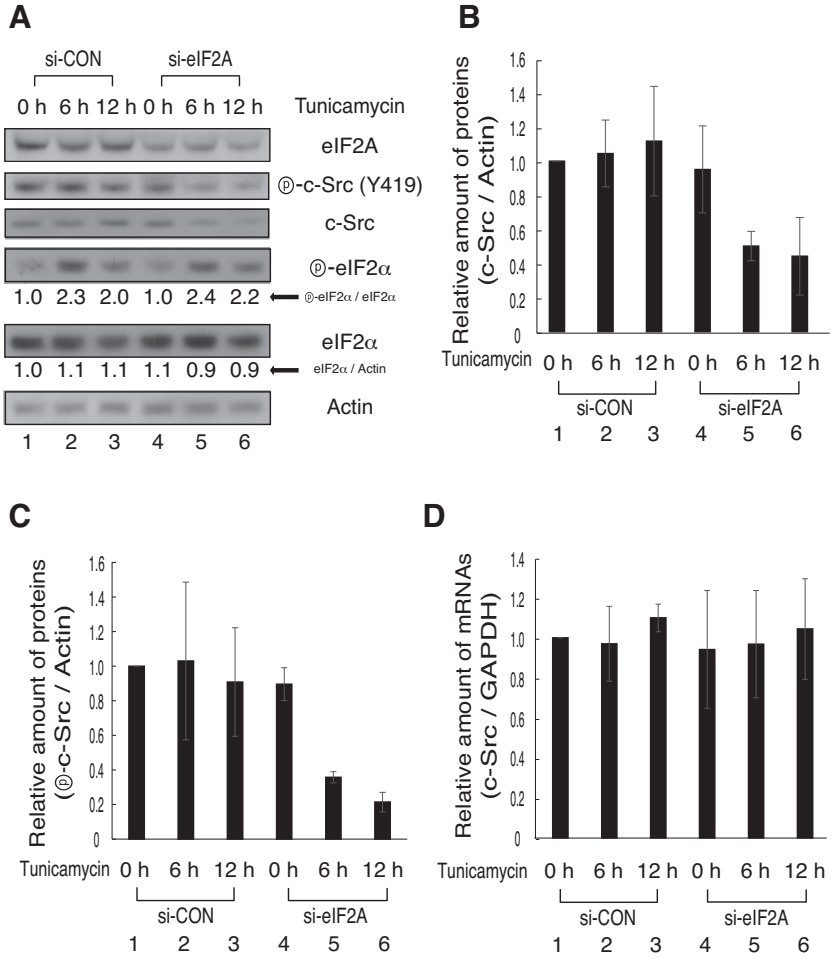


**Figure 7.** eIF2A is required for translation of endogenous c-Src mRNA under stress conditions. (**A**) Effects of a control siRNA (lanes 1–3) or a siRNA against eIF2A (4–6) on the level of endogenous c-Src proteins under stress condition. Huh-7 cells were transfected with a siRNA against eIF2A or a negative control siRNA and then incubated with tunicamycin (5 μg/ml) for the indicated times. The amounts of proteins [eIF2A, phosphorylated c-Src (Y419), c-Src, phosphorylated eIF2α subunit, eIF2α subunit and actin] were monitored by western blotting using the indicated antibodies. Relative amount of phosphorylated eIF2α was calculated by dividing the average value of phosphorylated eIF2α by the average value of total eIF2α. The amount of eIF2α was normalized to the amount of actin. Relative amount of proteins was calculated and depicted using the average values from three independent Western blotting experiments. The amount of each protein at a specific condition was normalized to that in mock-treated cells transfected with a negative control siRNA which were set to 1. Representative blots of the western blot analyses are depicted. (**B**) The relative amount of total c-Src protein is depicted in a graph. The amount of c-Src protein at a specific condition was normalized to that in mock-treated cells transfected with a negative control siRNA which were set to 1. (**C**) The relative amount of active c-Src (phospho-c-Src) protein is depicted in a graph. The amount of phospho-c-Src protein at a specific condition was normalized to that in mock-treated cells transfected with a negative control siRNA which were set to 1. (**D**) The relative amount of c-Src mRNA is depicted in a graph. The amount of c-Src mRNA was normalized to that of GAPDH mRNA. The amount of c-Src mRNA at a specific condition was normalized to that in mock-treated cells transfected with a negative control siRNA which were set to 1. (B–D) The experiments were performed three times. Columns and bars represent mean and standard deviation values, respectively.

that of active c-Src are 5–6 h and 1–3 h, respectively. Almost complete repression of cell proliferation under stress conditions by the depletion of eIF2A (Figure 8) is likely due to the limitation of active c-Src protein (Figure 7). Interestingly, the level of active c-Src protein was partially restored up to 40% of untreated cells at 24 h after tunicamycin treatment (Figure 8D). The restoration of active c-Src is likely attributed to nullification of stress responses by the continuous stimuli (41). The nullification of stressed response is clearly observed by the restoration of phospho-eIF2α level at 24 h after tunicamycin treatment (Figure 8D, lanes 2 and 4).

Phylogenetic analysis revealed that c-Src proteins are well conserved in vertebrates (50). Moreover, the primary sequences and the predicted secondary structures of domain II and SL I of c-Src genes of vertebrates are well conserved (Supplementary Figure S3). Especially the sequences and secondary structures of SL I of vertebrate c-Src genes are very well conserved (>72% of sequences are identical among the distantly related vertebrates; Supplementary Figure S3B). This may suggest that the domain II and SL I regions of vertebrate c-Src mRNAs contain important cis-acting element(s) controlling translation of c-Src proteins and that the eIF2A-mediated translation of c-Src mRNAs occurs in a large variety of vertebrates.

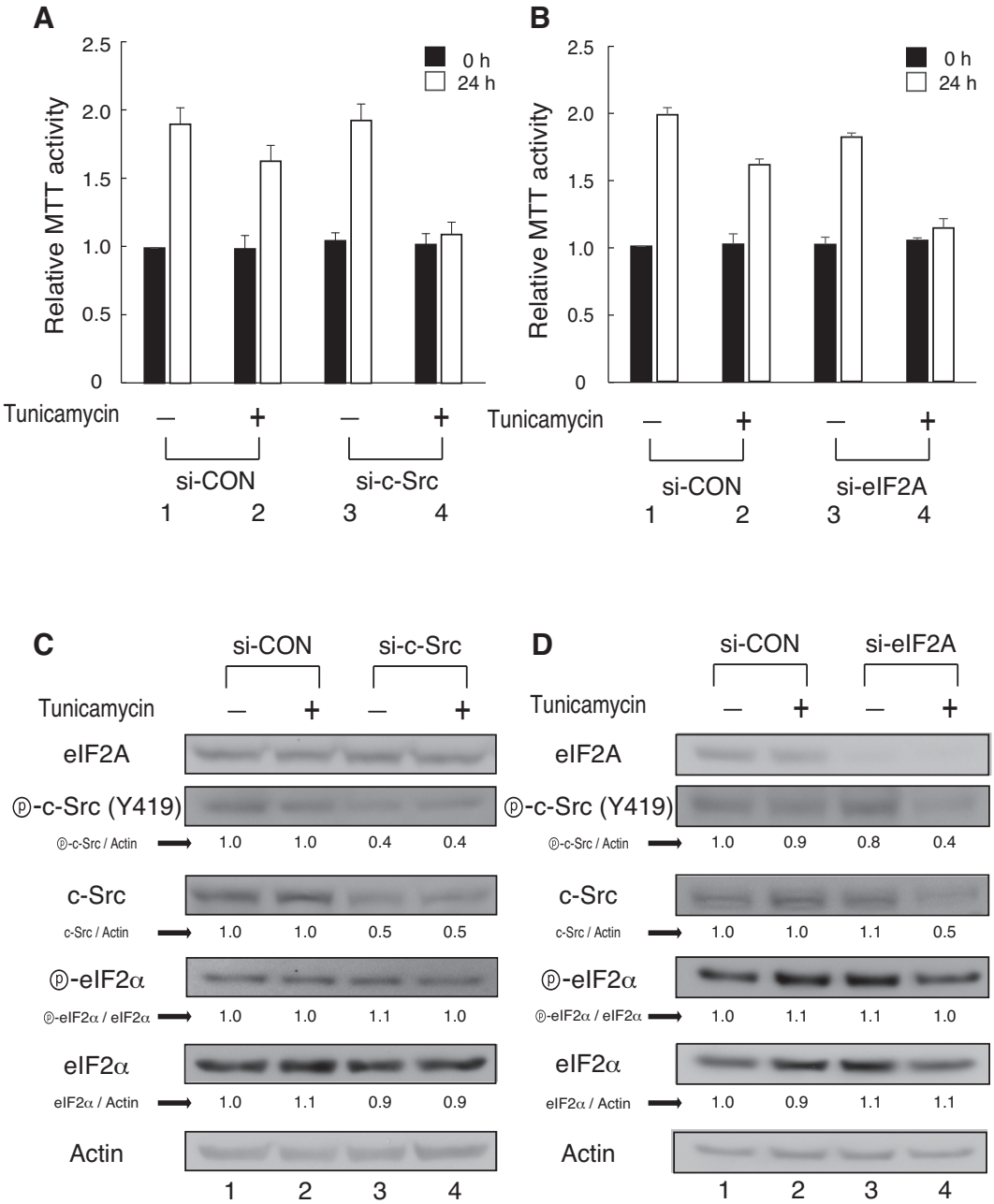


**Figure 8.** Cell growth is inhibited by the depletion of eIF2A or c-Src under stress conditions. (**A**) Effects of a control siRNA (lanes 1 and 2) or a siRNA against c-Src (lanes 3 and 4) on the cell proliferation. Huh-7 cells were transfected with a negative control siRNA or a siRNA against c-Src and then cultivated for 24 h. The cells were further cultivated for 0 or 24 h in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of tunicamycin (5 μg/ml). MTT assay was performed with these Huh-7 cells. The experiments were performed three times. The relative MTT activities were normalized to those in cells treated with the negative control siRNA under normal conditions without additional incubation (0 h) which was set to 1. Open and solid columns represent relative MTT activity with (24 h) or without (0 h) additional incubation, respectively. Columns and bars represent mean and standard deviation values, respectively. (**B**) Effects of a control siRNA (lanes 1 and 2) or a siRNA against eIF2A (lanes 3 and 4) on the proliferation of cells. A negative control siRNA or a siRNA against eIF2A were transfected into Huh-7 cells and then cells were cultivated for 24 h. The cells were further cultivated for 0 or 24 h in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of tunicamycin (5 μg/mL). MTT assay was performed with these Huh-7 cells. The experiments were performed three times. The relative MTT activities were normalized to those in the cells treated with the negative control siRNA under normal conditions without additional incubation (0 h) which was set to 1. Open and solid columns represent relative MTT activity with (24 h) or without (0 h) additional incubation, respectively. Columns and bars represent mean and standard deviation values, respectively. (**C** and **D**) The amounts of proteins [eIF2A, phosphorylated c-Src (Y419), c-Src, phosphorylated eIF2α subunit, eIF2α subunit and actin] were monitored by Western blotting using the indicated antibodies. Relative amount of phosphorylated eIF2α was calculated by dividing the value of phosphorylated eIF2α by the value of total eIF2α. The amount of eIF2α was normalized to the amount of actin. The amount of each protein at a specific condition was normalized to that in mock-treated cells transfected with a negative control siRNA which were set to 1. Relative amount of proteins was calculated and depicted using the average values from three independent Western blotting experiments.

Interestingly, there seems to be a striking difference in regulation of eIF2A expression between yeast and mammalian cells. In yeast, the level of eIF2A mRNA decreases under stress conditions (51). On the other hand, there was no detectable changes in the levels of eIF2A protein with or without ER stress in the cells studied in this study (Figure 2C, 5G, 7A, 8C and D). Furthermore, there was a report suggesting that the level of mammalian eIF2A protein increases under stress conditions (34). This report and our results indicate that eIF2A is required for translation of specific mammalian mRNAs refractory to the translational repression induced by stress responses. In fact, yeast eIF2A was also shown to augment translation of URE2 mRNA under ethanol stress (51). Considering both mammalian and yeast studies, we can conclude that eIF2A plays an important role in augmenting translation of specific mRNAs under stress conditions in both yeast and mammalian cells.

The effects of eIF2A depletion on c-Src IRES activities under normal and stress conditions were clearly different from each other. Depletion of eIF2A greatly reduced the c-Src IRES activity under stress conditions. On the contrary, depletion of eIF2A did not affect the c-Src IRES activity under normal conditions (Figure 2). This result indicates that the c-Src IRES utilizes eIF2 as a $tRNA_i$ carrier under normal conditions whereas utilizes eIF2A instead of eIF2 as an exclusive $tRNA_i$ carrier under stress conditions. Interestingly, the localization of eIF2A changes depending on the environmental conditions. The majority of eIF2A proteins are localized in the nucleus under normal conditions whereas they are mostly localized in the cytoplasm under stress conditions (27). This suggests that the $tRNA_i$ is loaded onto the 40S ribosomes by eIF2A associated with specific mRNAs mainly under stress conditions due to the differential localization of eIF2A and the inhibition of eIF2 function by the phosphorylation of α-subunit. The molecular basis of the switch of eIF2A localization remains to be elucidated.

It is noteworthy that the filter binding assay shown in Figure 6 was performed with uncharged $tRNA_i$s since charged and uncharged $tRNA_i$s have similar affinity for eIF2A (27). The charging of $tRNA_i$ with methionine may occur before or after association of eIF2A. Alternatively, an unidentified protein might assist preferential binding of charged $tRNA_i$ to eIF2A. The detailed mechanism of aminoacylation of $tRNA_i$ on eIF2A remains to be elucidated.

Through the analyses of the best known eIF2A-dependent mRNAs c-Src (human) and HCV (viral), following common features of eIF2A-dependent translation were conjectured: (1) A specific RNA–protein interaction between an mRNA and eIF2A is required for eIF2A-dependent translation. The SL I of c-Src and the SL-IIId of HCV IRESes participate in the interaction with eIF2A and in the eIF2A-mediated translation. This indicates that the mRNA–eIF2A interactions provide the specificity of target mRNAs in eIF2A-dependent translation. (2) The eIF2A-binding sites are close to the start codon. Both the SL-IIId of HCV and the SL I of c-Src mRNAs are close to the start codon, which may make it possible for eIF2A to recruit the $tRNA_i$ onto the 40S ribosomal subunit [Figure 6 and (27)]. However, it is noteworthy that the SL-IIId of HCV is located upstream of the start codon, but the SL I of c-Src is located downstream of the start codon. The detailed process of eIF2A-meadiated $tRNA_i$-loading onto the 40S ribosomal subunit remains to be elucidated. (3) The eIF2A-mediated translation of HCV and c-Src mRNAs occurs only under stress conditions when the activity of eIF2 is compromised. Translation of these mRNAs is likely to be executed by eIF2 under normal conditions since knockdown of eIF2A did not affect translation of these mRNAs under normal conditions [Figure 2B and (27)].

Through the knockdown experiments of eIF2A and c-Src, we found that both eIF2A and c-Src proteins are required for proliferation of cells under stress conditions. However, knockdown of eIF2A did not affect cell proliferation under normal conditions. Considering the above features of eIF2A, this gene is a potential new target for development of anti-cancer drug which has little side effect.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

## ACKNOWLEDGEMENTS

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*Conflict of interest statement.* None declared.

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