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Roles of YB-1 under arsenite-induced stress: Translational activation of HSP70 mRNA and control of the number of stress granules

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ABSTRACT

Background: When cells become stressed, they form stress granules (SGs) and show an increase of the molecular chaperone HSP70. The translational regulator YB-1 is a component of SGs, but it is unclear whether it contributes to the translational induction of HSP70 mRNA. Here we examined the roles of YB-1 in SG assembly and translational regulation of HSP70 mRNA under arsenite-induced stress.
Method: Using arsenite-treated NG108-15 cells, we examined whether YB-1 was included in SGs with GluR2 mRNA, a target of YB-1, and investigated the interaction of YB-1 with HSP70 mRNA and its effect on translation of the mRNA. We also investigated the distribution of these mRNAs to SGs or polysomes, and evaluated the role of YB-1 in SG assembly.
Results: Arsenite treatment reduced the translation level of GluR2 mRNA; concomitantly, YB-1-bound HSP70 mRNA was increased and its translation was induced. Sucrose gradient analysis revealed that the distribution of GluR2 mRNA was shifted from heavy-sedimenting to much lighter fractions, and also to SG-containing non-polysomal fractions. Conversely, HSP70 mRNA was shifted from the non-polysomal to polysome fractions. YB-1 depletion abrogated the arsenite-responsive activation of HSP70 synthesis, but SGs harboring both mRNAs were still assembled. The number of SGs was increased by YB-1 depletion and decreased by its overexpression.
Conclusion: In arsenite-treated cells, YB-1 mediates the translational activation of HSP70 mRNA and also controls the number of SGs through inhibition of their assembly.
General significance: Under stress conditions, YB-1 exerts simultaneous but opposing actions on the regulation of translation via SGs and polysomes.

1. Introduction

Both formation of stress granules (SGs) and upregulation of stress proteins are important for cellular defense against various forms of acute stress such as heat shock and exposure to oxidative agents. SGs are assembled through aggregation of TIA-1 protein, leading to accumulation of translation-silenced mRNAs during the stress response [1–3]. These SGs contain several translational initiation factors and many RNA-binding proteins [4]. However, mRNA of the stress protein HSP70, a molecular chaperone, is not included in SGs, and synthesis of the protein is upregulated [2]. HSP70 is known to regulate SG formation [3], but the mechanism responsible for exclusion of its mRNA from SGs is unknown.

YB-1 is a multifunctional protein distributed in not only intra- but also extracellular domains, and interacts with many other proteins involved in cellular phenomena. It also binds to nucleic acids to control gene expression in both a transcriptional and a translational manner [5–9]. In the cytoplasm, it binds to polysomal or non-polysomal mRNAs and regulates the levels of their translation. The protein acts as either a translational activator or an inhibitor depending on its amount that binds to the target mRNA [10,11]. We have previously shown that in neural cells and skeletal muscle cells, YB-1 regulates the translation of specific mRNAs in response to neural activity [12,13]. It has also been reported that cell condition-dependent activation of protein kinase Akt alters the RNA binding of YB-1 and affects translational activation in both cancer and neuronal cells [14–19]. Importantly, YB-1 is of relevance in many aspects of cancer progression, such as cell proliferation, multiple drug resistance and metastasis [9,20].

Interestingly, under stress conditions, YB-1 is also incorporated into SGs, and can sometimes be used as a marker of these granules [21–24]. Therefore, it can be speculated that YB-1 plays some role in the translational regulation of stress responses. It has recently been reported that YB-1 contributes to inhibition of translational initiation through association with tRNA-derived fragment (tiRNA), which is cleaved by stress-activated ribonuclease angiogenin [25–27]. However, it is still unclear whether YB-1 is necessary for SG formation, or whether it plays a role in translational control of mRNAs in the granules, and no previous study has investigated whether YB-1 contributes to translational regulation of HSP70 mRNA under conditions of stress.

In the present study using neural NG108-15 cells, we found that YB-1 binds to mRNA for HSP70, as well as that for GluR2, which has been

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identified as a target mRNA of YB-1 in neural cells [12]. We investigated the roles of YB-1 in translational control of GluR2 and HSP70 mRNAs, and in SG assembly, during arsenite-mediated oxidative stress. Translation of GluR2 mRNA was inhibited in cells that formed SGs in response to arsenite treatment, whereas HSP70 mRNA was actively translated in the cells. Sucrose gradient analysis revealed that under stress conditions, these YB-1-bound mRNAs were distributed into different translation-regulating complexes, polysomes and SGs. In YB-1-depleted cells, translational induction of HSP70 mRNA during arsenite-mediated stress was not observed, but translation of GluR2 mRNA was still reduced and both mRNAs were immunoprecipitated with TIA-1. We further found that the number of SGs produced was affected by the amount of intracellular YB-1. Our results suggest that under stress conditions, YB-1 is involved in upregulation of HSP70 mRNA translation, but is not necessary for repression of GluR2 mRNA translation, and in fact has a role in controlling the number of SGs through an inhibitory effect on their assembly.

2. Materials and methods

2.1. Cell culture and arsenite treatment

Mouse neuroblastoma × rat glioma hybrid NG108-15 cells were grown in Dulbecco's modified Eagle medium with 10% fetal bovine serum. To induce arsenite stress, the cells were incubated with 0.75 mM sodium arsenite (Sigma-Aldrich) for 30 min. For cycloheximide (Sigma-Aldrich) or MG-132 (Wako Pure Chemicals) treatment, the reagents were added to the cells at a final concentration of 20 μg/ml or 1 μM, respectively, prior to arsenite treatment.

2.2. Protein preparation and Western blot analysis

After a wash with PBS, the cells were homogenized in TKM buffer, containing 50 mM triethanolamine (pH 7.8), 50 mM KCl, 5 mM $MgCl_2$, 0.25 M sucrose, 1 mM PMSF, protein inhibitors (complete cocktail without EDTA, Roche), 1 mM DTT and RNase inhibitor (0.2 unit/μl, Takara). The homogenate was centrifuged at 3000 rpm for 10 min and the supernatant was used as the cytosol fraction. For Western blot analysis, proteins were separated by SDS-PAGE and transferred to a PVDF membrane. After treatment with the first antibody, the membrane was incubated with the second antibody conjugated with horseradish peroxidase (GE Healthcare Life Sciences). The protein signals were detected with an ECL kit (GE Healthcare Life Sciences). Rabbit anti-YB-1 antibody was prepared using two synthesized amino-terminal peptides as antigens [28]. Goat anti-GluR2 antibody, goat anti-TIA-1 antibody, rabbit anti-HSP70 antibody and rabbit anti-β-actin antibody were purchased from Santa Cruz Biotechnology. Rabbit anti-S6 antibody and rabbit anti-phospho-YB-1 (Ser102) antibody were purchased from Cell Signaling Technology. Rabbit anti-GFP antibody was purchased from Life Technologies.

2.3. RNA extraction and RT-PCR

Total RNA extraction was performed using guanidine isothiocyanate followed by phenol extraction. RNA in the immunoprecipitated complex was prepared by SDS–phenol–chloroform extraction. The RNA was purified by ethanol precipitation and dissolved in water. The first-strand cDNA was synthesized with reverse transcriptase MMLV (Takara) using an oligo (dT) primer. The double-strand cDNA was synthesized using a primer pair specific for each mRNA, and the nucleotide sequences were verified by dideoxy-mediated sequencing. The amounts of the RT-PCR products were analyzed using a Bio-Rad Gel Documentation system (Gel Doc XR Plus Image Lab system). The primer pairs used for the RT-PCR were: 5′-TCCGGTGGATCCGCAGACCGTAACCAT-3′ (forward) and 5′-TCTGCAGTCGACTCGACGCGCATAGGG-3′ (reverse) for YB-1 mRNA, 5′-GAAGATTGGGTACTGGAGTGAAGTG-3′ (forward) and 5′-TTAATGGAGCAATGGCAATATCAGC-3′ (reverse) for GluR2 mRNA, and 5′-ATCGAGGTGACCTTCGACATCGACG-3′ (forward) and 5′-TGGCACTTGTCCAGCACCTTCTTCT-3′ (reverse) for HSP70 mRNA, and 5′-GATGACCCAGATCATGTTTGAG-3′ (forward) and 5′-TCAACGTCACACTTCATGATGG-3′ (reverse) for β-actin mRNA.

2.4. Immunoprecipitation analysis

Immunoprecipitation was performed using Dynabeads protein G (Invitrogen) in accordance with the manufacturer's protocol. Anti-YB-1 antibody or anti-TIA-1 antibody was bound to the beads, and the cytosol fraction was then incubated with the beads for 4 h at 4 °C. After washing with PBS containing 0.1% BSA, the immune complex was eluted with buffer containing 20 mM Tris–HCl, pH 7.5, 140 mM NaCl, and 2% SDS. Proteins were analyzed by Western blotting and co-immunoprecipitated mRNAs were extracted for use as a template for RT-PCR.

2.5. Immunocytochemistry

The cells were fixed in 4% paraformaldehyde in PBS for 10 min and treated with 0.5% Triton X-100 in PBS for 10 min. The cells were then incubated with mouse anti-TIA-1 antibody (Santa Cruz Biotechnology) or rabbit anti-YB-1 antibody in PBS containing 5% skimmed milk at room temperature for 2 h. The cells were washed with PBS, and then incubated with Alexa Fluor 488-conjugated donkey anti-mouse IgG antibody (Molecular Probes) for TIA-1 or Alexa Fluor 555-conjugated goat anti-rabbit IgG antibody (Molecular Probes) for YB-1 at room temperature for 1 h. After washing with PBS, fluorescent signals were viewed with an Olympus inverted microscope linked to a DP-70 imaging system. In YB-1-GFP-overexpressing cells, SGs were detected by goat anti-TIA-1 antibody (Santa Cruz Biotechnology) and Alexa Fluor 555-conjugated donkey anti-goat IgG antibody (Molecular Probes). As a negative control for the immunostainings, prior to the reaction, antibodies were incubated with the peptides, YB-1 [28] and TIA-1 (Santa Cruz Biochemistry), that had been utilized for antibody generation.

2.6. DNA transfection and RNA interference

The cells were transfected with a plasmid DNA (pYB-1-GFP) or a siRNA using Lipofectamine LTX and plus reagent (Invitrogen). The fusion gene pYB-1-GFP was constructed using a rat cDNA clone [12]. The siRNA specific for YB-1 (M-04834-01) and a control siRNA (D-001210-01) were both purchased from Thermo Scientific.

2.7. Sucrose gradient centrifugation

The cytosol fraction was prepared from the cell lysate in TKM buffer, and Nonidet P-40 (NP-40) was added to 0.5%. The mixture was loaded on a 20–50% sucrose gradient and centrifuged at 28,000 rpm for 2.5 h at 4 °C using a SW40Ti rotor (Beckman Coulter). The gradient was fractionated, and an equal volume of each fraction was used for detection of proteins by Western blotting. To examine the distribution of GluR2 mRNA and HSP70 mRNA on the gradient, RNA was extracted from the fractions and then RT-PCR was performed.

3. Results

3.1. GluR2 mRNA is taken into SGs with YB-1 under arsenite-induced stress conditions and its translation is suppressed

We previously identified GluR2 mRNA as a target mRNA of the translational regulator YB-1 in neural cells [12]. To examine how the translation of GluR2 mRNA is regulated under the stress conditions in which SGs are assembled, NG108-15 cells were treated with 0.75 mM sodium arsenite for 30 min, and then the mRNA and protein levels of both

GluR2 and YB-1 were analyzed by RT-PCR and Western blotting, respectively (Fig. 1A). The same analysis was carried out for β-actin as an internal control. The level of GluR2 protein was decreased by arsenite-mediated stress without any significant change in the amount of its mRNA. YB-1 expression was unchanged in terms of both mRNA and protein levels. The amounts of β-actin protein and mRNA were also unchanged. We further examined the possibility that the YB-1 molecule might be modified, for example by cleavage and phosphorylation at Ser102 by Akt, in arsenite-treated cells (Fig. 1B). However, no change in the molecular weight was observed and the level of phosphorylated YB-1 was not altered significantly, suggesting that YB-1 itself is not modified by arsenite treatment. Under these conditions, SGs were observed by immunocytochemistry using a specific antibody against the SG marker protein TIA-1 (Fig. 1C). YB-1 was distributed mainly in the cytoplasm under these conditions and, as has already been reported, was co-localized with the granules. To confirm the presence of GluR2 mRNA in the granules, we performed immunoprecipitation analysis using anti-TIA-1 antibody (Fig. 1D). As compared with the control cells, under arsenite-induced stress, YB-1 was apparently co-precipitated with TIA-1, and RT-PCR using RNA that had been extracted from the immune complex revealed that GluR2 mRNA was also contained in the SGs. These results suggested that under the present stress conditions, GluR2 mRNA is taken up rapidly into SGs with YB-1 and its translation is inhibited.

3.2. Interaction between YB-1 and HSP70 mRNA under arsenite-induced stress

It has been shown that the mRNA of a molecular chaperone, HSP70, is excluded from SGs even under stress conditions in which many species of mRNA are taken up by the granules and their translation suppressed [2]. We subjected NG108-15 cells to arsenite stress as described above, and then analyzed the amounts of HSP70 mRNA and protein using RT-PCR and Western blotting, respectively (Fig. 2A). Although the level of HSP70 mRNA was not significantly changed, an increase of HSP70 protein was observed. As shown in Fig. 2B, the ratio of arsenite-dependent increase of HSP70 protein was not altered remarkably either in the presence or absence of a proteasome inhibitor

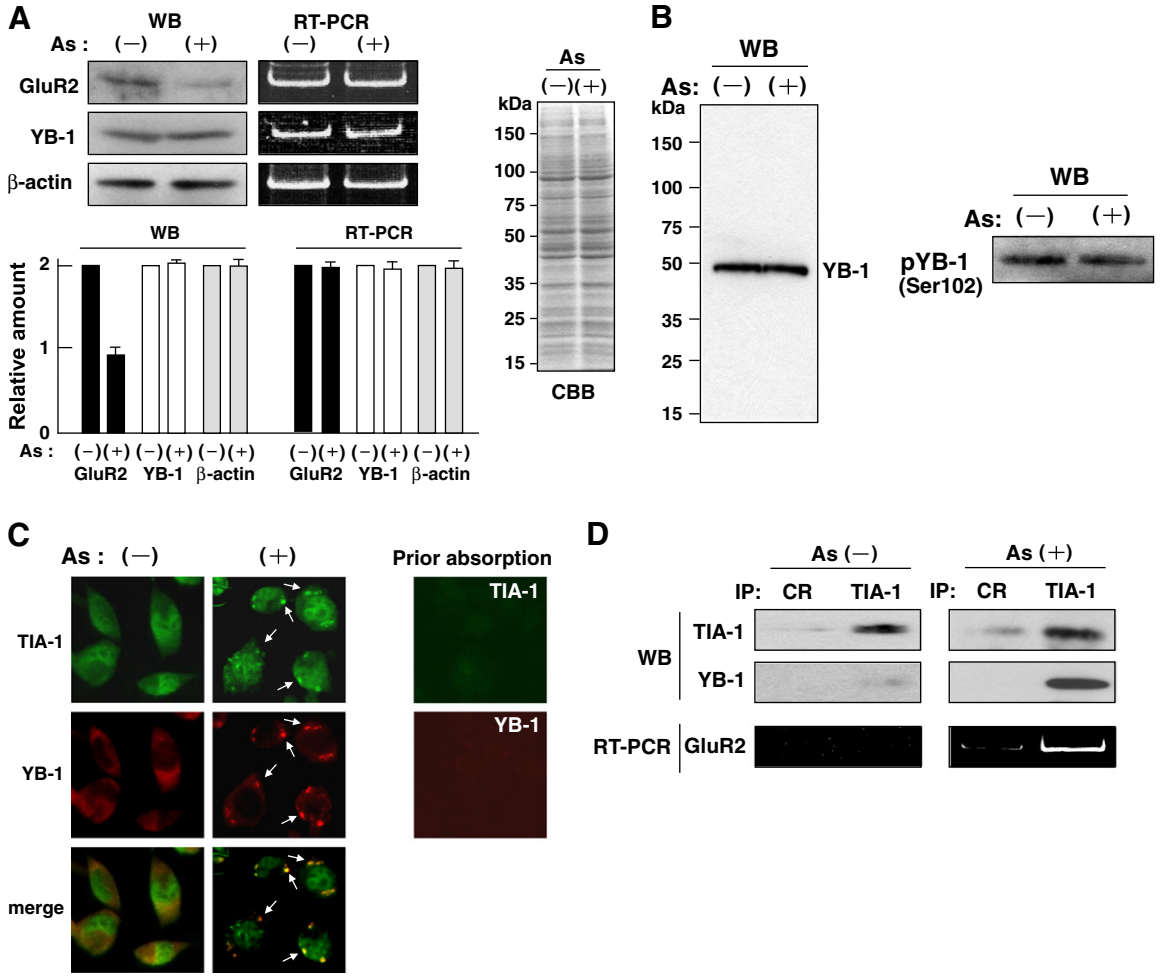

Fig. 1. GluR2 mRNA is included in SGs with YB-1 under arsenite-mediated stress conditions and its translation is reduced. (A) Translation levels of GluR2 mRNA and YB-1 mRNA were examined. The amounts of protein and mRNA of both GluR2 and YB-1 were analyzed by Western blotting and RT-PCR, respectively. Signal intensities of the proteins and the PCR products stained with ethidium bromide were analyzed by densitometry and the Bio-Rad Gel Documentation system, respectively. The amounts of each protein or each mRNA in arsenite-treated cells relative to those in control cells are indicated. The data represent an average of three independent experiments. The amounts of β-actin protein and mRNA are also indicated as an internal control. Proteins in the lysate were analyzed for their integrity and quantity after electrophoresis on gel stained with CBB. Each lane corresponds to that of Western blotting. (B) YB-1 itself is not modified by arsenite treatment. An immunoblot of YB-1 shows the whole molecular range, and phosphorylation of the protein at Ser102 was tested using a specific antibody. (C) Left panel: co-localization of TIA-1 and YB-1 in SGs upon treatment with arsenite. Cells were double stained with both anti-TIA-1 antibody and anti-YB-1 antibody. Alexa Fluor 488-conjugated second antibody (green) and Alexa Fluor 555-conjugated second antibody (red) were used for TIA-1 and YB-1, respectively. A merged image is also shown. Arrows show typical YB-1-containing SGs. Right panel: reduction of immunoreactivity by prior absorption of the antibodies with the peptides that were utilized as antigens. (D) Left panel: experiment using control cells is indicated. Neither YB-1 protein nor GluR2 mRNA was co-precipitated by anti-TIA-1 antibody. Right panel: GluR2 mRNA was co-precipitated with YB-1 protein by anti-TIA-1 antibody, but not by control IgG, from arsenite-treated cells. TIA-1 and YB-1 in the immune complex were analyzed by Western blotting. RNA was extracted from the complex and GluR2 mRNA was detected by RT-PCR.

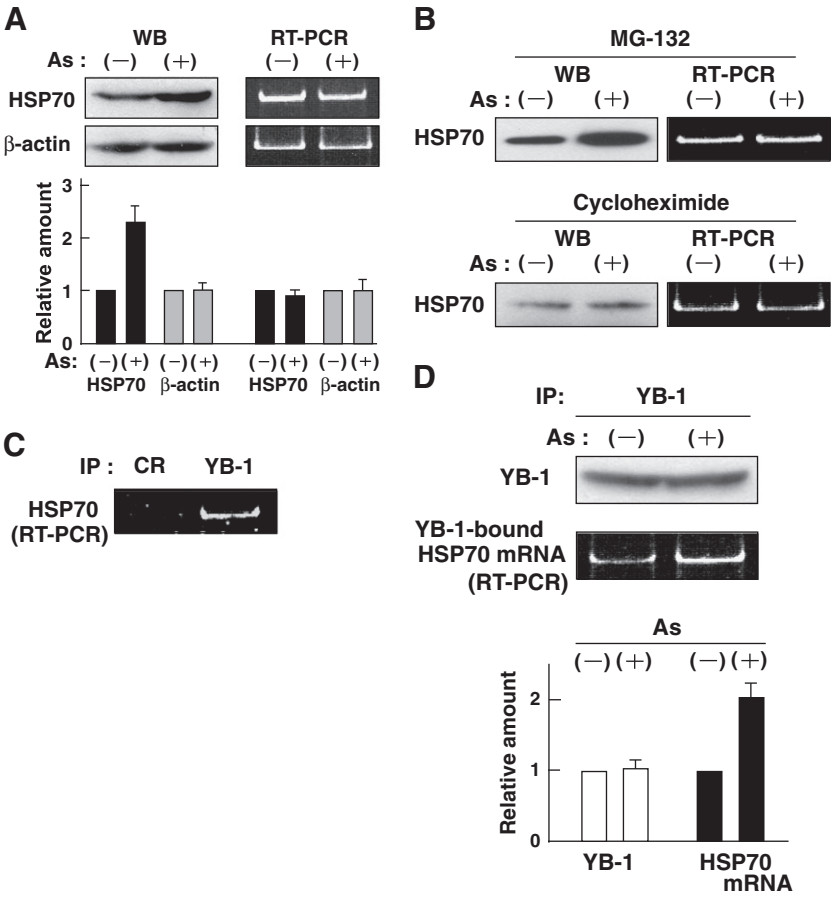

Fig. 2. YB-1 associates with HSP70 mRNA which shows stress-mediated translational induction. (A) Cells were treated with arsenite and then the levels of HSP70 protein and mRNA were analyzed by Western blotting and RT-PCR, respectively. Signal intensity of the protein was analyzed by densitometry. Signal intensity of the PCR products was analyzed using the Bio-Rad Gel Documentation system. The amounts of HSP70 protein and its mRNA in arsenite-treated cells relative to those in control cells are indicated. The data represent an average of three independent experiments. The amounts of β-actin protein and mRNA are also indicated as an internal control. (B) Expression of HSP70 in cells treated with arsenite in the presence of the proteasome inhibitor MG-132 (1 μM) or the protein synthesis inhibitor cycloheximide (20 μg/ml). Cells were treated with the reagents for 30 min prior to arsenite treatment. Protein and mRNA were detected by Western blotting and RT-PCR, respectively. (C) YB-1 associates with HSP70 mRNA in NG108-15 cells. The cytosol fraction of the cells was immunoprecipitated with anti-YB-1 antibody or control IgG and RNA was extracted from the immune complex. RT-PCR was then carried out using specific primers for HSP70 mRNA. (D) The amount of YB-1-bound HSP70 mRNA was increased in response to arsenite-induced stress. Cells were treated with arsenite and the cytosol fraction was immunoprecipitated with anti-YB-1 antibody. The amounts of HSP70 mRNA and YB-1 in the immune complex were analyzed by RT-PCR and Western blotting, respectively. The amounts of YB-1 and YB-1-bound HSP70 mRNA in arsenite-treated cells relative to those in control cells are indicated. The data are an average of three independent experiments.

MG-132 (see Fig. 2A). On the other hand, this increase of HSP70 protein was abolished and any decrease of the protein was not observed in the presence of the protein synthesis inhibitor cycloheximide, indicating that translation of HSP70 mRNA was induced within a short period (30 min) upon exposure to the stress, and that HSP70 protein was not degraded under these conditions. It is noteworthy that YB-1 functions not only as a translational inhibitor but also as a translational activator according to cellular conditions [10,11]. To investigate the possibility that YB-1 binds to HSP70 mRNA, immunoprecipitation analysis using anti-YB-1-antibody was performed (Fig. 2C). HSP70 mRNA was detected in the immune complex by RT-PCR, suggesting interaction of YB-1 with HSP70 mRNA. Furthermore, the amount of YB-1-bound HSP70 mRNA was apparently increased under the stress conditions (Fig. 2D). As the efficiency of immunoprecipitation by anti-YB-1 antibody was not altered by arsenite treatment, and the amounts of both YB-1 protein and HSP70 mRNA were unchanged in the cells irrespective of the presence of stress (see Figs. 1A and 2A), it appeared that the HSP70 mRNA molecules to which YB-1 binds were increased in response to the stress. It is suggested that this change in the interaction between YB-1 and HSP70 mRNA leads to the induction of mRNA translation.

3.3. YB-1 is necessary for the stress-mediated translational induction of HSP70 mRNA but not for the translational inhibition of GluR2 mRNA

We previously demonstrated that cellular condition-dependent translational regulation of YB-1-binding mRNA is observed as a change in the association of YB-1 with polysomes [12,13,19]. Hence, the association of YB-1 with polysomes in the arsenite-treated cells was examined using sucrose gradient centrifugation (Fig. 3A). The distributions of YB-1, TIA-1 and S6 ribosome proteins on the gradient were analyzed using specific antibodies. TIA-1 was detected only in the non-polysomal fractions, irrespective of arsenite treatment that induced SG formation (see Fig. 1B), indicating that the density of SGs was much lower than that of the translational initiation complex. A similar observation has been reported previously [29]. In the control cells, YB-1 was distributed from free mRNP fractions to heavy-sedimenting polysome fractions, but the distribution was shifted to much lighter polysome fractions with S6 ribosomal protein by arsenite treatment, suggesting the inhibition of ribosome recruitment to mRNAs and induction of SG formation. Indeed, as shown in Fig. 3B, under the stress conditions, GluR2 mRNA was lost in the heavy-sedimenting fractions (nos. 8–11), and was increased in the lighter (nos. 4–6) and non-polysomal fractions (nos. 1 and 2). In

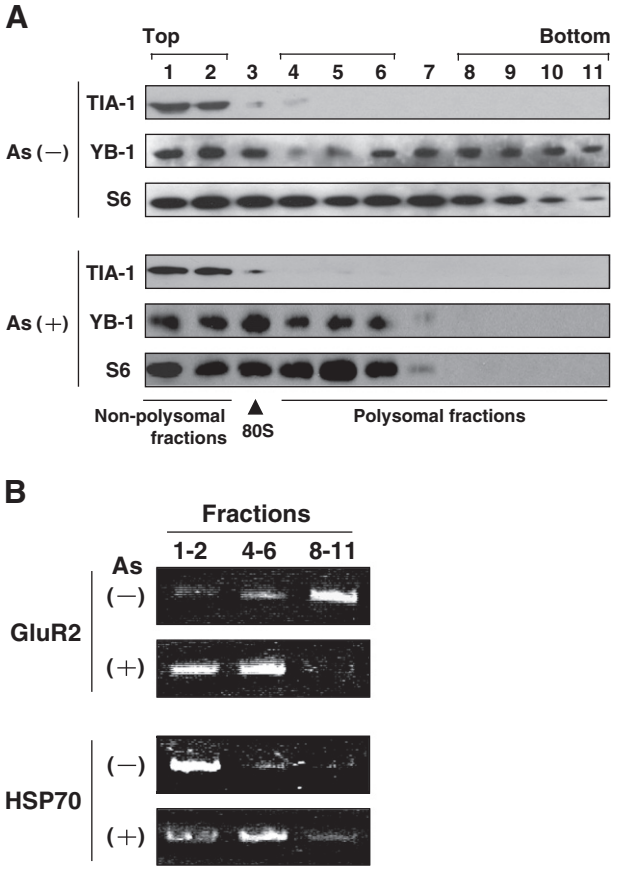

Fig. 3. Distributions of GluR2 mRNA and HSP70 mRNA on a sucrose gradient are shifted to opposite fractions in response to stress. (A) Sucrose gradient analyses of TIA-1, YB-1, and S6 ribosome proteins from the cells treated with or without arsenite. The cytosol fractions were centrifuged on a 20–50% sucrose gradient and the distribution of each protein was analyzed by Western blotting using an equal volume of each fraction. The position of an 80S ribosomal particle is indicated. (B) Change in the distribution of GluR2 mRNA and HSP70 mRNA on the sucrose gradient in response to arsenite-induced stress. Non-polysomal fractions (nos. 1 and 2), lighter-sedimenting polysome fractions (nos. 4–6), and heavy-sedimenting polysome fractions (nos. 8–11) of each gradient were collected. After RNA had been extracted from each mixture, GluR2 mRNA and HSP70 mRNA were detected by RT-PCR.

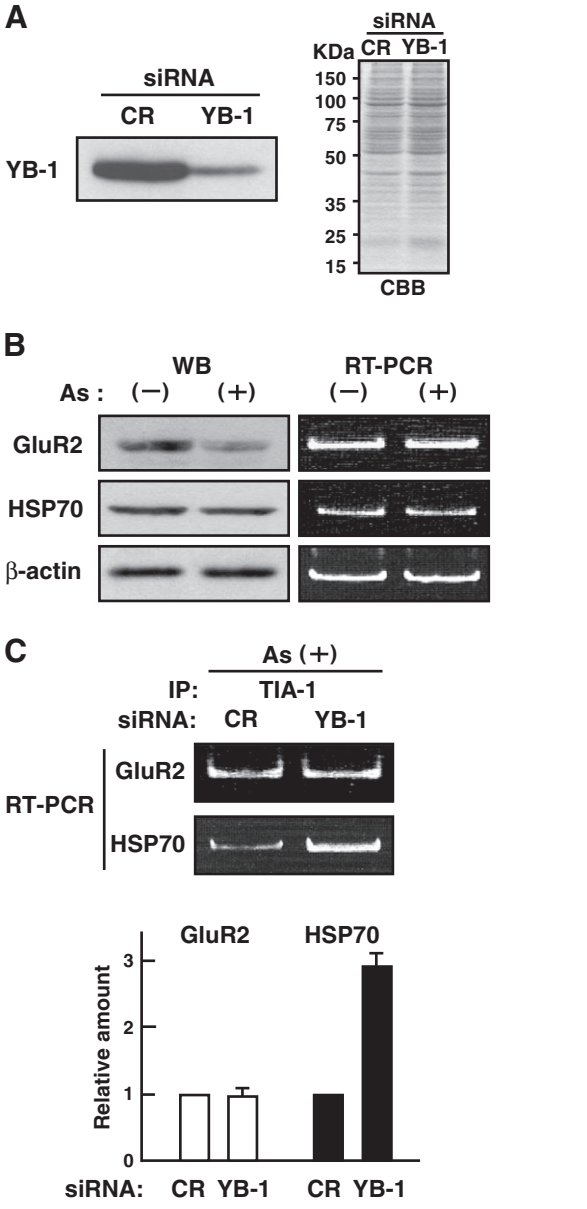

Fig. 4. Depletion of YB-1 abolishes arsenite-mediated translational induction of HSP70 mRNA but not translational reduction of GluR2 mRNA under stress conditions. (A) Cells were treated with a specific siRNA against YB-1 mRNA, or with control siRNA for 2 days, and the level of YB-1 protein was analyzed by Western blotting. Proteins in the lysate were analyzed for their integrity and quantity after electrophoresis on gel stained with CBB. Each lane corresponds to that of Western blotting. (B) Translation levels of GluR2 mRNA and HSP70 mRNA in the YB-1-depleted cells were examined after arsenite treatment. The amounts of protein and mRNA for both GluR2 and HSP70 were analyzed by Western blotting and RT-PCR, respectively. The amounts of β-actin protein and mRNA are also indicated as an internal control. (C) Both YB-1-depleted cells and control cells were treated with arsenite, and then the cytosol fraction was immunoprecipitated with anti-TIA-1 antibody. RNA was extracted from the immune complex, and GluR2 mRNA and HSP70 mRNA were detected by RT-PCR. The amounts of GluR2 mRNA and HSP70 mRNA in YB-1-depleted cells relative to those in control cells are indicated. The data are an average of three independent experiments.

contrast, with arsenite treatment, the amount of HSP70 mRNA in the non-polysomal fractions was decreased and a corresponding amount of the mRNA appeared in the polysome fractions (nos. 4–6).

The results described above are consistent with the opposite changes in the translational activities of GluR2 mRNA and HSP70 mRNA induced by arsenite treatment. Together with the observations shown in Figs. 1 and 2, it is indicated that YB-1 associates with both SGs and polysomes, simultaneously, in arsenite-treated cells. This suggests that under these stress conditions, YB-1 is assigned to different translation-regulating machineries with each target mRNA in order to mediate protein synthesis.

We next attempted to deplete YB-1 using a specific siRNA, and then examined the levels of both mRNA and protein levels of GluR2 and HSP70 after treatment of the cells with arsenite for 30 min. The level of YB-1 was markedly decreased by the siRNA (Fig. 4A). As shown in Fig. 4B, in the YB-1-depleted cells, no arsenite-mediated increase of HSP70 protein occurred. Both protein and mRNA levels of β-actin, used as an internal control, were unchanged. On the other hand, a significant decrease of GluR2 protein was still observed. The amounts of HSP70 mRNA and GluR2 mRNA were unchanged. Together with the data in Fig. 2, these findings suggested that YB-1 is necessary for the stress-mediated translational induction of HSP70 mRNA, but not for the translational reduction of GluR2 mRNA, even though YB-1 is a component of SGs. We examined the effects of YB-1 depletion on the amounts of GluR2 mRNA and HSP70 mRNA within SGs under stress conditions (Fig. 4C). GluR2 mRNA was immunoprecipitated by anti-TIA-1 antibody, irrespective of the YB-1 level. Interestingly, in the YB-1-depleted cells, an apparent increase of HSP70 mRNA was detected in the immune complex. Together with the data in Fig. 3, these results indicate that YB-1 plays a role in excluding HSP70 mRNA from SGs and recruiting ribosomes to the mRNA. Although HSP70 mRNA was present in SGs after YB-1 depletion, no significant decrease in the

amount of HSP70 protein was observed (Fig. 4B), suggesting that, unlike GluR2 protein, a certain level HSP70 was maintained in the cells to function as a molecular chaperone under stress conditions and YB-1 does not contribute to stabilization of HSP70 protein.

3.4. YB-1 exerts an inhibitory effect on SG formation and affects the number of granules

We examined the necessity of YB-1 for SG formation. After depletion of YB-1 using siRNA, the cells were treated with arsenite for 30 min and SGs were detected by immunocytochemistry with anti-TIA-1 antibody. As expected from the results in Fig. 4, SGs were formed in the YB-1-depleted cells (Fig. 5A) and the percentage of SG-positive cells was similar to that of the control cells that had been treated with non-relevant siRNA (Fig. 5B). This indicated that YB-1 is not necessary for assembly of SGs. However in SG-positive cells, the number of granules was apparently larger after YB-1 depletion than in the control cells (Fig. 5A and C), suggesting that in fact YB-1 has a negative effect on granule formation.

To confirm the inhibition of SG assembly by YB-1, we next evaluated the effect of YB-1 overexpression on granule formation by transfecting the cells with a YB-1-GFP expression vector (pYB-1-GFP) or a control GFP vector (pEGFP). The considerable amount of YB-1-GFP or GFP was expressed by transfection, and the level of endogenous YB-1 was not altered significantly (Fig. 6A). Cells were then treated with arsenite for 30 min and SGs were detected using anti-TIA-1 antibody in both sets of transfected cells (Fig. 6B and C). The control GFP and YB-1-GFP were distributed in both the cytoplasm and the nucleus. In the control cells, SGs were formed irrespective of the level of GFP expression. On the other hand, the number of SG-positive cells was apparently decreased in the cells overexpressing YB-1-GFP and in the untransfected cells, SG assembly was observed. Together with the results shown in Figs. 4 and 5, these observations suggested that although YB-1 is known to be a component of SGs, the protein acts as an inhibitor of granule assembly to control their numbers in cells under stress conditions.

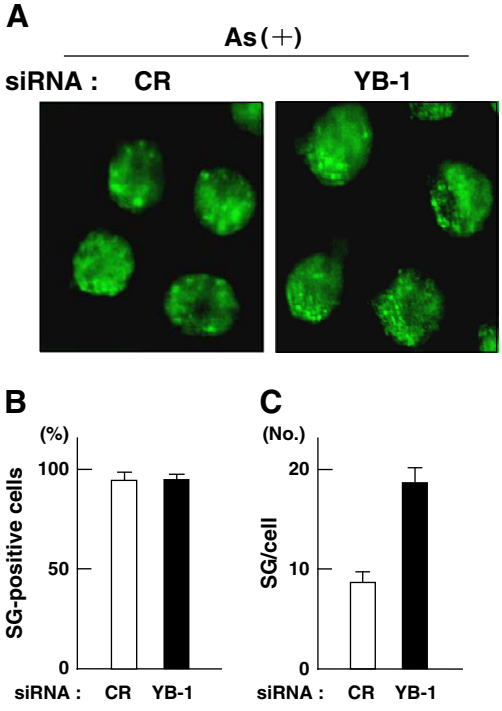

Fig. 5. Depletion of YB-1 does not affect the number of SG-positive cells but increases the number of SGs in the cells after arsenite treatment. (A) After siRNA treatment, both YB-1-depleted cells and control cells were treated with arsenite and then immunocytochemistry was performed using anti-TIA-1 antibody. Alexa Fluor 488-conjugated second antibody was used. (B) Approximately 100 TIA-1-expressing cells were counted under each condition in (A) and the percentages of SG-positive cells were compared. The data represent the mean and standard error obtained from three independent experiments. (C) The number of SGs in the SG-positive cells was evaluated. The data represent the mean and standard error obtained in three independent experiments.

4. Discussion

Although YB-1 interacts with both GluR2 mRNA and HSP70 mRNA under conditions of arsenite-mediated oxidative stress, GluR2 mRNA was recruited to SGs with YB-1, whereas HSP70 mRNA was distributed to polysome fractions with YB-1 in a sucrose gradient. This localization of each mRNA in different translation-regulating complexes is consistent with opposing translational regulation of the two mRNAs, suggesting that YB-1 is involved in the stress-mediated sorting of mRNAs. However, even in YB-1-depleted cells, GluR2 mRNA was incorporated into SGs and its translation was still repressed. On the other hand, the stress-mediated translational induction of HSP70 mRNA was abrogated by YB-1 depletion, and the RNA was co-precipitated with TIA-1, indicating that HSP70 mRNA was also taken up by SGs. These results indicate that under arsenite-induced stress, YB-1 is needed to prevent HSP70 mRNA from being sequestered by SGs to form HSP70 mRNA-containing polysomes, but is not required for granule formation or for incorporation of GluR2 mRNA into them.

The level of HSP70, a molecular chaperone, increases in response to cellular stresses and protects proteins from misfolding or abnormal aggregation. Furthermore, molecular chaperones regulate the aggregation of TIA-1 [2,3]. It is suggested that HSP70 is also involved in SG disassembly once any stress has been removed. However, overexpression of HSP70 blocks TIA-1 aggregation [3]. Therefore the level of HSP70 needs to be under a certain degree of control, and it is possible that YB-1 plays an important role in this process. Translational regulation by YB-1 depends on its molar ratio relative to the mRNA with which it binds [10,11,13,19]. Indeed, we observed a change in the level of interaction between YB-1 and HSP70 mRNA upon exposure to arsenite stress (Fig. 2). Under the stress conditions, therefore, the overall structure of HSP70 mRNA might be modulated through alteration of the number of YB-1 molecules on the mRNA. The YB-1 depletion experiment revealed that the protein is not involved in protection of HSP70 protein from degradation within a short period (30 min) of arsenite treatment. However, cells require at least 3 h to disassemble SGs after arsenite removal [29]. It is possible that YB-1 may also contribute to stabilization of HSP70 protein within a much longer time span until cells have recovered sufficiently from stress-induced damage.

YB-1 is often used as a marker of SGs [21–24]. However, our observations in this study indicate that YB-1 has an inhibitory effect on SG assembly and that the amount of YB-1 affects the number of SGs in arsenite-treated cells. Assembly of SGs occurs upon auto-aggregation of the TIA-1 protein, and many SG-associated proteins have been identified [2,4]. There is interesting evidence that Staufen, a protein component of SGs, impairs granule assembly [30]. Together with our present data, it appears that several species in SG-associated proteins are recruited into the granules to regulate their number and/or their degree of aggregation with other components. Like the molecular chaperone, HSP70, these regulator proteins would also be necessary for disassembly of SGs so that repressed translation reactions can resume after cellular conditions have returned to normal. It has been shown that YB-1 protects cancer cells from anticancer drug toxicity through the expression of many genes that promote malignant progression [20,31–35]. YB-1 thus participates in resistance to apoptosis-inducing conditions. The effects of YB-1 depletion and/or overexpression on cell survival under arsenite-induced stress will need to be clarified in future studies.

Recently, using a technique for labeling endogenous mRNAs, it was demonstrated that mRNAs associate with and dissociate from SGs in arsenite-stressed living cells [36]. In addition, it has previously been reported that protein components of SGs show continuous shuttling in and out of the granules during stress [37]. We demonstrated here that

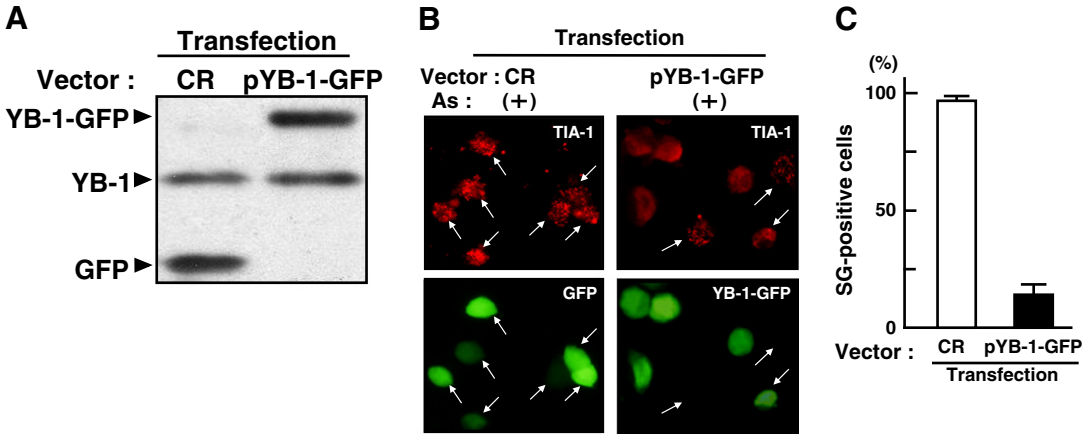

Fig. 6. Overexpression of YB-1 inhibits SG formation in arsenite-treated cells. (A) Cells were transfected with the YB-1-GFP expression vector (pYB-1-GFP), or with a control GFP vector, and then the levels of YB-1-GFP, endogenous YB-1 and GFP were analyzed by Western blotting using anti-YB-1 antibody and anti-GFP antibody, simultaneously. (B) Both pYB-1-GFP-transfected cells and control cells were treated with arsenite and then immunocytochemistry was performed to detect SGs using goat anti-TIA-1 antibody and Alexa Fluor 555-conjugated donkey anti-goat IgG antibody. Expressed control GFP and YB-1-GFP are also shown. Arrows indicate SG-positive cells. (C) Approximately 100 GFP- or YB-1-GFP-expressing cells were counted under each condition in (B), and the percentage of SG-positive cells was evaluated. The data represent the mean and standard error obtained from three independent experiments.

YB-1 is distributed in different translational regulatory complexes: SGs with GluR2 mRNA and polysomes with HSP70 mRNA. There is a possibility that YB-1 is involved in such an association/dissociation shuttle of its target mRNAs in SGs.

Our data also revealed that the level of YB-1 was not affected at either the mRNA or protein level, even under stress conditions. Unlike many proteins whose synthesis is rapidly arrested, or are degraded in a short time under stress, YB-1 is necessary for regulating the translational response to various situations through SG assembly or disassembly. Moreover, as we demonstrated here, the protein is also involved in HSP70 mRNA polysome formation during stress. Therefore, the amount of YB-1 in the cell appears to be kept constant at all stages of the stress. It has been reported that YB-1 controls its own synthesis at the translation level [38]. The mechanism by which the cellular amount of functional YB-1 remains constant under stress remains to be elucidated. It has been reported that YB-1 is secreted through a non-classical pathway and participates in Notch-3-mediated cell signaling circuits with autoregulation [8,9,39,40]. Therefore, there is a need to verify whether signaling through interaction of secreted YB-1 with Notch-3 participates in controlling the amount of YB-1 and/or regulation of protein sorting into stress granules and active polysomes under arsenite-induced stress. It is also noteworthy that YB-1 is highly expressed in the brain of developing and young mice, whereas its level is very low in the adult and aged brain [28,41]. Such a decrease of the YB-1 level with aging may reduce the degree to which neural cells are protected from various stresses.

An illustrative scheme of a model for the modulation of SG assembly and translation of HSP70 mRNA by YB-1 is indicated (Fig. 7). During arsenite-induced stress, although YB-1 is recruited into SGs with translationally-suppressed mRNAs, it functions as an inhibitor of granule assembly and affects the number of SGs in cells. Furthermore, we propose that YB-1 also binds to mRNA of a molecular chaperone, HSP70, playing an important role in exclusion of the mRNA from SGs and recruiting ribosomes for translation of the mRNA during the stress. It would be important to clarify the function of YB-1 in recovery of protein synthesis after stress removal.

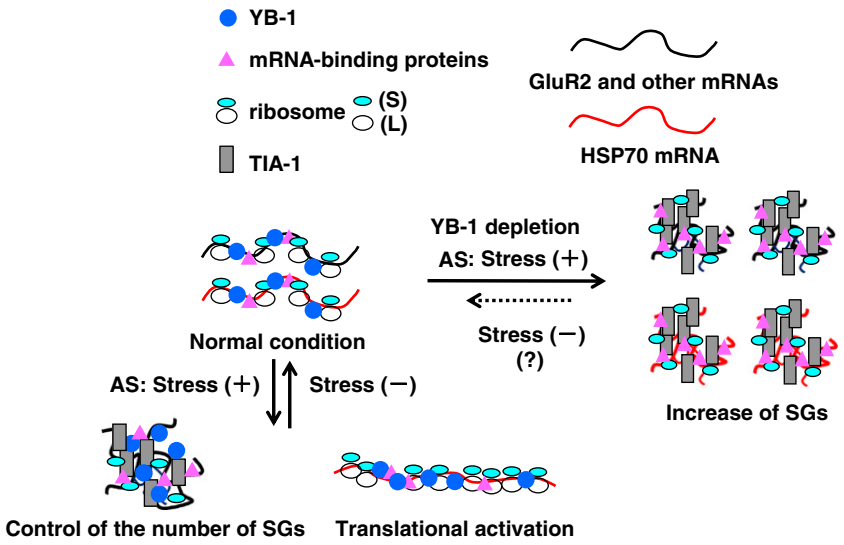

Fig. 7. YB-1 modulates SG assembly and translation of HSP70 mRNA. Under arsenite-mediated stress, YB-1 acts as a preexisting molecular chaperone in both SG assembly and formation of HSP70 mRNA-containing active polysomes. In YB-1-depleted cells, the number of SGs is increased. The function of YB-1 in disassembly of SGs after removal of the stress remains to be elucidated.

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