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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.

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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 $MgCl_2$, 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

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