RNA-binding Proteins TIA-1 and TIAR Link the Phosphorylation of eIF-2α to the Assembly of Mammalian Stress Granules

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Abstract. In response to environmental stress, the related RNA-binding proteins TIA-1 and TIAR colocalize with poly(A)$^+$ RNA at cytoplasmic foci that resemble the stress granules (SGs) that harbor untranslated mRNAs in heat shocked plant cells (Nover et al., 1989; Nover et al., 1983; Scharf et al., 1998). The accumulation of untranslated mRNA at SGs is reversible in cells that recover from a sublethal stress, but irreversible in cells subjected to a lethal stress. We have found that the assembly of TIA-1/R$^+$ SGs is initiated by the phosphorylation of eIF-2α. A phosphomimetic eIF-2α mutant (S51D) induces the assembly of SGs, whereas a nonphosphorylatable eIF-2α mutant (S51A) prevents the assembly of SGs. The ability of a TIA-1 mutant lacking its RNA-binding domains to function as a transdominant inhibitor of SG formation suggests that this RNA-binding protein acts downstream of the phosphorylation of eIF-2α to promote the sequestration of untranslated mRNAs at SGs. The assembly and disassembly of SGs could regulate the duration of stress-induced translational arrest in cells recovering from environmental stress.

Key words: RNA-binding proteins • stress • translational control • eIF-2α

Tᴴᴇ general translational arrest that accompanies environmental stress is initiated by the phosphorylation of eIF-2α, an essential component of the heterotrimeric eIF-2 complex that loads the initiator tRNA (Met-tRNA^{Met}) onto the 40S ribosomal subunit (Berlanga et al., 1998; Gray and Wickens, 1998; Srivastava et al., 1998; Harding et al., 1999). The eIF-2 complex binds GTP/GDP, and its activity is regulated by eIF-2B, a guanine nucleotide exchange factor. Phosphorylation of eIF-2α increases the affinity of eIF-2 for eIF-2B, and prevents the exchange of GDP for GTP, thereby inhibiting the formation of preinitiation complexes. Although other initiation factors (e.g., eIF-4E) can also contribute to stress-induced translational arrest, the ability of nonphosphorylatable mutants of eIF-2α to abrogate stress-induced translational arrest (Murtha-Riel et al., 1993) indicates the central importance of eIF-2α in this process.

Stress-induced inhibition of translational initiation allows elongating ribosomes to run off nonheat shock mRNAs, resulting in the disassembly of polyribosomes. In cells allowed to recover from stress in the presence of actinomycin D, synthesis of nonheat shock proteins is rapidly resumed, indicating that preexisting mRNAs are not degraded (Storti et al., 1980; Lindquist, 1981; Panniers and Henshaw, 1984). Studies of cultured Peruvian tomato cells have shown that untranslated mRNAs accumulate at discrete cytoplasmic foci known as heat stress granules (HSGs)[1] (Nover et al., 1983, 1989; Scharf et al., 1998). In cells that are returned to ambient temperature, these mRNAs rapidly move from HSGs to polyribosomes. If mRNAs sequestered at HSGs are unavailable for translational reinitiation, the assembly and disassembly of these structures might influence the duration of stress-induced translational arrest. Heat shock mRNAs that are selectively translated during stress are excluded from HSGs (Nover et al., 1989), consistent with the proposed role for these structures in translational repression. The further observation that low molecular weight heat shock proteins are prominent components of HSGs (Scharf et al., 1998) suggests that they may be homologous to the mammalian stress granules to which HSP27 (a mammalian low molecular weight heat shock protein) is recruited in heat-stressed chicken embryo fibroblasts (Collier and Schlesinger, 1986; Collier et al., 1988) and HeLa cells (Arrigo et al., 1988). Although stress-induced nascent transcripts are excluded from mammalian SGs (Collier et al., 1988) (consistent with the exclusion of HSP mRNAs from tomato

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1. *Abbreviations used in this paper:* HA, hemagglutinin; HSG, heat shock granule; HSP, heat shock protein; PABP-I, poly (A)$^+$ binding protein I; RNP, ribonuclear protein, SG, stress granule.

HSGs), the association of untranslated mRNAs with mammalian stress granules (SGs) has not been reported.

We have identified three RNA-binding proteins (TIA-1, TIAR, and poly(A)$^+$ binding protein I [PABP-I]) that co-aggregate with poly(A)$^+$ RNA at mammalian SGs. TIA-1, TIAR, and PABP-I are members of the RNA recognition motif type family of RNA-binding proteins (Tian et al., 1991; Kawakami et al., 1992, 1994). PABP-I resides in the cytoplasm where it promotes the stability and translation of polyadenylated mRNAs (Adam et al., 1986; Query et al., 1989; Burd et al., 1991; Matunis et al., 1993). In contrast, TIA-1 and TIAR are concentrated in the nucleus at steady state but normally shuttle between the nucleus and the cytoplasm (Kedersha, N., and P. Anderson, unpublished observations). Although TIA-1 and TIAR have been implicated in signaling cascades regulating entry into apoptosis (Tian et al., 1991, 1995; Kawakami et al., 1992, 1994; Taupin et al., 1995), their precise function has not been determined. Mutant mice lacking TIAR exhibit partial embryonic lethality and defective germ cell maturation, implicating this protein in selective aspects of vertebrate development (Beck et al., 1998). Here, we report that TIA-1 and TIAR act downstream of the stress-induced phosphorylation of eIF-2α to promote the recruitment of untranslated mRNAs to SGs. The ability of phospho-eIF-2α (Srivastava et al., 1998), TIA-1 (Tian et al., 1991), and TIAR (Kawakami et al., 1992) to trigger apoptosis suggests that the assembly and disassembly of SGs might influence the survival of cells subjected to environmental stress.

Materials and Methods

Cell Lines

DU145 and COS-7 cells were obtained from the American Type Culture Collection. Cells were maintained in 10% FBS in DME.

Antibodies and Reagents

Murine mAbs to TIA-1 (ML-29, IgG1) were raised against recombinant protein at ImmunoGen, Inc., screened by ELISA, and shown to recognize TIA-1 but not TIAR, by immunofluorescent staining. Anti-TIAR (murine monoclonal 6E3, IgG2a) and anti–TIA-1/R (murine monoclonal 3E6, IgG2a) have been described previously (Taupin et al., 1995). Antibodies from commercial sources include HSP-27 (StressGen Biotechnologies Corp.), anti-HA (murine mAb clone 16B12, IgG1; Berkeley Antibody Co.), anti–ribosome P antibody (ImmunoVision, Springdale, AR), anti–β-galactosidase mAb (Promega Corp), and anti-T7 (Novagen). Isotype-specific secondary antibodies were obtained from Southern Biotechnology Associates. Hoechst dye (33258), nocodazole, cytochalasin B, cycloheximide, puromycin, anisomycin, and protease inhibitors were obtained from Sigma Chemical Co. Anti–La antibodies were obtained from Dr. Peter Schur (Brigham and Women's hospital, Boston, MA). mAb against eIF-2α was a gift from Dr. Richard Panniers (National Institutes of Health, Bethesda, MD). Anti–PABP-I mAb (10E10) was a gift from Dr. Gideon Dreyfuss (University of Pennsylvania, School of Medicine, Philadelphia, PA).

Stress Treatments and Immunofluorescence

Cells were plated on 11-mm glass coverslips in either 24-well or 4-well plates, grown for 2–5 d until cells were fully spread, and the desired degree of confluency was obtained. Cells were subjected to heat shock by floating the plate in a 44°C pan of water in a CO$_2$ incubator for times indicated in the figure legends, and immediately fixed. UV treatment was administered by removing the media from the cells, placing the plate in a Stratolinker, exposing the cells to 100 mJoules, and then immediately adding back media to the wells. Control cells were mock-treated (e.g., me-

dia removed without irradiation) and found to be unaffected. For immunofluorescence, a wide range of fixation conditions were tested to determine the optimal conditions for each antibody used. Cells were briefly rinsed in PBS and incubated for 10 min in 2% paraformaldehyde in PBS. This was removed and the cells were immediately immersed in −20°C methanol for 10 min, rinsed in PBS, and incubated in blocking buffer (5% normal goat serum in PBS) for 1 h before the addition of primary antibodies. Primary antibodies were diluted (purified IgG from murine mAbs 3E6 and ML-29 were used at 2.5 μg/ml, anti-HA ascites fluid was used at 1/2,000, anti-T7 ascites fluid was used at 1/5,000, anti–ribosome P Ig was used at 1/5,000, anti-La autoimmune serum was used at 1/1,000, and anti-PABP (10E10) was used at 1/1,000), and cells were incubated in the diluted antibody for 1–12 h, washed several times in PBS, and incubated for 1 h in diluted isotype-specific secondary antibodies (1/200 for FITC-labeled secondary antibodies; 1/2,000 for Texas red–labeled secondary antibodies) in blocking solution supplemented with Hoechst dye 33258 at 50 ng/ml. Cells were washed three times for 10 min in PBS, mounted in vinol mounting media (Fukui et al., 1987), and viewed through a Nikon Eclipse 800 microscope equipped with epifluorescence optics and appropriate filters for detection of FITC, Texas red, or Hoechst dye. Controls were routinely performed to insure the isotype specificity of each secondary antibody used, and to monitor that possible spillover between channels did not occur. Specimens were photographed using either T-max 400 film (Kodak) or Fujichrome 400 film, scanned into Adobe Photoshop, and compiled.

In situ hybridization to detect poly(A)$^+$ RNA was performed essentially as described previously (Spector et al., 1998), with a slight modification to allow double staining for TIAR/TIA-1. In brief, cells were fixed in 2% paraformaldehyde in PBS for 10 min, permeabilized in −20°C methanol for 10 min, and washed twice in 2× SSC. Hybridization was performed in a humid chamber at 43°C, using a biotinylated oligo-dT probe (50-mer) for 4–16 h. Cells were washed three times with 2× SSC and incubated in a 1:2,000 dilution of Alexa 488-labeled streptavidin (Molecular Probes) in 4× SSC containing 0.1% Triton X-100 for 45 min at room temperature. Cells were washed in 4× SSC, and then probed with anti–TIAR/TIA-1 antibody (mAb 3E6, which recognizes both TIAR and TIA-1) in 2× SSC containing 0.1% TX-100 and also containing biotinylated goat anti-streptavidin (1/2,000 dilution; Vector Labs) to amplify the biotinylated dT signal. After a 1-h incubation, cells were again washed in 4× SSC and subjected to a final incubation with a solution containing 1/2,000 dilution of goat anti–mouse IgG Texas red (Southern Biotechnology Associates), 1/2,000 streptavidin Alexa 488, and 0.5 μg/ml Hoechst dye in 2× SSC/0.1% Triton X-100. After 1 h, cells were sequentially washed in 4× SSC and 2× SSC, and mounted. Similar results for poly(A)$^+$RNA were obtained when cells were treated for in situ without counterstaining with anti–TIA-1/R. Controls included cells mock-hybridized in the absence of probe or cells pretreated with NaOH to hydrolyze RNA.

Plasmid Constructions and Transfections

Full-length TIA-1 subcloned into pMT2 was described previously (Tian et al., 1991). TIA-1ΔRRM was constructed using a PCR strategy. The COOH-terminal region of TIA-1 spanning met219 through the last amino acid (glutamine 386) was amplified from pMT2-TIA-1 for 25 cycles (94°C for 1 min, 50°C for 1 min, and 74°C for 1 min) using Tli polymerase (Promega Corp.) and primers with EcoRI and XbaI cloning sites (GGG-AATTCATGCGTCAGACTTTTTCACCA and GCTCTAGATTCA-CTGGGGTTTCATACCCTGC), respectively. The insert was cut with EcoRI and XbaI and cloned in-frame with a hemagglutinin (HA) tag in pMT2 that was similarly cut. The final clone was verified by sequencing.

Full-length pMT2-HA-TIA-1, pMT2-HA-TIA-1ΔRRM, and T7-tagged hnRNPA1 cloned into the pCGT7-A1 vector (Caceres et al., 1994) (a gift from Dr. Adrian Krainer, Cold Spring Harbor Laboratory) were transiently transfected into COS cells using SuperFect according to the manufacturer's recommendations. After 24–48 h, cells were cultured in the absence or presence of arsenite (0.5 mM, 30 min) before processing for immunofluorescence microscopy using mAbs reactive with either TIA-1 (mAb ML29) or the T7 tag. Plasmids pETF-eIF-2α, pETF-eIF-2-αS51A, pETF-eIF-2-αS51D, and pETF vector were provided by Dr. Randal Kaufman (University of Michigan, Ann Arbor, MI).

Transient Transfection Assay for SG Formation

The effects of wild-type and mutant eIF-2α on spontaneous and arsenite-induced SG formation were determined by cotransfecting COS cells with

a reporter plasmid (pCDNA3-β-galactosidase) and the pETF vector encoding wild-type, mutant eIF-2α, or no insert. COS cells were transfected in 6-well plates using SuperFect according to the manufacturer's directions. Immediately after transfection, the cells were trypsinized and divided among several wells of 24-well plates to insure constant transfection efficiency of parallel samples. Cells were allowed to grow for 24–48 h, processed for fluorescence microscopy, and SDS-PAGE. SG formation was assessed using two-color staining for β-galactosidase and TIA-1 using mAb ML-29. Cells expressing β-galactosidase were counted and scored for SG formation. At least 100 transfected cells were scored per well, and the results of three independent experiments were averaged to produce the data shown in Fig. 7. Samples processed for SDS-PAGE were harvested by trypsin and lysed in SDS-PAGE buffer. Equal amounts of total protein were resolved on SDS-PAGE, blotted onto nitrocellulose, and probed with anti–β-galactosidase (1/2,000) and anti–eIF-2α (1/1,000).

Results

TIA-1 and TIAR Colocalize at Stress Granules in Response to Stress

Although TIA-1 and TIAR are concentrated in the nuclei of the DU145 human prostate cell line (Fig. 1, A and B), both proteins rapidly accumulate in the cytoplasm in response to mild heat shock (20 min, 44°C; Fig. 1, D and F). In the cytoplasm, TIA-1 and TIAR colocalize at discrete phase dense particles (Fig. 1, D–F, arrows), which are similar to the SGs that form in heat shocked plant and animal cells (Nover et al., 1983; Collier and Schlesinger, 1986; Arrigo et al., 1988; Collier et al., 1988; Scharf et al., 1998; Nover et al., 1989). In addition, TIA-1/R$^+$ SGs are also formed in cells treated with osmotic shock (1 M sorbitol for 60 min, followed by a 30-min recovery in isotonic media), UV irradiation (data not shown) and sodium arsenite (a potent inducer of oxidative stress). Stimuli that do not induce the formation of SGs in DU145 or HeLa cells include: serum starvation, inhibitors of RNA polymerases (actinomycin D, α-amanitin, or DRB), microtubule or actin depolymerizing agents (nocodazole or cytochalasin B), and inflammatory cytokines (TNF-α) or antibody-mediated Fas ligation (data not shown).

The cytoplasmic accumulation of TIA-1 and TIAR in response to certain stress stimuli could result from inactivation of the cytoplasm-to-nucleus arm of the shuttling machinery. As such, SGs might be sites at which many shuttling proteins accumulate in a general response to stress. We tested this possibility by comparing the subcellular localization of hnRNP A1, an RNA-binding protein that shuttles between the nucleus and the cytoplasm (Pinol-Roma and Dreyfuss, 1992), before and during the application of stress. We used arsenite rather than heat shock in these and subsequent experiments (see Figs. 4 and 5) to allow the delivery of graded doses of stress (Brostrom and Brostrom, 1998). Recombinant TIA-1 and T7-tagged recombinant hnRNP A1 were transiently transfected into COS cells using SuperFect. After 24 h, cells were cultured in the absence (Fig. 2, A–C) or presence (Fig. 2, D–F) of arsenite (0.5 mM, 30 min) before processing for immunofluorescence microscopy. Whereas arsenite-induced oxidative stress results in the accumulation of recombinant TIA-1 at SGs (Fig. 2 D), it does not result in the accumulation of recombinant hnRNP A1 at SGs (Fig. 2 E). The stress-induced aggregation of endogenous TIA-1/R (Fig. 2 F, showing the same field as in E) in the hnRNP A1–transfected cells confirms that SGs formed under these conditions specifically exclude hnRNP A1. These results indicate that accumulation at SGs is not a general property of RNA-binding proteins that shuttle between the nucleus and cytoplasm. Additional proteins tested and found not to accumulate at SGs during heat shock include the following: the RNA-binding proteins, La (Meerovitch et al., 1993) and nmt-55/p54^{nrb} (Traish et al., 1997), the major vault protein, ubiquitin-conjugating enzyme UBC9, focal adhesion kinase, the adaptor protein shc, actin, tubulin, vi-
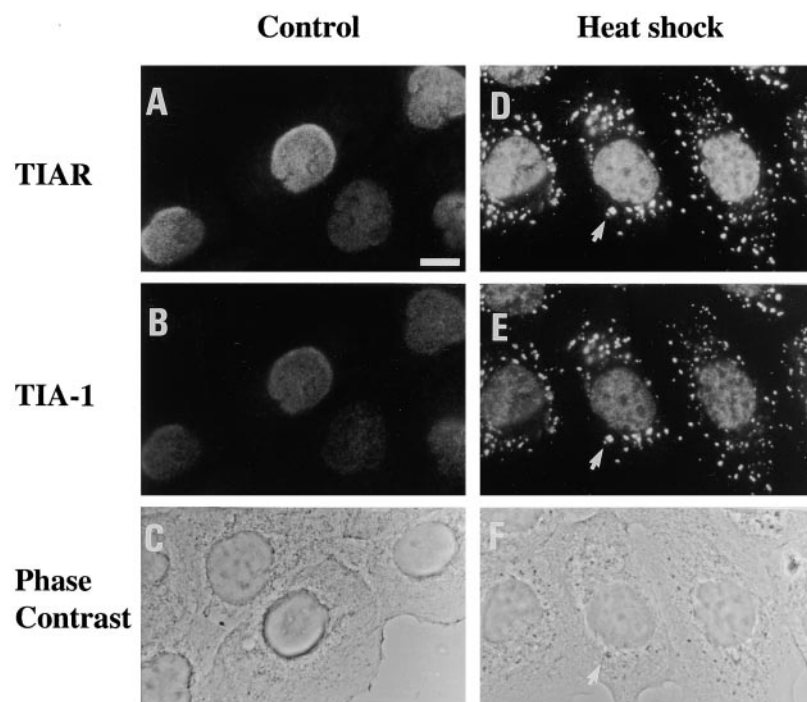

Control　　**Heat shock**

TIAR

TIA-1

Phase Contrast

Figure 1. TIA-1 and TIAR coaggregate at SGs. DU 145 cells were untreated (A–C, same field) or exposed to mild heat shock (44°C for 20 min, D–F, same field). Cells were immediately fixed and processed for two-color immunofluorescence using the TIAR-specific antibody 6E3 (A and D) and the TIA-1–specific antibody ML29 (B and E), and photographed separately. TIA-1 and TIAR colocalize at SGs (arrows), which are also visible by phase-contrast microscopy (C and F). Bar, 10 μm.
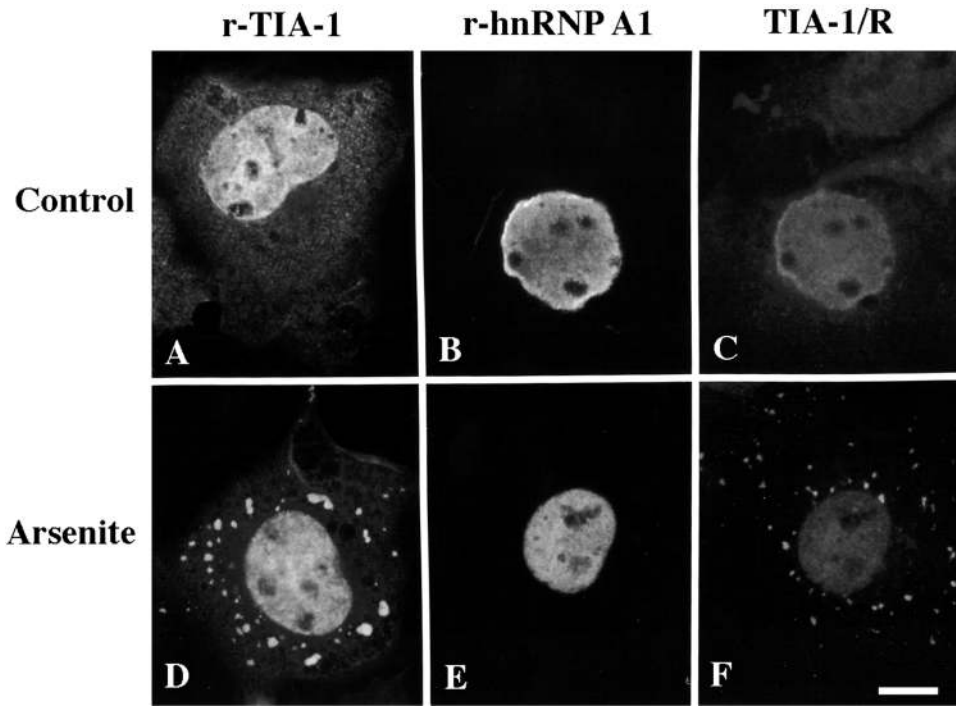
r-TIA-1   r-hnRNP A1   TIA-1/R

Control

A   B   C

Arsenite

D   E   F

Figure 2. Recombinant TIA-1, but not hnRNP A1, accumulates at SGs in arsenite-treated COS transfectants. COS cells were transfected with plasmid vectors encoding recombinant TIA-1 (A and D) or T7-tagged recombinant hnRNP A1 (B, E, C, and F) using Superfect. After 24 h, cells were cultured in the absence (A–C) or presence (D–F) of arsenite (0.5 mM, 30 min) before processing for visualization of recombinant proteins using mAbs reactive with TIA-1 (anti-3E6, the level of expression of recombinant TIA-1 greatly exceeds that of endogenous TIA-1/R, allowing the identification of transfected cells; A and D), T7 (B and E) or endogenous TIA-1/R (C and F, anti-3E6). Paired fields (B and C and E and F) are shown. Bar, 10 μm.

mentin, keratin, and protein phosphatase PP2A (data not shown). In addition, markers for various membrane-bound compartments including the ER (BIP, calreticulin, and calnexin), the Golgi apparatus (lectin vinca villosa), and the mitochondria (MitoTracker dye) showed no consistent association with SGs, suggesting that SGs are free, self-assembling structures rather than arising from a preexisting membranous or cytoskeletal structure.

HSP27, Poly(A)⁺ RNA, and PABP-I Are Components of SGs

Low molecular weight heat shock proteins are prominent components of SGs in both plant and animal cells (Arrigo et al., 1988; Collier et al., 1988; Scharf et al., 1998). To clarify the relationship between TIA-1/R$^+$, tomato, and mammalian SGs, we used immunofluorescence microscopy to compare the subcellular localization of TIA-1/R and HSP27 in DU145 cells before and after heat shock. Whereas TIA-1/R are concentrated in the nucleus in the absence of stress (Fig. 3 A), heat shock induces the formation of TIA-1/R$^+$ SGs (Fig. 3 B). As previously reported (Arrigo et al., 1988; Loktionova et al., 1996), heat shock induces the translocation of HSP27 from the cytoplasm to the nucleus (Fig. 3, compare C with D). A subpopulation of the HSP27 that remains in the cytoplasm coaggregates with TIA-1/R at SGs (Fig. 3 D). Whereas virtually all cytoplasmic TIA-1/R associates with SGs
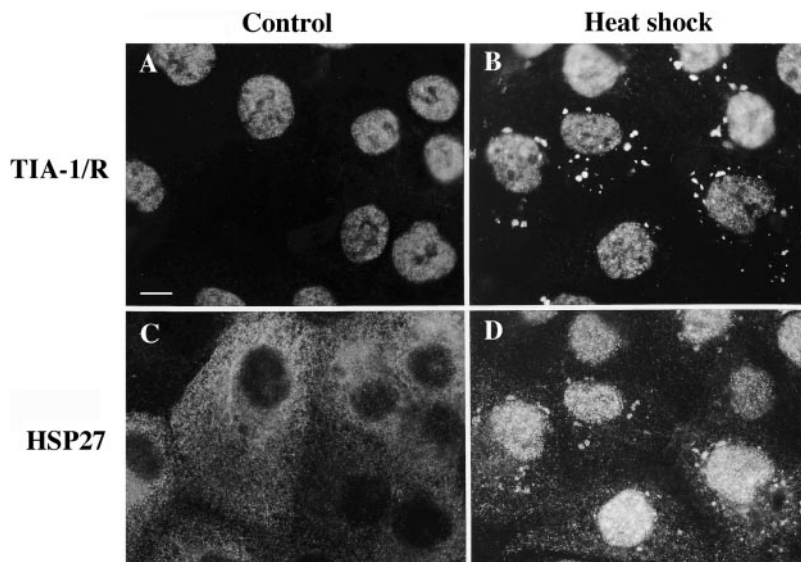

Control   Heat shock

A   B

TIA-1/R

C   D

HSP27

Figure 3. Recruitment of HSP27 to SGs. DU145 cells were untreated (A and C) or exposed to heat shock (45°C for 20 min, B and D) and stained for both TIA-1/R (A and B, anti-3E6) and HSP27 (C and D). Bar, 10 μm.

formed in response to many different stresses, little or no HSP27 associates with SGs formed in response to arsenite or UV irradiation (data not shown). These results suggest that HSP27$^+$ SGs constitute a subset of TIA-1/R$^+$ SGs, and argue against a direct role for HSP27 in nucleating SG formation.

In cultured tomato cells, SGs are sites at which untranslated mRNA accumulates in response to stress (Nover et al., 1989). Therefore, we used in situ hybridization to compare the subcellular localization of poly(A)$^+$ RNA and TIA-1/R in DU145 cells before and after arsenite-induced oxidative stress. In untreated cells, both poly(A)$^+$ RNA (Fig. 4 A) and TIA-1/R (Fig. 4 D) are distributed between the nucleus and the cytoplasm. After exposure to a sublethal concentration of arsenite (0.5 mM) for 1 h (Fig. 4 B), poly(A)$^+$ RNA accumulates at discrete cytoplasmic aggregates that are coincident with TIA-1/R$^+$ SGs (Fig. 4 E). When arsenite-treated cells are allowed to recover for 3 h in arsenite-free media, aggregates of poly(A)$^+$ RNA (Fig. 4 C) and TIA-1/R (Fig. 4 F) coincidentally disappear. No signal was observed in cells processed for in situ hybridization in the absence of oligo-dT or in cells stained with an isotype-matched control mAb (data not shown). In situ hybridization revealed that poly(A)$^+$ RNA also accumulates at SGs formed in response to sublethal heat shock (data not shown).

In contrast to the reversible recruitment of poly(A)$^+$ RNA to TIA-1/R$^+$ SGs in cells treated with a sublethal dose of arsenite, cells exposed to a lethal dose of arsenite exhibit SGs that persist until the apoptotic death of the cell. Fig. 5 shows the colocalization of poly(A)$^+$ RNA

(Fig. 5 A) and TIA-1/R (Fig. 5 D) at SGs induced by arsenite (2 mM, 1 h). After 3 h in the absence of arsenite, poly(A)$^+$ RNA (Fig. 5 B) and TIA-1/R (Fig. 5 E) remain at SGs. At this time, Hoechst staining reveals nuclear DNA condensation characteristic of apoptosis (Fig. 5 C), whereas the nuclei of untreated cells appear normal (Fig. 5 F). Thus, the persistence of SGs correlates with the level of stress. We used antibodies reactive with ribosomal P antigen to confirm that the large ribosomal subunit is not a component of SGs, consistent with the idea that these particles are sites at which untranslated mRNAs accumulate in stressed cells (data not shown).

The identification of poly(A)$^+$ RNA as a component of SGs suggested that proteins known to associate with poly(A)$^+$ RNA might also be components of SGs. Therefore, we compared the subcellular localization of PABP-I in DU145 cells in the absence or presence of arsenite. As expected, TIA-1/R resides in the nucleus (Fig. 6 A), whereas PABP-I resides in the cytoplasm (Fig. 6 C, same field as A) in unstressed cells. In response to arsenite-induced oxidative stress, both TIA-1/R (Fig. 6 B) and PABP-I (Fig. 6 D, same field as B) coaggregate at SGs. Whereas the cytoplasmic aggregation of TIA-1/R is nearly quantitative, a significant amount of PABP-I is excluded from these cytoplasmic aggregates, consistent with its association with mRNAs that continue to be translated during stress (i.e., heat shock mRNAs). Sucrose gradient fractionation (data not shown) was performed on both stressed and unstressed cells, and confirmed that PABP-I exhibited a shift from large polysomes in unstressed cells to monosomes and small polysomes in stressed cells, while
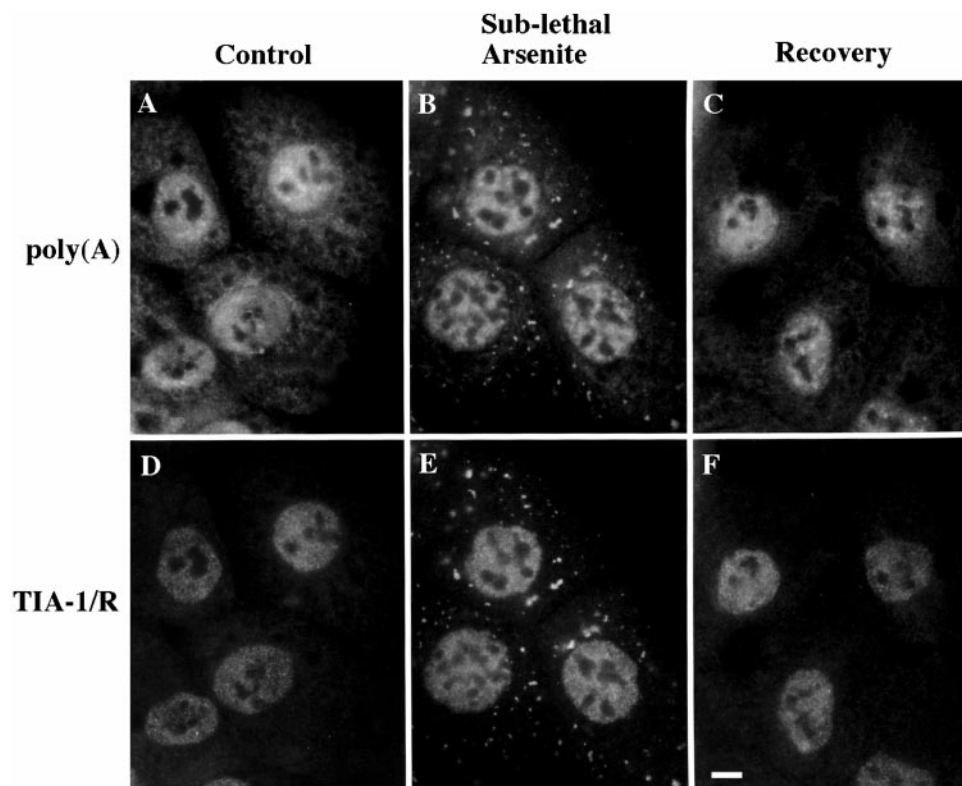

Figure 4. TIA-1/R and poly(A)$^+$ RNA coaggregate at SGs. Untreated (A and D), arsenite-treated and immediately fixed (0.5 mM for 1 h, B and E), or arsenite-treated as in B and E and allowed to recover in arsenite-free media for 3 h (C and F) DU145s were processed for in situ immunofluorescence to detect poly(A) (A–C), and counterstained for TIA-1/R (D–F, anti-3E6). Paired fields (A and D, B and F, C and F) are shown. Bar, 10 μm.
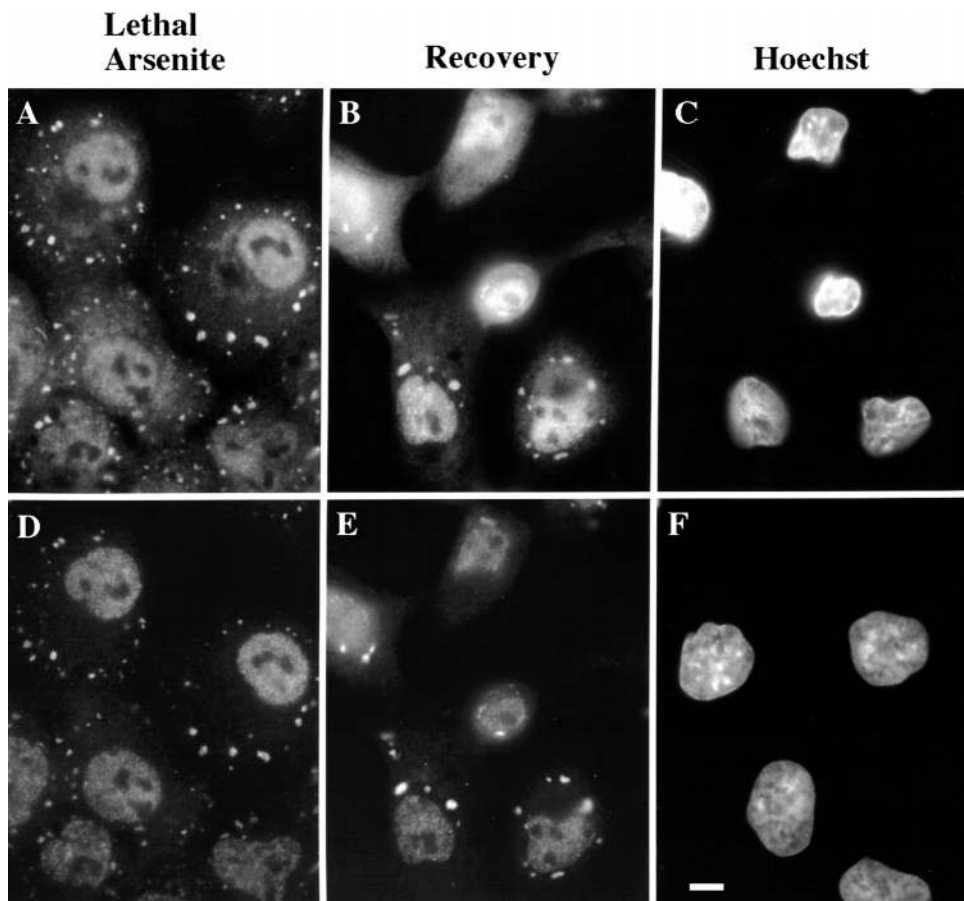
Figure 5. Lethal stress induces the irreversible assembly of SGs. DU145 cells were cultured in the absence (F) or presence (A–E) of arsenite (2 mM, 1 h), immediately fixed (A and D) or allowed to recover for 3 h in the absence of arsenite (B, C, and E) before processing for visualization of poly(A)$^+$ RNA (A and B), TIA-1/R (D and E), or DNA (C and F, Hoechst dye). Bar, 10 μm.

both TIAR and TIA-1 were found in the light mRNP fractions smaller than monosomes in both stressed and unstressed cells.

Phosphorylation of eIF-2α Is Necessary and Sufficient for the Assembly of TIA-1/R$^+$ SGs

The rapid assembly and disassembly of poly(A)$^+$ RNA–containing SGs in DU145 cells subjected to arsenite treat-ment or heat shock suggests that SGs are sites at which untranslated mRNAs accumulate in response to stress. The primary determinant of stress-induced translational arrest is the phosphorylation of eIF-2α that prevents the assembly of the 43S preinitiation complex. Therefore, we monitored the assembly of SGs in COS transfectants expressing wild-type or mutant eIF-2α. As shown in Fig. 7 A, COS cells were cotransfected with cDNAs encoding β-galactosidase (as a marker of transfected cells) and either vec-
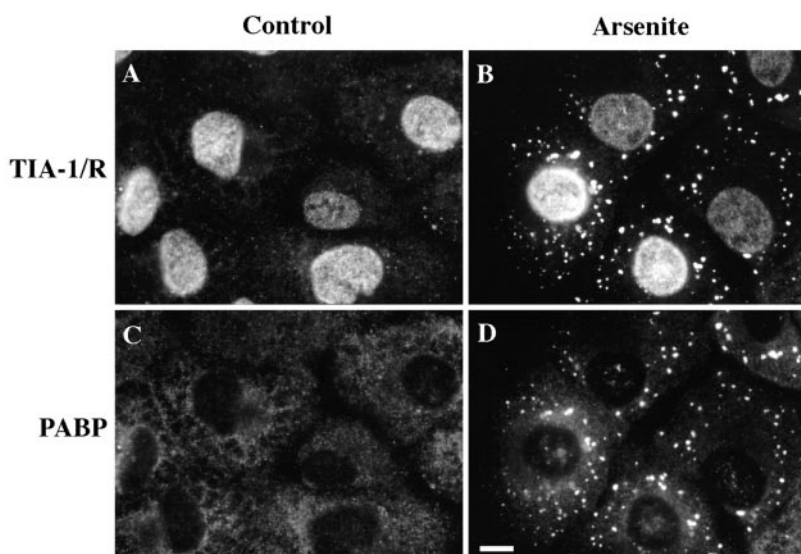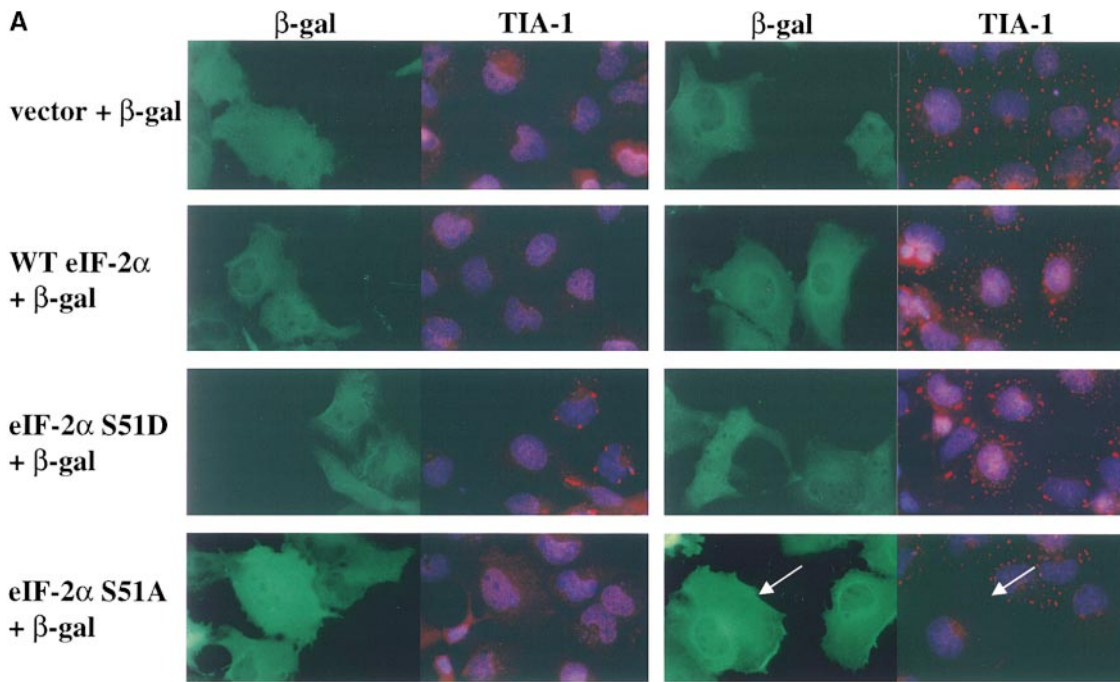

Figure 6. PABP-I is a component of SGs. Control (A and C) or arsenite-treated (0.5 mM for 40 min, B and D) DU145 cells were fixed, permeabilized, and processed for two-color immunofluorescence using antibodies to TIA-1/R (A and B, anti-3E6) or PABP-I (C and D). Both TIA-1/R and PABP-I are clearly recruited to the SGs upon stress (B and D, paired views of same field). Bar, 10 μm.

A

	β-gal	TIA-1	β-gal	TIA-1
vector + β-gal				
WT eIF-2α + β-gal				
eIF-2α S51D + β-gal				
eIF-2α S51A + β-gal				

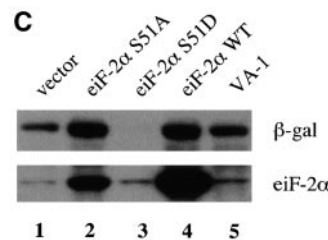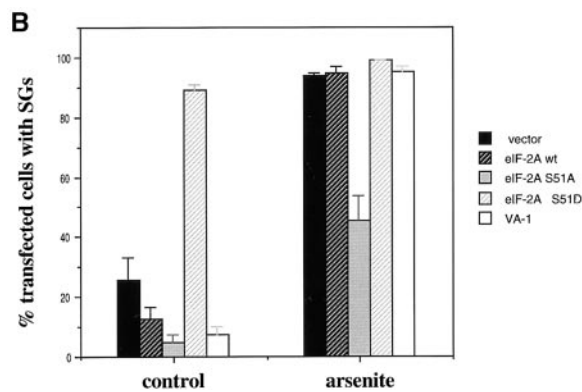Control   Arsenite

B

C

Figure 7. Effect of wild-type and mutant recombinant eIF-2α on the assembly of TIA-1/R$^+$ SGs. (A) COS cells were transiently cotransfected with plasmids encoding β-galactosidase and either vector alone, wild-type eIF-2α, eIF-2α (S51D), or eIF-2α (S51A) as indicated at the left of the figure. After 48 h, cells were cultured in the absence (Control, left paired panels) or presence (Arsenite, right paired panels) of arsenite (1 mM, 1 h) before fixation and processing for two-color immunofluorescence microscopy using mAbs specific for β-galactosidase (green, anti–β-galactosidase) or TIA-1 (red, TIA-1–specific antibody ML-29). In each case, paired views of the same field are presented to allow the identification of transfected and untransfected cells. Arrows in the bottom right panel point out transfected cells in which arsenite-induced assembly of SGs is prevented by the expression of eIF-2α (S51A). (B) Phosphorylation of eIF-2α is required for SG formation. Transiently transfected COS cells were treated and stained as described and were scored (at least 100 transfected cells per treatment) for the presence or absense of TIAR/1$^+$ SGs as detected with mAb 3E6, and expressed as a percentage of the total cells scored per treatment. The results of 3 independent experiments were averaged. (C) Effects of mutant eIF-2α on expression of β-galactosidase. COS cells were transiently transfected with the indicated plasmids and total cell lysates were analyzed by Western blot. Upper panel, β-galactosidase; lower panel, eIF-2α.

tor alone, wild-type eIF-2α, or mutant eIF-2α, and then processed for two-color immunofluorescence microscopy to visualize β-galactosidase (green) and TIA-1 (red). In the absence of arsenite, cells transfected with the S51D phosphomimetic eIF-2α mutant formed TIA-1/TIAR$^+$ SGs, whereas most cells transfected with vector alone, wild-type eIF-2α or the S51A nonphosphorylatable eIF-2α mutant did not (Fig. 7 A, left). In situ hybridization confirmed that the SGs assembled in S51D-transfected cells include poly(A)$^+$ RNA (data not shown). The induction of SGs by S51D correlates with the ability of the S51D mutant to inhibit protein translation of cotransfected β-galac-

tosidase as seen by immunofluorescence (β-galactosidase staining in the S51D cells was appreciably dimmer) and confirmed by Western blot (see below). When transfected cells were treated with arsenite, S51A nonphosphorylatable mutant eIF-2α prevented the formation of TIA-1/TIAR$^+$ SGs (arrows, right), whereas wild-type and S51D mutants did not (Fig. 7 A, right).

The percentage of transfected cells containing TIA-1/R$^+$ SGs (averaged over three independent experiments) is presented in Fig. 7 B. Cells transfected with the reporter β-galactosidase plasmid and empty pETF vector displayed SGs in 25% of the transfected COS cells, even without ex-

posing the cells to arsenite (Fig. 7 B). In cells transfected with wild-type eIF2-α or the nonphosphorylatable eIF2-α mutant S51A, the percentage of spontaneous SGs dropped to <13% and 5%, respectively. We reasoned that the presence of spontaneous SGs in the cells transfected with vector alone could be due to phosphorylation of endogenous eIF-2α by PKR, which phosphorylates eIF-2α in response to dsRNA and is activated by transfection with many plasmids (Davies et al., 1989). To test this hypothesis, we cotransfected a plasmid encoding VA-1 (designated pAdvantage), an adenoviral pol III RNA that inhibits PKR and is used to enhance the translational efficiency of transfected plasmids (Davies et al., 1989). Cotransfection with VA-1 reduced the transfection-induced assembly of SGs to 8% from 25% (Fig. 7 B, column 5), but did not prevent SG formation when the cells were subjected to arsenite treatment, suggesting that kinases other than PKR are likely to phosphorylate eIF-2-α in response to arsenite treatment.

In addition to serving as a marker of transfected cells, the expression of β-galactosidase was quantified by Western blot analysis (Fig. 7 C, top) to assess the degree of translational enhancement or repression mediated by the cotransfected wild-type and mutant forms of eIF-2α and VA-1 RNA. The amount of β-galactosidase obtained was significantly increased when either mutant S51A (Fig. 7 C, lane 2) or wild-type (Fig. 7 C, lane 4) eIF-2α was cotransfected relative to the amount obtained with cotransfected empty vector (Fig. 7 C, lane 1). The cotransfection of the PKR inhibitor VA-1 RNA (Fig. 7 C, lane 5) modestly increased β-galactosidase expression relative to the vector control. In contrast, cotransfection of S51D mutant eIF-2α reduced the expression of β-galactosidase to undetectable levels by Western blot (Fig. 7 C, top, lane 3), although protein was detectable in individual cells by immunofluorescence. In addition to repressing the expression of β-galactosidase, S51D mutant eIF-2α dramatically reduced its own expression (Fig. 7 C, bottom, lane 3), although it was weakly detectable in individual cells by immunofluorescence (data not shown). Taken together, the data indicate that the phosphorylation of eIF-2α induces SGs and reduces expression of transfected proteins.

Mutant TIA-1 Prevents the Recruitment of Poly(A)$^{+}$ RNA to SGs

The potential for TIA-1 to directly promote the assembly of SGs was determined by comparing the localization of TIA-1/R and poly(A)$^{+}$ RNA in COS cells expressing HA-tagged recombinant TIA-1ΔRRM, a truncation mutant that is incapable of binding RNA (Tian et al., 1991), in the absence or presence of arsenite. Recombinant wild-type TIA-1 is distributed between the nucleus and the cytoplasm in a distribution similar to the endogenous protein and accumulates at SGs in cells treated with arsenite (Fig. 2). In contrast, recombinant HA-TIA-1ΔRRM is concentrated in the cytoplasm, where it forms homogeneous microaggregates (Fig. 8 A). In situ hybridization reveals that the distribution of poly(A)$^{+}$ RNA is similar in transfected and untransfected cells (Fig. 8 C, arrows point to transfected cells). In cells subjected to arsenite-induced stress, the distribution of recombinant HA-TIA-1ΔRRM is not significantly altered (Fig. 8 B). Whereas poly(A)$^{+}$ RNA accumulates at SGs in untransfected cells (Fig. 8 D), SG formation is prevented in cells transfected with HA-TIA-1ΔRRM (Fig. 8, B and D, paired views of the same field). The aggregation of endogenous TIAR (revealed using mAb 6E3, which recognizes an epitope within the RRM domains deleted in the mutant construct) is also prevented in cells transfected with HA-TIA-1ΔRRM (Fig. 8, E and F, paired views of the same field, arrows point to transfected cells). Even in the absence of stress, the expression of HA-TIA-1ΔRRM coaggregates all detectable full-length endogenous TIAR and TIA-1 into the cytoplasmic microaggregates. These results indicate that TIA-1ΔRRM blocks both the recruitment of endogenous TIAR/1 and of poly(A)$^{+}$ RNA to SGs in response to arsenite, thereby acting as a transdominant inhibitor of SG formation.

Discussion

Heat stress granules are phase-dense particles that appear in the cytoplasm of plant (Nover et al., 1983, 1989; Scharf et al., 1998) and animal (Collier and Schlesinger, 1986; Arrigo et al., 1988; Collier et al., 1988) cells exposed to supra-
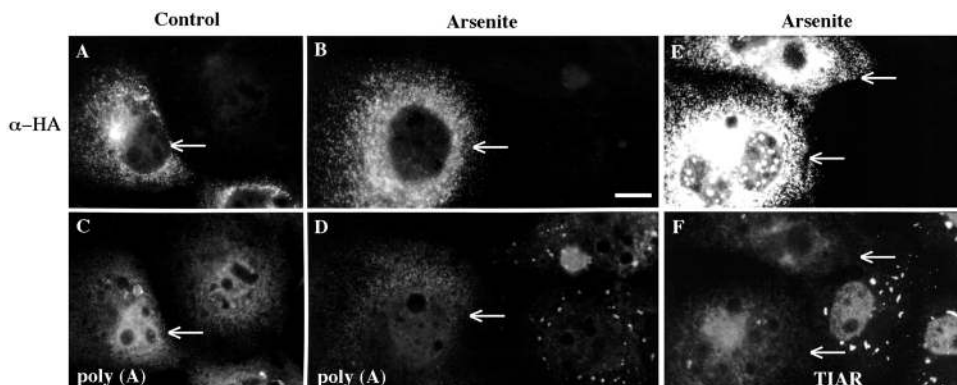

Figure 8. Effect of HA-TIA-1ΔRRM on arsenite-induced assembly of SGs. COS cells were transiently transfected with recombinant HA-TIA-1ΔRRM, allowed to express protein for 48 h, and either untreated (A and C, paired views of same fields) or treated (B and D–F, paired views of same fields) with 0.5 mM sodium arsenite for 1 h before fixation and double staining for poly(A)$^{+}$ RNA by in situ hybridization (C and D), and either HA-TIA-1ΔRRM (A, B, E) or endogenous TIAR (F) by immunofluorescence microscopy using mAbs reactive with the HA tag (A, B, and E) or endogenous TIAR (6E3). Arrows point out transfected cells. Bar, 10 μm.

ambient temperatures. In cultured Peruvian tomato cells, SGs have been shown to harbor untranslated mRNAs that accumulate as a consequence of stress-induced translational arrest (Nover et al., 1989; Scharf et al., 1998). We have used in situ hybridization to show that poly(A)$^+$ RNA is also a component of mammalian SGs formed in response to a variety of environmental stresses (i.e., heat, UV, hyperosmolarity, arsenite). Intact ribosomes are not components of poly(A)$^+$ RNA–containing SGs, which is consistent with the hypothesis that mammalian SGs, like their plant counterparts, harbor untranslated mRNAs. The phase-dense cytoplasmic aggregates formed in heat shocked HeLa cells have been reported to contain HSP27 (Collier and Schlesinger, 1986; Arrigo et al., 1988; Collier et al., 1988). We have confirmed that HSP27 is a component of SGs formed in heat shocked DU145 cells. Surprisingly, HSP27 is not a component of SGs formed in arsenite-treated or UV-irradiated DU145 cells, indicating that HSP27 is not a general component of all SGs (operationally defined as the cytoplasmic foci at which untranslated mRNAs accumulate in response to stress). In addition to HSP27, we have identified the RNA-binding proteins TIA-1, TIAR, and PABP-I as components of SGs. TIA-1 and TIAR are invariant components of (poly(A)$^+$RNA–containing)-SGs formed by diverse stimuli in every cell type examined (i.e., DU145, MCF7, COS, NIH3T3, primary fibroblasts, HeLa, C6 glimoma, CaOV3, SW1573, and rat-1 cells; Kedersha, N., and P. Anderson, unpublished results), suggesting that these proteins are core components of SGs that might recruit untranslated mRNAs to these cytoplasmic foci in response to many stimuli other than heat shock.

Our results redefine mammalian SGs as RNP aggregates or cytoplasmic foci at which untranslated mRNAs are actively sequestered by TIA-1/TIAR in response to the stress-induced phosphorylation of eIF-2α. This expanded definition includes the SGs induced by heat shock that include low molecular weight HSPs as well as SGs induced by arsenite and UV irradiation, which do not include low molecular weight HSPs as major components. By this definition, SGs are discrete cytoplasmic foci that arise as a result of altered RNA metabolism. Many nuclear structures such as nuclear speckles, nuclear dots, coiled bodies, GEMS, and nucleoli constitute prominent nuclear substructures whose morphology is a byproduct of RNA metabolism (Spector, 1993). The SG appears to be an analogous cytoplasmic structure whose morphology is dependent on events downstream of the stress-induced phosphorylation of eIF-2α. A family of stress-activated eIF-2α kinases appears to monitor different types of cellular stress. Whereas PKR is a double-stranded RNA-dependent kinase that is activated by viral infection and selected environmental stresses (Williams, 1997), PERK/PEK is a resident ER protein that is activated by changes in glycoprotein metabolism (Shi et al., 1998; Harding et al., 1999). A vertebrate orthologue of yeast, GCN2 kinase has been proposed to regulate the cellular response to amino acid starvation (Olsen et al., 1998). Finally, a heme-regulated eIF-2α kinase that ensures the balanced synthesis of globin chains and heme during erythrocyte maturation might also monitor the stress response in nonerythroid cells (Berlanga et al., 1998). Whereas VA-1, an adenoviral

RNA that specifically inactivates PKR (Mathews and Shenk, 1991), inhibits transfection-induced assembly of SGs, it does not inhibit arsenite-induced assembly of SGs. This result suggests that PKR, and at least one other eIF-2α kinase can regulate the assembly of SGs. PERK/PEK is not significantly activated by arsenite (Harding et al., 1999), suggesting that heme-regulated eIF-2α kinase, GCN2, or an unidentified eIF-2α kinase is responsible for the VA-1–resistant assembly of SGs in cells treated with arsenite. It will be important to compare the assembly of SGs in response to environmental stresses that target different eIF-2α kinases.

The ability of TIA-1ΔRRM to function as a transdominant inhibitor of the arsenite-induced recruitment of poly(A)$^+$ RNA to SGs implicates TIA-1 (and possibly TIAR) as an effector of SG assembly. The two functional domains of TIA-1 (and TIAR) confer the requisite properties required for the assembly of SGs: self-aggregation and RNA binding (Dember et al., 1996). The COOH terminus of TIA-1 (i.e., TIA-1ΔRRM) is structurally related to both high molecular weight glutenin (27% amino acid sequence identity over the last 90 amino acids of TIA-1) (Shani et al., 1994), and prion protein (38% amino acid identity over 50 amino acids with the introduction of one gap), both of which are glutamine-rich proteins prone to self-aggregation (Prusiner, 1989; Prusiner et al., 1990). Recombinant TIA-1ΔRRM forms cytoplasmic microaggregates when overexpressed in COS cells, indicating that this prion-related domain can self-aggregate in vivo. Moreover, these microaggregates recruit endogenous TIA-1 and TIAR in the absense of stress (data not shown), and prevent the relocalization of TIA-1, TIAR, and poly(A)$^+$ RNA into SGs in response to arsenite-induced oxidative stress. By trapping endogenous TIA-1/R in TIA-1ΔRRM microaggregates, it is likely that the mutant blocks the recruitment of poly(A)$^+$ RNA to SGs by disrupting the normal shuttling of TIA-1/R, thereby preventing its association with nuclear mRNA transcripts.

In addition to promoting the assembly of SGs, other studies suggest that TIA-1 and TIAR contribute to the translational arrest of specific mRNAs. Recently, TIAR has been shown to be a component of a translational repressor complex that binds to an AU-rich element in the 3′ untranslated region of TNF-α transcripts (Gueydan et al., 1999). In resting macrophages, TNF-α transcripts are abundantly expressed, but excluded from polysomes (Crawford et al., 1997; Han et al., 1990). In response to lipopolysaccharide, TNF-α transcripts move to polysomes, allowing the translation and secretion of TNF-α protein (Crawford et al., 1997). It remains to be determined whether posttranscriptional regulation of TNF-α occurs at the level of translational initiation, polyadenylation, or mRNA stability. Other recent results indicate that AU-rich element–binding proteins related to TIA-1 and TIAR can promote the assembly of cytoplasmic aggregates that might be sites of cytoplasmic deadenylation or readenylation (Antic and Keene, 1998; Ford et al., 1999). These results suggest that the ability of TIA-1 and TIAR to promote the aggregation, and/or translational repression, of mRNA may be part of a general translational control mechanism. It is likely that the assembly of microscopi-

cally visible SGs in response to stress constitutes an extreme example of this regulatory mechanism.

A role for SGs as effectors of stress-induced translational arrest could explain the imperfect correlation between phosphorylation of eIF-2α and translational arrest that exists in some experimental systems. Thus, translational arrest in heat shocked HeLa cells occurs in the absence of detectable phospho-eIF-2α (Duncan and Hershey, 1989). More remarkably, heat shock–induced phosphorylation of eIF-2α is transient under conditions in which translational arrest is persistent (DeBenedetti and Baglioni, 1986). These results suggest that phospho-eIF-2α is required for the initiation, but not the maintenance, of translation arrest. If dephosphorylation of eIF-2α restores the initiation machinery in the continued presence of stress, sequestration of mRNA at the SG may become a major determinant of translational arrest. Indeed, this mechanism might also explain in part how heat shock transcripts can be translated in stressed cells, inasmuch as heat shock transcripts are selectively excluded from heat SGs in tomato cells (Nover et al., 1989; Scharf et al., 1998). As we have now identified eIF-2α phosphorylation as the molecular event that initiates SG formation, and have more completely defined the composition and nature of the SG, the issue of whether mammalian SGs directly influence the duration and specificity of stress-induced translational arrest can be addressed in future studies.

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