Mammalian stress granules represent sites of accumulation of stalled translation initiation complexes

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Kimball, Scot R., Rick L. Horetsky, David Ron, Leonard S. Jefferson, and Heather P. Harding. Mammalian stress granules represent sites of accumulation of stalled translation initiation complexes. Am J Physiol Cell Physiol 284: C273–C284, 2003. First published October 3, 2002; 10.1152/ajpcell.00314.2002.—In eukaryotic cells subjected to environmental stress, untranslated mRNA accumulates in discrete cytoplasmic foci that have been termed stress granules. Recent studies have shown that in addition to mRNA, stress granules also contain 40S ribosomal subunits and various translation initiation factors, including the mRNA binding proteins eIF4E and eIF4G. However, eIF2, the protein that transfers initiator methionyl-tRNAi (Met-tRNAi) to the 40S ribosomal subunit, has not been detected in stress granules. This result is surprising because the eIF2-GTP-Met-tRNAi complex is thought to bind to the 40S ribosomal subunit before the eIF4G-eIF4F-mRNA complex. In the present study, we show in both NIH-3T3 cells and mouse embryo fibroblasts that stress granules contain not only eIF2 but also the guanine nucleotide exchange factor for eIF2, eIF2B. Moreover, we show that phosphorylation of the α-subunit of eIF2 is necessary and sufficient for stress granule formation during the unfolded protein response. Finally, we also show that stress granules contain many, if not all, of the components of the 48S preinitiation complex, but not 60S ribosomal subunits, suggesting that they represent stalled translation initiation complexes.

eIF4E; eIF4G; eIF3; unfolded protein response; PERK

ONE OF THE BEST-CHARACTERIZED mechanisms for regulating mRNA translation in eukaryotic cells involves phosphorylation of the α-subunit of eukaryotic initiation factor, eIF2 (reviewed in Ref. 16). During initiation, eIF2 forms a complex with GTP and initiator methionyl-tRNAi (met-tRNAi), and this ternary complex subsequently binds to the 40S ribosomal subunit to form the 43S preinitiation complex (reviewed in Refs. 10 and 24). Through the action of a translation initiation factor complex referred to as eIF4F, which is comprised of eIF4A, eIF4E, and eIF4G, mRNA is bound to the 43S preinitiation complex, resulting in formation of the 48S preinitiation complex (reviewed in Ref. 20). During a later step in initiation, the GDP bound to eIF2 is hydrolyzed in an eIF5-mediated process and initiation factors are released from the ribosome. Before binding Met-tRNAi and reforming the ternary complex, the GDP bound to eIF2 must be exchanged for GTP, a reaction that is catalyzed by the guanine nucleotide exchange factor, eIF2B. One mechanism for regulating the activity of eIF2B involves phosphorylation of the α-subunit of eIF2 on Ser51, an event that converts eIF2 from a substrate into a competitive inhibitor of eIF2B (reviewed in Ref. 10). Thus, by inhibiting eIF2B, phosphorylation of eIF2α results in a global inhibition of protein synthesis.

Hyperphosphorylation of eIF2α occurs under a variety of conditions that result in disruption of normal cell homeostasis. For example, conditions that impede correct folding of newly synthesized proteins in the lumen of the endoplasmic reticulum (ER), i.e., the so-called unfolded protein response, result in activation of the eIF2α kinase, PERK (also referred to as PEK) (28). PERK is a trans-ER membrane protein with a luminal domain that exhibits homology to IRE1 and a cytoplasmic domain homologous to other eIF2α kinases (9, 29). Accumulation of misfolded proteins in the lumen of the ER, such as occurs in response to thapsigargin treatment, promotes the release of the chaperone protein, BiP, from the IRE1 homology domain, allowing oligomerization of the protein and subsequent activation of the cytoplasmic kinase domain (2). The activated kinase then phosphorylates eIF2α, causing inhibition of eIF2B and a reduction in protein synthesis rate.

Recent studies by Kedersha and coworkers (14, 15) show that in cells treated with arsenite or subjected to heat shock, i.e., conditions that promote eIF2α phosphorylation, mRNA is sequestered into punctate cytoplasmic aggregates that are referred to as stress granules. Such structures have been observed in plant, yeast, and animal cells (5, 14, 15, 23), and it has been proposed that they represent sites where mRNA is targeted for either translation, degradation, or seques-
tration into untranslated mRNP complexes (15). In addition to mRNA, stress granules contain the RNA binding proteins TIA-1 and TIAR, poly(A) binding protein (PABP), eIF3, the mRNA cap binding protein eIF4E, eIF4G, and the 40S ribosomal proteins S3 and S19 (13). However, stress granules reportedly lack eIF2 and the 60S ribosomal proteins L5 and L37 (13). The lack of eIF2 in stress granules is surprising because binding of the eIF4G-eIF4E-mRNA complex to the 40S ribosomal subunit does not occur in the absence of eIF2 (25).

A recent study attempted to answer the question of whether or not phosphorylation of eIF2α is sufficient to induce stress granule formation by expression of a phosphomimetic variant of eIF2α, where Ser51 was changed to aspartic acid (eIF2αS51D) (15). Overexpression of the variant protein promoted stress granule formation. In contrast to the wild-type protein, some, but not all, stress granules were found to contain eIF2αS51D, although wild-type eIF2α was not found (13). However, in the study by Kedersha and coworkers (15), the extent to which the exogenously expressed eIF2αS51D was incorporated into three subunit eIF2 holoproteins was not assessed. In addition, the phosphorylation state of endogenous eIF2α in cells expressing eIF2αS51D was not examined in that study. Thus, the exogenously expressed α-subunit variant may not have been extensively incorporated into the eIF2 holoprotein but instead may have been acting through a mechanism distinct from that invoked by phosphorylation of the wild-type protein. The mechanism through which phosphorylation of eIF2α induces stress granule formation, therefore, remains obscure, and the question of whether or not eIF2α phosphorylation is sufficient to induce stress granule formation was incompletely answered.

In the present study, activation of PERK and phosphorylation of eIF2α were shown to be required for stress granule formation during the unfolded protein response. Moreover, in cells expressing the cytoplasmic PERK kinase domain as a fusion protein with the extracellular and transmembrane domains of the T-lymphocyte coreceptor CD4 (CD4-PERK) (2), activation of the cytoplasmic membrane-associated kinase stimulated both eIF2α phosphorylation and stress granule formation. Thus, phosphorylation of eIF2α is necessary and sufficient for stress granule formation during the unfolded protein response. In contrast to the results of Kedersha et al. (13), both eIF2 and eIF2B were associated with stress granules. Finally, in thapsigargin-treated cells, eIF2 colocalized in granules with other key regulatory translation initiation factors, poly(A)^+ RNA, and the 40S ribosomal proteins S3 and S6. In contrast, neither elongation factor eEF2 nor the 60S ribosomal proteins L5 or L37 accumulated in stress granules under any of the tested stress conditions. Overall, the results suggest that stress granules represent sites at which stalled initiation complexes accumulate.

**MATERIALS AND METHODS**

**Materials.** Cy2-conjugated AffiniPure donkey anti-mouse IgG (multiple labeling grade) and Cy3-conjugated AffiniPure donkey anti-rabbit IgG (multiple labeling grade) were purchased from Jackson ImmunoResearch Laboratories. The Cy2 MAb and Cy3 MAb labeling kits were obtained from Amersham Pharmacia Biotech. Affinity purified anti-eIF2α (pS51) antibody was purchased from Biosource International, and antiribosomal protein S6 and anti-eEF2 antibodies were purchased from Cell Signaling Technology. Antiribosomal protein S3, L5, and L37 antibodies and anti-TIA-1 antibody were kind gifts from Dr. Nancy L. Kedersha, Brigham and Women’s Hospital. Antibodies to the α-subunit of eIF2, the ϵ-subunit of eIF2B, eIF4E, and eIF4G were prepared in our laboratory.

**Cell culture.** Cells were plated on coverslips in a six-well dish at an initial density of 200,000 (NIH/3T3) or 100,000 [cell lines derived from SV40-transformed mouse embryonic fibroblasts (SVT-MEF)] cells/well. Two days later, coverslips were removed and cells were fixed and permeabilized as described below. Cells remaining in the well were harvested in SDS sample buffer at 90°C for measurement of eIF2α phosphorylation as described below.

**Measurement of protein synthesis.** Protein synthesis in NIH/3T3 or SVT-MEF cells was monitored by measuring the incorporation of [35S]methionine and [35S]cysteine into protein as described previously (17).

**Cell fixation and permeabilization.** Cells on coverslips were fixed in either 1% or 3% paraformaldehyde, as indicated in the figure legends, for 20 min at room temperature and the coverslips were washed twice with phosphate-buffered saline (PBS). The fixed cells were permeabilized by incubation in 0.2% Triton X-100 in PBS. The coverslips were then incubated for 1 h in 10% bovine serum albumin in PBS containing 0.1% Triton X-100.

**Microscopy.** After fixation and permeabilization, cells were incubated with primary antibody overnight at 4°C. For visualization of eIF2α or eIF2B, fixed, permeabilized cells were incubated with monoclonal anti-eIF2α or anti-eIF2B antibodies that had been covalently labeled with Cy2 or Cy3, respectively, using a kit (CyDye MAb labeling kit) from Amersham Pharmacia Biotech. For visualization of other proteins, coverslips were incubated with primary antibody followed by a 1-h incubation with a secondary antibody coupled to either Cy2 or Cy3, as noted in the figure legends. Phosphorylated eIF2α was visualized by incubation of permeabilized cells with an affinity-purified anti-phospho-eIF2α antibody (Biosource International) that had been covalently labeled with Cy3.5 using a CyDye kit. Coverslips were then mounted on glass slides using Aquamount. Slides were examined using a Nikon E-800 fluorescence microscope equipped with a Nikon PCM-2000 multilens laser confocal system equipped with argon ion and helium/neon lasers. All images were acquired using a Nikon 60X plan apo objective and were processed as TIFF files in Canvas (Deneba) using standard image processing techniques.

**Measurement of eIF2α phosphorylation.** Cells were maintained in culture as described above, with the exception that they were harvested by scraping in SDS sample buffer at 90°C. The relative amount of eIF2α in the phosphorylated form was quantitated by protein immunoblot analysis using an affinity-purified antibody that specifically recognizes eIF2α phosphorylated at Ser51 (eIF2α(P)) (Biosource International). For this analysis, samples were resolved by electrophoresis using a 12.5% SDS-polyacrylamide gel and the proteins in the gel were electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane. The mem-
branes were incubated with the rabbit polyclonal antibody that specifically recognizes eIF2α(P), and blots were developed as described previously (18). The horseradish peroxidase coupled to the anti-rabbit secondary antibody was then inactivated by incubating the membrane in 15% hydrogen peroxide for 30 min at room temperature. The total amount of eIF2α in the samples was determined by reprobing the blot with a monoclonal antibody that recognizes equally the phosphorylated and unphosphorylated forms of eIF2α followed by an anti-mouse secondary antibody. Values obtained using the anti-eIF2α(P) antibody were normalized for the total amount of eIF2α present in the sample.

RESULTS

Previous studies have shown that arsenite treatment and heat shock, both of which promote phosphorylation of eIF2, result in the accumulation of TIA-1, TIAR, and poly(A)\(^+\) RNA into stress granules (14, 15). To establish whether activation of the eIF2α kinase PERK can likewise promote stress granule formation, NIH/3T3 cells were incubated in the presence or absence of thapsigargin and the distribution of TIA-1 and poly(A)\(^+\) RNA was assessed by immunofluorescence microscopy. In cells maintained in the absence of thapsigargin, TIA-1 was present in both the nucleus and cytoplasm (Fig. 1). However, in the presence of thapsigargin, TIA-1 accumulated in punctate foci in the cytoplasm, a distribution reminiscent of that reported for stress granules. Likewise, poly(A)\(^+\) RNA was shown to accumulate in punctate foci in thapsigargin-treated but not control cells, suggesting that the cytoplasmic foci are indeed stress granules. Because conditions that promote stress granule formation also stimulate eIF2α phosphorylation, the possibility that eIF2 might colocalize with TIA-1 in stress granules was examined. As shown in Fig. 1, both the α-subunit of eIF2 and the ε-subunit of eIF2B accumulate in stress granules in response to thapsigargin treatment. Moreover, both translation factors colocalize with TIA-1, showing that under these conditions both eIF2 and eIF2B accumulate in stress granules.

Although previous studies have reported that a portion of eIF2 localizes to the nucleus (6, 12), the high proportion of eIF2α present in the nucleus observed in Fig. 1 was surprising. A possible explanation for this finding is that under the fixation conditions used above, some of the cytoplasmic eIF2 was lost, leading to an apparently low cytoplasmic content. To assess this possibility, cells were fixed with 3% rather than 1% paraformaldehyde and then probed with anti-eIF2α or anti-eIF2Bε antibodies. As shown in Fig. 2, following fixation with 3% paraformaldehyde, the cytoplasmic signal is greatly increased relative to the nuclear signal for both proteins. Importantly, cytoplasmic foci are still observed in stressed cells fixed with 3% paraformaldehyde. However, due to the overall stronger cytoplasmic staining, foci are not as easily visualized. Therefore, for the remainder of the studies reported herein, cells were fixed with 1% paraformaldehyde.

To determine whether or not the eIF2 that accumulates in stress granules is phosphorylated, an affinity-purified antipeptide antibody that only recognizes eIF2α when it is phosphorylated on Ser51 [eIF2α(P)] was covalently labeled with Cy3.5 and used to probe fixed cells. As shown in Fig. 3, thapsigargin causes accumulation of eIF2α(P) in granules. Whether or not all of the eIF2α in the granules is phosphorylated could not be determined by using these antibodies.

Phosphorylation of eIF2α in cells treated with thapsigargin occurs through activation of PERK. If eIF2α phosphorylation is required for stress granule formation, then cells lacking PERK should not exhibit either eIF2α phosphorylation or granule formation. To examine the requirement for eIF2α phosphorylation in the response, stress granule formation in wild-type SVT-MEF (PERK\(^{+/+}\)) cells was compared with formation in SVT-MEF cells containing a chromosomal disruption of the PERK gene (PERK\(^{-/-}\) cells; Ref. 8). As in NIH/3T3 cells, thapsigargin treatment of SVT-MEF (PERK\(^{+/+}\)) cells results in formation of granules that contain eIF2α (Fig. 4, bottom). In contrast, granule formation is not observed in SVT-MEF (PERK\(^{-/-}\)) cells treated with thapsigargin. Similarly, thapsigargin treatment inhibits protein synthesis and enhances eIF2α phosphorylation in SVT-MEF (PERK\(^{+/+}\)), but not SVT-MEF (PERK\(^{-/-}\)), cells (Fig. 4, top). Like thapsigargin, arsenite inhibits protein synthesis by stimulating eIF2α phosphorylation but does not induce the unfolded protein response or activate PERK (8). As shown in Fig. 4, arsenite inhibits protein synthesis, enhances eIF2α phosphorylation, and promotes stress granule formation to a similar extent in SVT-MEF (PERK\(^{-/-}\)) as in SVT-MEF (PERK\(^{+/+}\)) cells. This result suggests that it is eIF2α phosphorylation, and not ER stress, that promotes foci formation.

If eIF2α phosphorylation is sufficient to induce stress granule formation, then induction of eIF2α phosphorylation through a mechanism that does not otherwise impose a stress on the cell should cause stress granule assembly. To further define the sufficiency of eIF2α phosphorylation in the induction of stress granule formation, studies were performed using NIH/3T3 cells stably expressing fusion proteins consisting of the extracellular and transmembrane domains of the CD4 coreceptor with either the wild-type PERK kinase domain (CD4-PERK(wt)) or a kinase-dead K618A variant (CD4-PERK(ka)). As shown in Fig. 5 (top), treating cells expressing CD4-PERK(wt) with anti-CD4 antibody causes a reduction in protein synthesis to 67% of the untreated control value. In contrast, anti-CD4 antibody has no effect on protein synthesis in CD4-PERK(ka)-expressing cells. Similarly, anti-CD4 antibody causes a greater than fivefold increase in eIF2α phosphorylation in cells expressing CD4-PERK(wt) but has no effect in cells expressing CD4-PERK(ka). Furthermore, treating cells expressing CD4-PERK(wt), but not CD4-PERK(ka), with anti-CD4 antibody results in accumulation of eIF2α in stress granules (Fig. 5, bottom). The lack of effect of anti-CD4 antibody in cells expressing CD4-PERK(ka) is not due to an arti-
Fig. 1. eIF2α and eIF2Bε colocalize with TIA-1 in stress granules. NIH/3T3 cells were incubated for 50 min in the presence or absence of 1 μM thapsigargin. Cells were fixed in 1% paraformaldehyde, permeabilized, and then incubated with a Cy2-labeled monoclonal anti-eIF2α antibody as described in MATERIALS AND METHODS. Slides were examined using a Nikon E-800 fluorescence microscope equipped with a Nikon PCM-2000 laser confocal system. Images were merged using Deneba Canvas software. Permeabilized cells were incubated with Cy2-labeled monoclonal anti-eIF2α and polyclonal anti-TIA-1 antibodies followed by a Cy3-labeled anti-rabbit IgG antibody (A) or Cy3-labeled monoclonal anti-eIF2Bε and polyclonal anti-TIA-1 antibodies followed by a Cy2-labeled anti-rabbit IgG antibody (B), or slides were analyzed by in situ hybridization using biotinylated oligo(dT) followed by Cy3.5-labeled streptavidin (C) as described in MATERIALS AND METHODS. thap., Thapsigargin.
fact of the transfection procedure because thapsigargin inhibits protein synthesis, enhances eIF2α phosphorylation, and causes granule formation equally in cells expressing either CD4-PERK(wt) or CD4-PERK(ka) (Fig. 5). Thus activation of exogenously expressed PERK in the absence of ER stress promotes stress granule formation.

Because both eIF2 and eIF2B localize to stress granules, the localization of the two proteins with each other and with other initiation factors was examined in control and thapsigargin-treated cells. As shown in Fig. 6A, the cellular distribution of eIF2Bε exhibits a pattern similar to that of eIF2α, and thapsigargin treatment causes accumulation of both proteins in stress granules. Labeling cells simultaneously with both antibodies reveals colocalization of eIF2α and eIF2Bε in foci. To determine whether other translation initiation factors exhibit a similar redistribution during the unfolded protein response, the subcellular distribution of two proteins that regulate mRNA binding to the 43S preinitiation complex, eIF4G and eIF4E, were examined. In contrast to eIF2α and eIF2Bε, eIF4G and eIF4E are predominantly localized to the cytoplasm, with less intense staining observed in the nucleus (Fig. 6, B and C). Thapsigargin treatment promotes the accumulation of eIF4G and eIF4E in foci and colocalization of eIF4G with eIF2α and eIF4E with eIF4G, indicating that all four of the proteins are present together in foci.

During formation of the translation initiation complex, the met-tRNAi-eIF2-GTP ternary complex and the eIF4G-eIF4E-mRNA complex both bind to the 40S ribosomal subunit (reviewed in Ref. 24). Because this is the only known mechanism through which eIF2 and eIF4F associate, the possibility that 40S ribosomal subunits might localize to stress granules in thapsigargin-treated cells was examined. As shown in Fig. 7, A and B, in thapsigargin-treated cells, two proteins that are integral members of the 40S ribosomal subunit, S3 and S6, colocalize with eIF2α into cytoplasmic foci, suggesting that 40S ribosomal subunits accumulate in stress granules under such conditions. In contrast, neither 60S ribosomal proteins L5 or L37 nor eEF2 are detected in stress granules.
DISCUSSION

In previous studies (14, 15), arsenite was shown to promote the accumulation of poly(A)-containing RNA, PABP, and two other RNA binding proteins, TIA-1 and TIAR, into what were referred to as stress granules. Results of a more recent study (13) revealed that stress granules also contain the translation initiation factors eIF3, eIF4E, and eIF4G, but eIF2, eIF2B, and eIF5 were not detected in stress granules. Finally, ribosomal proteins S3 and S19 were detected in stress granules, but L5 and L37 were not, suggesting that 40S, but not 60S, ribosomal subunits are components of stress granules. Thus stress granules contain most of the components of the 48S preinitiation complex. However, because eIF2 was not detected, it was proposed that stress granules represent sites of accumulation of inactive 48S preinitiation complexes (13).

In the present study, stress granule formation induced by arsenite, the unfolded protein response, and activation of the protein kinase domain of PERK at the plasma membrane all promoted the accumulation of eIF2a and eIF2Bε into stress granules. These results demonstrate that under a variety of conditions that promote eIF2a phosphorylation, eIF2 is associated with stress granules. The reason that Kedersha et al. (13) failed to detect either eIF2a or eIF2Bε in stress granules may be related to the conditions that they used to visualize the proteins. In the study by Kedersha et al. (13), eIF2a and eIF2Bε were visualized using primary antibodies followed by secondary antibodies coupled to fluorescent dyes. In contrast, in the present study, the eIF2a and eIF2Bε monoclonal antibodies were directly labeled with fluorescent dye to eliminate the need for a secondary antibody [note that in both the present study and the study by Kedersha and coworkers (13), the same eIF2Bε monoclonal antibody was used]. In this regard, we found that when eIF2a was visualized in thapsigargin-treated NIH/3T3 cells using the secondary antibody approach, little if any eIF2a was detected in stress granules (unpublished observations). The basis for this discrepancy is unknown but could be a result of steric hindrance, in which the secondary antibody is not able to bind to the primary antibody associated with the 48S preinitiation complex. A second reason that Kedersha et al. (13) might not have detected eIF2a in stress granules could be related to the different cell types that were utilized in the two studies. However, it should be noted that in the present study, eIF2a was detected in stress granules in a variety of cell types, including NIH/3T3 (Fig. 2), MEF (Fig. 5), and L6 myoblasts (unpublished observations), suggesting that localization of eIF2a to stress granules is not a finding unique to a single cell type.

The results of the present study extend earlier studies to show that induction of stress granules requires phosphorylation of eIF2a; stress in the absence of eIF2a phosphorylation does not cause stress granule formation. Thus, in PERK−/− cells, thapsigargin does not cause eIF2a phosphorylation but does induce other endpoints of the ER stress response (8). In fact, IRE1α activation occurs earlier and persists longer in PERK−/− cells than in wild-type cells following induction of ER stress (8), suggesting that PERK−/− cells undergo a heightened stress response compared with wild-type cells. Therefore, stress in the absence of eIF2a phosphorylation is not sufficient to induce assembly of
stress granules. Further evidence that eIF2α phosphorylation is sufficient to induce granule formation is provided by studies in which CD4-PERK was ectopically expressed. In such cells, ER stress does not activate the expressed kinase because it is localized to the cytoplasmic rather than ER membrane, but treatment with anti-CD4 antibody does (2). In the present study, it was found that activation of the ectopically expressed kinase also promotes stress granule formation and inhibits protein synthesis. In contrast, in cells expressing kinase-dead CD4-PERK, anti-CD4 antibody had no effect on eIF2α phosphorylation or granule formation. Because protein synthesis is not affected by anti-CD4 antibody treatment in cells expressing kinase-dead CD4-PERK, it is unlikely that either antibody treatment by itself or expression of the CD4 chimeric proteins are inducing significant levels of stress. Thus these results also suggest that eIF2α phosphorylation and not some other manifestation of the stress response is sufficient for stress granule for-
It should be noted that stress granule formation has been observed in the absence of eIF2α phosphorylation (13). In that study, agents that deplete intracellular energy stores (i.e., 2-deoxyglucose, FCCP, or oligomycin) were shown to cause stress granule formation in the absence of changes in eIF2α phosphorylation. However, the reduced ATP-to-ADP ratio that occurs in response to treatment with such agents would likely lead to a decrease in the GTP-to-GDP ratio. Because formation of the eIF2-GTP-met-tRNA₆S complex is a key step in the initiation of protein synthesis, these findings suggest that stress granule formation is an alternative mechanism for regulating protein synthesis in cells with reduced ATP levels.

Fig. 5. Regulation of protein synthesis, eIF2α phosphorylation, and stress granule formation in NIH/3T3 cells. NIH/3T3 cells stably expressing fusions of the extracellular domain of CD4 with either wild-type (CD4-PERK wt) or kinase-dead K618A (CD4-PERK ka) PERK kinase domains were incubated for 60 min in the presence or absence of anti-CD4 monoclonal antibody or 1 μM thapsigargin. Cells were grown in six-well dishes containing a coverslip in the bottom of each well. At 10 min before harvest, [35S]Easytag Express protein labeling mix was added to the culture medium. The coverslips were then removed from the wells, cells were harvested, and protein synthesis (top left) and relative eIF2α phosphorylation (top right) were determined as described in MATERIALS AND METHODS. Cells on the coverslips were permeabilized and incubated with Cy2-labeled monoclonal anti-eIF2α (bottom). Slides were examined and images processed as described in the legend to Fig. 1. Hatched bars, control cells; gray bars, anti-CD4 antibody-treated cells; open bars, thapsigargin-treated cells.

Fig. 6. Thapsigargin-induced colocalization of eukaryotic initiation factors in NIH/3T3 cells. NIH/3T3 cells were incubated for 50 min in the presence or absence of 1 μM thapsigargin. Cells were fixed and permeabilized, as described in the legend to Fig. 1. Permeabilized cells were incubated with Cy2-labeled monoclonal anti-eIF2α and Cy3-labeled monoclonal anti-eIF2Be antibodies (A), Cy2-labeled monoclonal anti-eIF2α and polyclonal anti-eIF4G antibodies followed by a Cy3-labeled anti-rabbit IgG antibody (B), or monoclonal anti-eIF4E and polyclonal anti-eIF4G antibodies followed by Cy2-labeled anti-mouse IgG and Cy3-labeled anti-rabbit IgG antibodies (C). Slides were examined and images processed as described in the legend to Fig. 1.
Fig. 7. 40S ribosomal subunits, but not 60S ribosomal subunits, are present in stress granules. NIH 3T3 cells were incubated for 50 min in the presence or absence of 1 μM thapsigargin. Cells were fixed and permeabilized, as described in the legend to Fig. 1. Permeabilized cells were incubated with Cy2-labeled monoclonal anti-eIF2α and polyclonal antiribosomal protein S3 (rpS3) antibodies followed by a Cy3-labeled anti-rabbit IgG antibody (A), Cy2-labeled monoclonal anti-eIF2α and polyclonal antiribosomal protein S6 (rpS6) antibodies followed by a Cy3-labeled anti-rabbit IgG antibody (B), Cy2-labeled monoclonal anti-eIF2α and polyclonal antiribosomal protein L5 (rpL5) antibodies followed by a Cy3-labeled anti-rabbit IgG antibody (C), Cy2-labeled monoclonal anti-eIF2α and polyclonal antiribosomal protein L37 (rpL37) antibodies followed by a Cy3-labeled anti-rabbit IgG antibody (D), or Cy2-labeled monoclonal anti-eIF2α and polyclonal anti-eukaryotic elongation factor 2 (eEF2) antibodies followed by a Cy3-labeled anti-rabbit IgG antibody (E). Slides were examined and images processed as described in the legend to Fig. 1.
ternary complex is sensitive to changes in the GTP-to-GDP ratio (see Ref. 11 and references therein), energy depletion would mimic eIF2α phosphorylation in that both result in reduced formation of the ternary complex. The latter point is key because it is most likely reduced ternary complex availability rather than eIF2α phosphorylation per se that is responsible for stress granule formation.

The finding that eIF2, eIF4E, eIF4G, poly(A)+ RNA, and 40S ribosomal subunits all localize to stress granules indicates that the structures contain 48S preinitiation complexes. However, because initiation factors are released (reviewed in Ref. 24) from the 48S preinitiation complex upon binding of the 60S ribosomal subunit to form the active initiation complex, and neither 60S ribosomal subunits nor eEF2 are observed in stress granules, it appears that granules do not contain either the final initiation complex (i.e., the complete 80S ribosome bound to mRNA) or polysomes. This result suggests that the preinitiation complexes present in stress granules may be defective at either the 60S subunit joining step or an earlier step, such as hydrolysis of GTP bound to eIF2. Thus, if joining of the 60S ribosomal subunit to the 48S preinitiation complex is slowed under stress conditions, then 48S preinitiation complexes would accumulate. In support of this premise, agents that inhibit protein synthesis by stall- ing the ribosome on mRNA, e.g., emetine and cycloheximide, minimize stress granule formation, whereas agents, e.g., puromycin, that inhibit protein synthesis by causing premature termination enhance granule formation (14). Thus it was proposed that puromycin enhances the formation of both preinitiation complexes and stress granules by increasing the availability of free (i.e., nonpolysome associated) 40S ribosomal subunits. In contrast, emetine and cycloheximide would cause sequestration of ribosomal subunits in polysomes, making them unavailable to form preinitiation complexes, which would lead to a reduction in stress granule formation. Thus the availability of free 40S ribosomal subunits is crucial for stress granule formation.

The finding that preinitiation complexes accumulate in stress granules suggests that such structures might represent assembly points for initiation complexes. In this regard, stress granules might functionally be the cytoplasmic equivalent of nuclear speckles. Speckles are intranuclear structures characterized by a localized concentration of proteins involved in pre-mRNA splicing. They persist for long periods of time, but the proteins within speckles are in continuous flux and exchange rapidly with proteins in the nucleoplasm (26). The function of speckles is controversial, but a variety of studies suggest that they may act as a storage site for splicing components and/or may represent an assembly site for splicing components. Furthermore, speckles become larger when transcription is inhibited but become smaller and more diffuse after stimulation of transcription (3, 4, 21, 30). Thus both stress granules and speckles may represent stalled complexes, translation initiation complexes in stress granules, and splicing complexes in speckles.

On the basis of the results of the present and previous studies, it seems likely that stress granules contain preinitiation complexes not actively involved in protein synthesis but, instead, similar to nuclear speckles, may represent a storage or assembly site for translation initiation complexes. The possibility that stress granules form at intracellular sites where initiation complexes normally assemble is particularly intriguing because the RNA binding proteins TIA-1 and TIAR accumulate in stress granules (Fig. 1 and Ref. 15). In this regard, both proteins continuously shuttle between the nucleus and cytoplasm (1), suggesting that they may serve as carrier proteins that help to ferry mRNA from the nucleus to the cytosolic location where initiation occurs. When translation initiation is inhibited by eIF2α phosphorylation, mRNA with the accompanying TIA-1 and TIAR proteins accumulates at the putative assembly sites, resulting in formation of visible stress granules. It has also been suggested that TIA-1 and TIAR may have a role in the transfer of mRNA from polysomes to stress granules (1), although the mechanism through which this might occur is unknown. Overall, the available evidence suggests that translation is initiated at discrete points within the cell as opposed to occurring randomly throughout the cytoplasm. Such a finding is not surprising based on the multitude of components (e.g., initiation factors, 40S ribosomes, and mRNA) that must be assembled to form the 48S preinitiation complex. Having the required components present in a localized environment should allow translation initiation to occur in a more efficient manner.

The mechanism(s) involved in the accumulation of preinitiation complexes in cytoplasmic foci in response to cellular stress is unknown. However, previous studies by others have shown that under conditions that increase eIF2α phosphorylation, eIF2α(P) and eIF2B accumulate on 48S preinitiation and 80S initiation complexes (19, 27). Furthermore, Mats and coworkers (19) have suggested that eIF2 is released from the ribosome upon its interaction with eIF2B. Thus phosphorylation of eIF2α may result in accumulation of initiation factors in inactive preinitiation complexes by inhibition of the step that occurs immediately before joining of the 60S ribosomal subunit, release of initiation factors from the 48S preinitiation complex. The resulting inactive preinitiation complexes would then accumulate at putative assembly sites, resulting in stress granule formation. In this regard, stress granules may be viewed as possible storage sites for mRNAs and translation initiation factors that allow the cell to rapidly reinitiate translation once it has recovered from the stress. One might also speculate that mRNAs that are preferentially translated during the unfolded protein response, such as ATF4 mRNA, may be excluded from foci under such conditions. The mechanism through which such mRNAs are preferentially expressed when eIF2α is phosphorylated involves the multiple short open reading frames (uORF)
present in the 5’-untranslated region of the mRNA. When eIF2α phosphorylation is low, the uORFs act as translational repressors and limited ATF4 expression occurs (7). In contrast, hyperphosphorylation of eIF2α permits the scanning translation initiation complex to bypass the translationally repressive uORF(s) and allows the initiation complex to access the AUG start codon of the protein and initiate translation (reviewed in Ref. 22).

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