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Inhibition of Ribosome Recruitment Induces Stress Granule Formation Independently of Eukaryotic Initiation Factor 2α Phosphorylation[D]

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Cytoplasmic aggregates known as stress granules (SGs) arise as a consequence of cellular stress and contain stalled translation preinitiation complexes. These foci are thought to serve as sites of mRNA storage or triage during the cell stress response. SG formation has been shown to require induction of eukaryotic initiation factor (eIF)2α phosphorylation. Herein, we investigate the potential role of other initiation factors in this process and demonstrate that interfering with eIF4A activity, an RNA helicase required for the ribosome recruitment phase of translation initiation, induces SG formation and that this event is not dependent on eIF2α phosphorylation. We also show that inhibition of eIF4A activity does not impair the ability of eIF2α to be phosphorylated under stress conditions. Furthermore, we observed SG assembly upon inhibition of cap-dependent translation after poliovirus infection. We propose that SG modeling can occur via both eIF2α phosphorylation-dependent and -independent pathways that target translation initiation.

INTRODUCTION

In response to various assaults, mammalian cells activate a protective mechanism to prevent damage of vital cellular processes required for homeostasis, once the stress is relieved (Nover *et al*., 1989). The rapid formation of stress granules (SGs) in the cytoplasm is one of the main mechanisms by which the cell inhibits translation of mRNAs encoding for "housekeeping" functions to prioritize the synthesis of chaperones and enzymes needed for the stress response (Anderson and Kedersha, 2002). In addition to mRNAs, SGs contain many RNA-binding proteins, including TIA-1, eukaryotic initiation factor (eIF)3, eIF4E, eIF4G, poly(A) binding protein (PABP), AU-rich element binding protein (HuR), tristetraprolin (TTP), fragile X mental retardation protein (FMRP), and Ras-GTPase activating protein (G3BP) (Anderson and Kedersha, 2006; Kedersha *et al*., 1999, 2002; Mazroui *et al*., 2003). Translation inhibitors that stabilize polysomes (e.g., cycloheximide or emetine) induce SG disassembly, whereas compounds that cause disassembly of polysomes (e.g., puromycin) promote SG formation (Kedersha *et al*., 2000). Based on these observations, it was concluded that SGs constitute dynamic entities where mRNAs assemble for quality control before being redirected for reinitiation, degradation, or storage (Kedersha *et al*., 2005).

Unlike mRNAs directed to sites of decay known as processing bodies (PBs), the messages found in SGs are stabilized (Anderson and Kedersha, 2006). However, SGs and PBs share several RNA-binding proteins, such as TTP and BRF1, which are known as mRNA decay stimulators, suggesting that under stress conditions, SGs communicate with PBs (Anderson and Kedersha, 2006). The observed mRNA protection in SGs under stress could be explained by either inhibition of these decay factors and/or the recruitment of RNA-stabilizing proteins, such as HuR to SGs (Gallouzi *et al*., 2000). All these observations indicate that during the cell stress response, a close collaboration between different mRNA processing events, such as decay, stabilization, and translation, is required to ensure cellular protection against a lethal outcome and a rapid recovery after stress.

The process that inhibits translation during the stress response, and which also acts as a stimulus for SG assembly, targets specifically the initiation phase of translation (Anderson and Kedersha, 2006). Indeed, it has been shown that arsenite (AS)- and heat shock-mediated SG formation induce the

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Abbreviations used: AS, arsenite; eIF, eukaryotic initiation factor; MEF, mouse embryo fibroblast; PABP, poly(A) binding protein; PB, processing body; PI, postinfection; SG, stress granule; siRNA, short interfering RNA; uORF, upstream open reading frame; UTR, untranslated region; wt, wild-type.

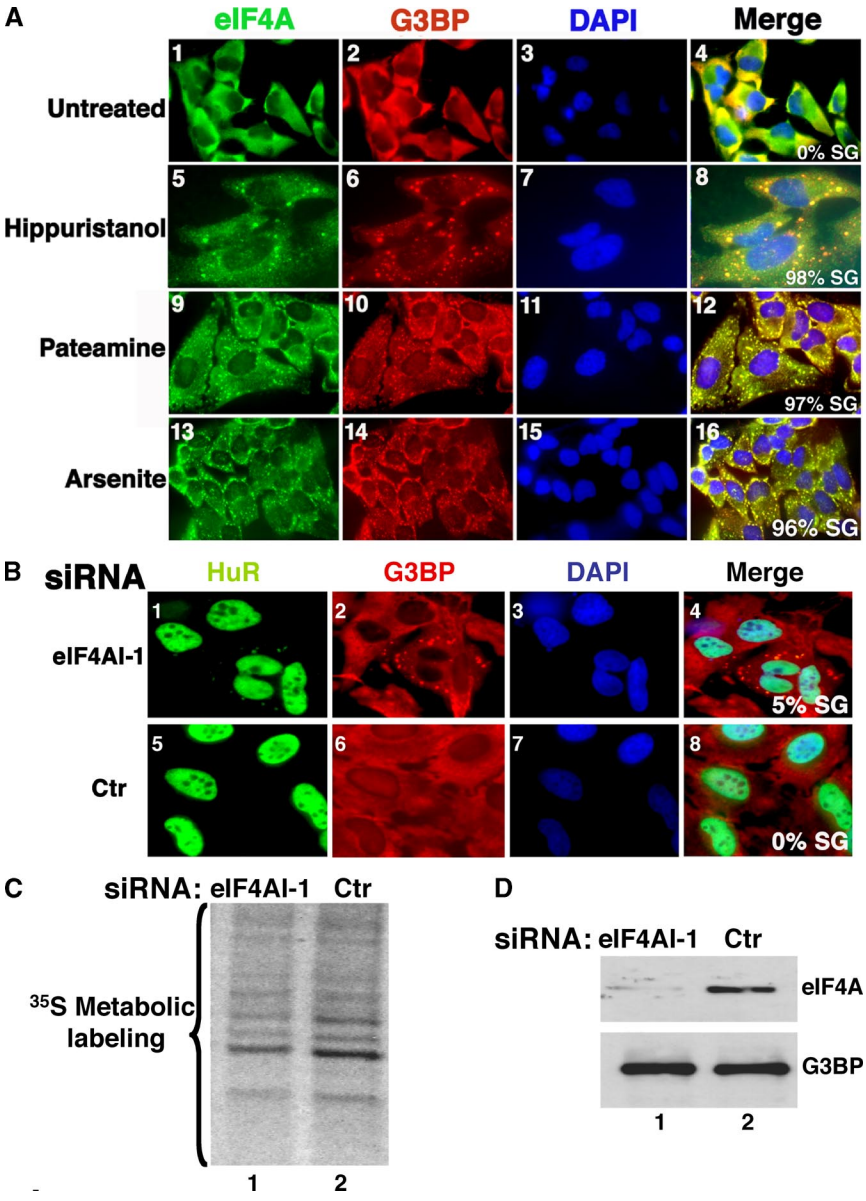

Figure 1. Perturbing eIF4A activity and levels induces SG formation. (A) eIF4A localizes to cytoplasmic granules upon inhibition of translation initiation. HeLa cells were treated with 1 μM hippuristanol (5–8), 0.1 μM pateamine (9–12), or 0.5 mM arsenite (13–16) for 30 min; permeabilized; and fixed. The primary antibodies used were a monoclonal anti-eIF4A antibody (5D3) and a polyclonal anti-G3BP antibody. The percentage of cells harboring SGs (>5 granules/cell), from three different fields and three different experiments containing a total of 450 cells, is indicated to the right bottom of 4, 8, 12, and 16. (B) Reduction of eIF4AI levels by siRNA induces SG formation. Cells were transfected with siRNA against eIF4AI (eIF4AI-1) or a control siRNA and fixed 2 d later. The distribution of HuR and G3BP was visualized by immunofluorescence. (C) Knockdown of eIF4A has a modest effect on cellular translation. Cells were treated with eIF4AI-1 or a control siRNA (Ctr) and 48 h later they were labeled for 30 min with 50 μCi/ml [^{35}S]methionine. (D) Western blot analysis of protein extracts prepared from cells treated with eIF4AI-1 or control siRNA (Ctr). The blot was first probed with a monoclonal anti-eIF4A antibody (5D3), stripped, and reprobed with an anti-G3BP antibody.

phosphorylation of eIF2α, leading to a reduction in the cellular levels of eIF2·GTP·Met-tRNA^{Met} ternary complexes, and a concomitant decrease in translation initiation rates. As a consequence, 40S ribosomes and some translation initiation factors are recruited to SGs. SG formation by mitochondrial poisons has been documented to occur in the absence of eIF2α phosphorylation (Kedersha *et al.*, 2002). This suggests that inhibition of translation initiation by stimuli that do not induce eIF2α phosphorylation may also be capable of inducing SG formation, a hypothesis that we address in this study.

Recently, two novel small molecule inhibitors of translation initiation, pateamine and hippuristanol, have been identified and characterized (Bordeleau *et al.*, 2005, 2006; Low *et al.*, 2005). Both compounds target eIF4A, an RNA helicase required for recruitment of ribosomes to cellular, and most viral, mRNAs (Rogers *et al.*, 2002). Pateamine stimulates eIF4A RNA-dependent ATPase, RNA binding, and helicase activity, whereas hippuristanol is an inhibitor of eIF4A RNA binding. eIF4A is the most abundant translation initiation factor, present at three copies per ribosome (Duncan *et al.*, 1987). There are two highly related eIF4A gene products, eIF4AI and eIF4AII (90–95% identical), both implicated in translation and functionally interchangeable in vitro (Conroy *et al.*, 1990; Yoder-Hill *et al.*, 1993). eIF4A exists as a free form (eIF4A$_f$) and as a subunit of eIF4F (eIF4A$_c$), a heterotrimeric complex that also contains eIF4E (the m^{7}GpppN cap binding protein) and the scaffolding protein eIF4G (Edery *et al.*, 1983; Grifo *et al.*, 1983). The helicase activity of eIF4A$_c$ is ~20-fold more efficient than eIF4A$_f$ (Pause and Sonenberg, 1992; Rogers *et al.*, 1999), suggesting that eIF4A$_c$ is the functional helicase required to unwind local secondary structure in the mRNA 5′ untranslated region during ribosome recruitment. A recent report indicates that exposure of cells to pateamine induces the formation of cytoplasmic granules containing TIA-1, eIF4A, and eIF4B (Low *et al.*, 2005). Whether the formation of these granules is an indirect consequence of eIF2α phosphorylation has not been investigated. To further characterize a potential relationship between the ribosome recruitment step of transla-

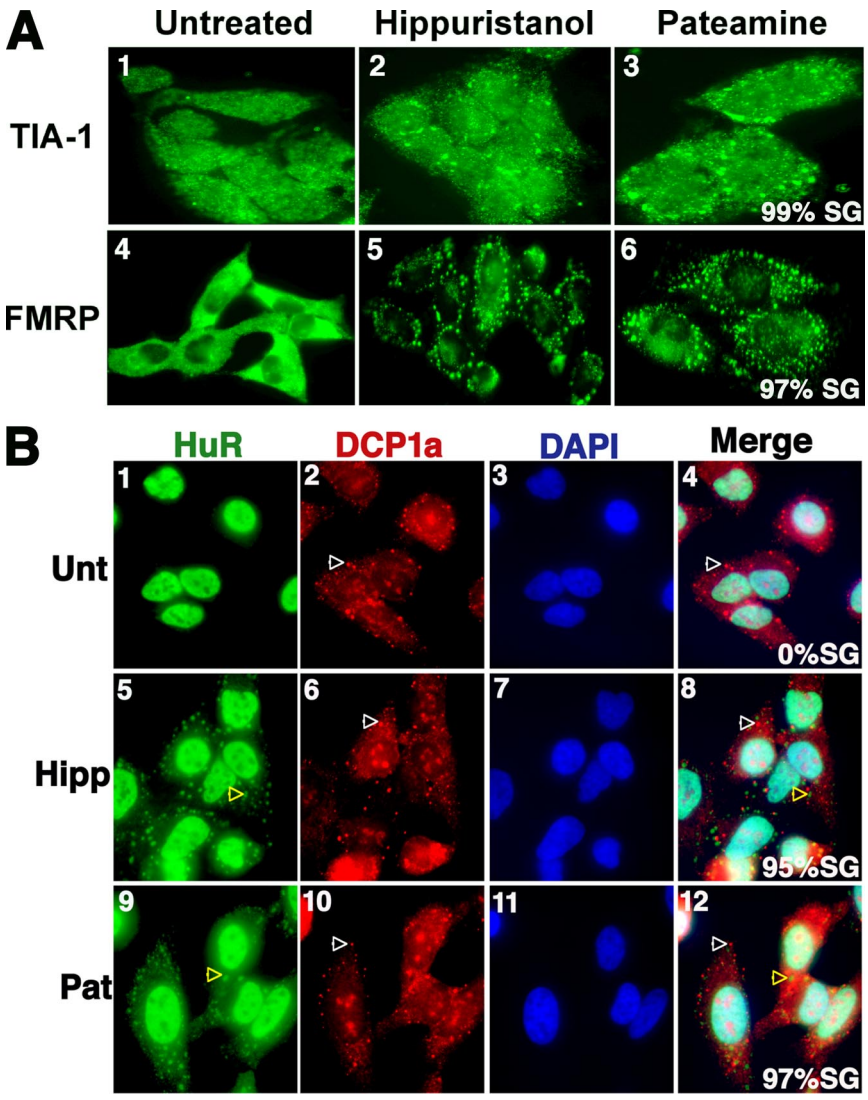

Figure 2. Granules induced by perturbation of eIF4A activity are similar to SGs and distinct from processing bodies. (A) Granules induced by perturbation of eIF4A activity contain TIA-1 and FMRP. HeLa cells were treated with 1 μM hippuristanol or 0.1 μM pateamine and then processed for immunofluorescence. The distribution of TIA-1 and FMRP was monitored with anti-TIA-1 and anti-FMRP (1C3) antibodies, respectively. The percentage of cells harboring SGs is indicated to the bottom right of 3 and 6. (B) Cellular distribution of HuR and DCP1α was visualized with anti-HuR (3A2) and anti-DCP1α antibodies, respectively. Yellow arrows indicate the location of granules induced by perturbing eIF4A activity, whereas the white arrows indicate the position of PBs.

tion initiation and SG formation, we made use of several strategies to interdict this phase of translation. Our data indicate that SG formation can occur as a consequence of impaired translation initiation independently of eIF2α phosphorylation.

MATERIALS AND METHODS

General Methods and Cell Line Maintenance

The compounds pateamine and hippuristanol were stored at −70°C in dimethyl sulfoxide as stocks of 10 mM. The characterization of these compounds has been documented previously (Bordeleau *et al.*, 2005, 2006). HeLa cells (obtained from American Type Culture Collection, Manassas, VA), wild-type (wt) mouse embryonic fibroblasts (MEFs), and MEFs harboring the mutation eIF2$\alpha^{S51A/S51A}$ were maintained in DMEM supplemented with 10% fetal bovine serum, penicillin, and streptomycin.

Antibodies

The monoclonal anti-eIF4A antibody has been described previously (Edery *et al.*, 1983). Phospho-specific anti-eIF2α and the pan anti-eIF2 were obtained from Cell Signaling Technology (Beverly, MA). Anti-HuR and anti-G3BP antibodies were described previously (Gallouzi *et al.*, 1998, 2000). The use of anti-Dcp1α anti-FMRP and antibodies has been documented previously (Sheth and Parker, 2003).

Small-interfering RNA (siRNA) Transfections

siRNA transfections were performed in HeLa cells essentially as described previously (Ferraiuolo *et al.*, 2004). Briefly, siRNA transfections were performed in HeLa cells by using Lipofectamine Plus reagent (Invitrogen, Carlsbad, CA). Twenty-four hours before transfection, cells were trypsinized to obtain 50–60% confluence on the day of transfection. For a six-well plate, 15 μl of siRNA duplex (20 μM annealed duplex; Dharmacon RNA Technologies, Lafayette, CO) was mixed with 100 μl of OPTI-MEM and 3.5 μl of Plus reagent and incubated for 15 min at room temperature. A mixture of 4 μl of Lipofectamine (Invitrogen) and 100 μl of OPTI-MEM was then added to the precomplexed RNA mix and incubated for an additional 15 min before adding to cells. Cells were harvested 48 h after transfection and processed for immunofluorescence, or proteins were extracted in 3× SDS-PAGE sample buffer and used for Western blots. The efficiency of knockdown was determined by quantitation of the signal on films using ImageQuant (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). The sense sequences of the siRNAs used in this study are eIF4AI-1, $^{5'}$GCCCAAUCUGGGACUGGGAdTdT$^{3'}$) (nucleotides [nt] 226–244), and eIF4AI-2, $^{5'}$UGAUAUGCUUAACCGGAGAdTdT$^{3'}$ (nt 488–506).

Fluorescence Microscopy

Cells were processed for immunofluorescence as described previously (Mazroui *et al.*, 2003). Essentially, cells were fixed in 3% paraformaldehyde and permeabilized with 0.1% Triton X-100/phosphate-buffered saline. Slides were incubated with primary antibodies diluted in 0.1% normal goat serum for 1 h at room temperature. After washing, slides were incubated with goat anti-mouse/rabbit IgG (H+L) secondary antibodies coupled to goat Alexa Fluor 488/594. Fluorescence microscopy was performed using a Zeiss AxioVision 3.1 microscope equipped with AxioCam HR (Carl Zeiss, Jena, Germany) digital

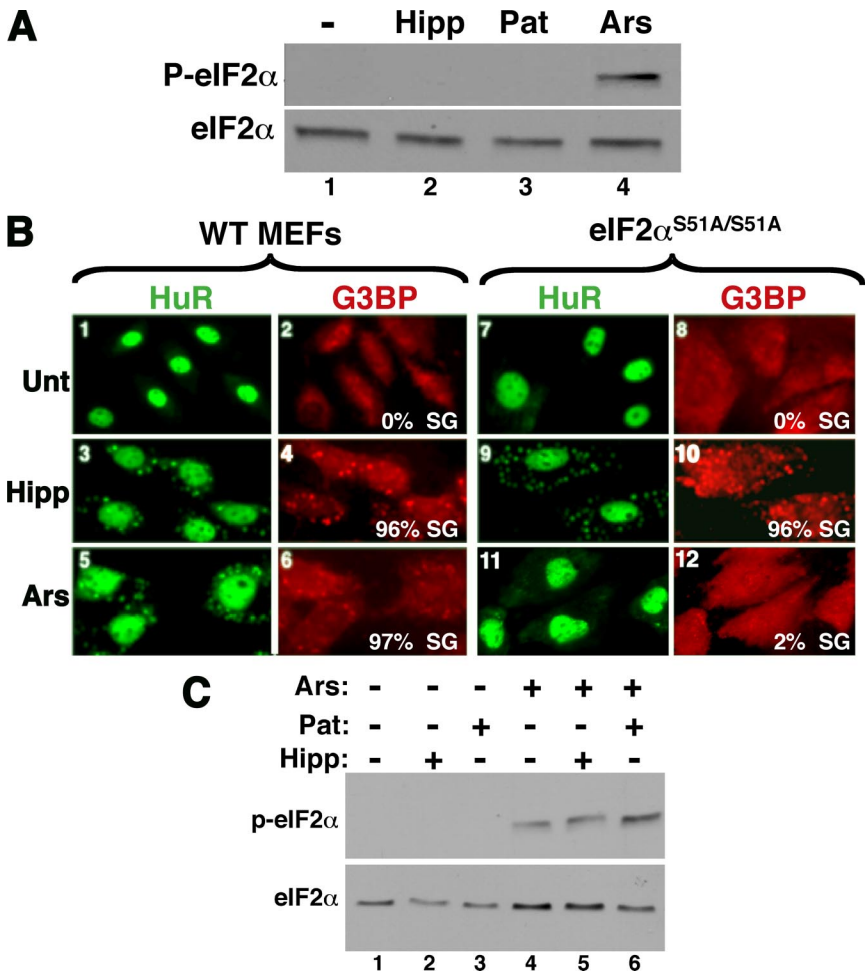

Figure 3. Assembly of eIF4A-inhibition induced granules is independent of eIF2α phosphorylation status. (A) Hippuristanol and pateamine do not induce phosphorylation of eIF2α. Cells were treated with 1 μM hippuristanol (lane 2), 0.1 μM pateamine (lane 3), or 0.5 mM of arsenite (lane 4) for 1 h. Protein extracts were prepared and analyzed by Western blotting using an anti-phospho eIF2α (top) or pan anti-eIF2α (bottom) antibody. (B) MEFs derived from wt and eIF2α$^{S51A/S51A}$ knockin mice were treated with 1 μM hippuristanol (3, 4, 9, and 10) or 0.5 mM arsenite (5, 6, 11, and 12) for 30 min. The localization of HuR (1, 3, 5, 7, 9, and 11) and G3BP (2, 4, 6, 8, 10, and 12) was assessed by immunofluorescence. The percentage of cells harboring SGs is indicated to the bottom right. (C) Exposure of cells to pateamine or hippuristanol does not block arsenite-mediated phosphorylation of eIF2α. Cells were exposed to hippuristanol (lanes 2 and 5) or pateamine (lanes 3 and 6) for 1.5 h. In some instances, arsenite was added to the cells 30 min after the addition of hippuristanol or pateamine, and the incubation was continued for 1 h. (lanes 4–6). Cell extracts were prepared and probed for eIF2α phosphorylation (top, p-eIF2α) as well as for total eIF2α levels (bottom, eIF2α).

camera. Images were compiled using Adobe Photoshop software (Adobe Systems, Mountain View, CA).

Poliovirus Infection

HeLa cells were incubated with the Mahoney strain of poliovirus type 1 (10 plaque-forming units/cell) in serum-free DMEM at room temperature for 30 min, after which time the medium was replaced with DMEM containing 10% fetal bovine serum. The infection was then allowed to proceed to the indicated times at 37°C. When present, guanidine HCl was used at a final concentration of 1.5 mM. Cells were then fixed and processed for immunofluorescence, or total protein was harvested for extraction and analyzed by Western blots.

RESULTS

In the course of characterizing the cellular effects of pateamine and hippuristanol, we noted that cells exposed to these compounds showed granules that were reminiscent of SGs induced by arsenite (Figure 1A, 1–16). SGs are thought to be sites of initiation factor and mRNA storage during translation inhibition, and the presence of eIF3, eIF4E, eIF4G, and PABP in these granules has been well documented (Kedersha *et al.*, 2002; McEwen *et al.*, 2005; McInerney *et al.*, 2005). AS, hippuristanol, and pateamine exposure induced the recruitment of eIF4A to these granules, as revealed by immunostaining using an anti-eIF4A monoclonal antibody (Figure 1A, compare 5, 9, and 13 with 1), confirming a previous report suggesting the presence of eIF4A in SGs (Low *et al.*, 2005). These granules harbor the classical SG marker G3BP (Tourriere *et al.*, 2003) (Figure 1A, compare 6, 10, and 14 with 2). In these experiments, translation was reduced by >95% when cells were treated with pateamine or hippuristanol, as judged by [^{35}S]methionine metabolic labeling (our unpublished data). These observations are consistent with previous reports linking SG formation and translation inhibition (Kedersha *et al.*, 2002; McEwen *et al.*, 2005; McInerney *et al.*, 2005).

Because both pateamine and hippuristanol target eIF4A, we addressed whether reducing eIF4A activity by an independent method would also trigger SG formation. To this end, HeLa cells were treated with an siRNA duplex directed to eIF4AI (eIF4AI-1) or a control siRNA (Figure 1B, 1–8). Treatment of cells with eIF4AI-1, but not the control siRNA, was sufficient to induce SG formation in ~5% of the cells visualized, as judged by the presence of HuR and G3BP in these granules (Figure 1B, compare 1 and 2 with 5 and 6). Metabolic labeling indicated that cellular protein synthesis was reduced by 40% (Figure 1C and as judged by quantitation of trichloroacetic acid-precipitable counts; our unpublished data). Western blot analysis of total cellular extracts revealed that the efficiency of eIF4AI knockdown in this experiment was 85% (Figure 1D, compare lane 1 with lane 2), consistent with what has been reported previously (Ferraiuolo *et al.*, 2004). These results were reproduced with a second siRNA targeting a different region of eIF4AI (eIF4AI-2), arguing that they are unlikely a consequence of off-target effects (Supplemental Figure 1). These results suggest that perturbing eIF4A activity with small molecules or by siRNA knockdown is sufficient to induce SG formation.

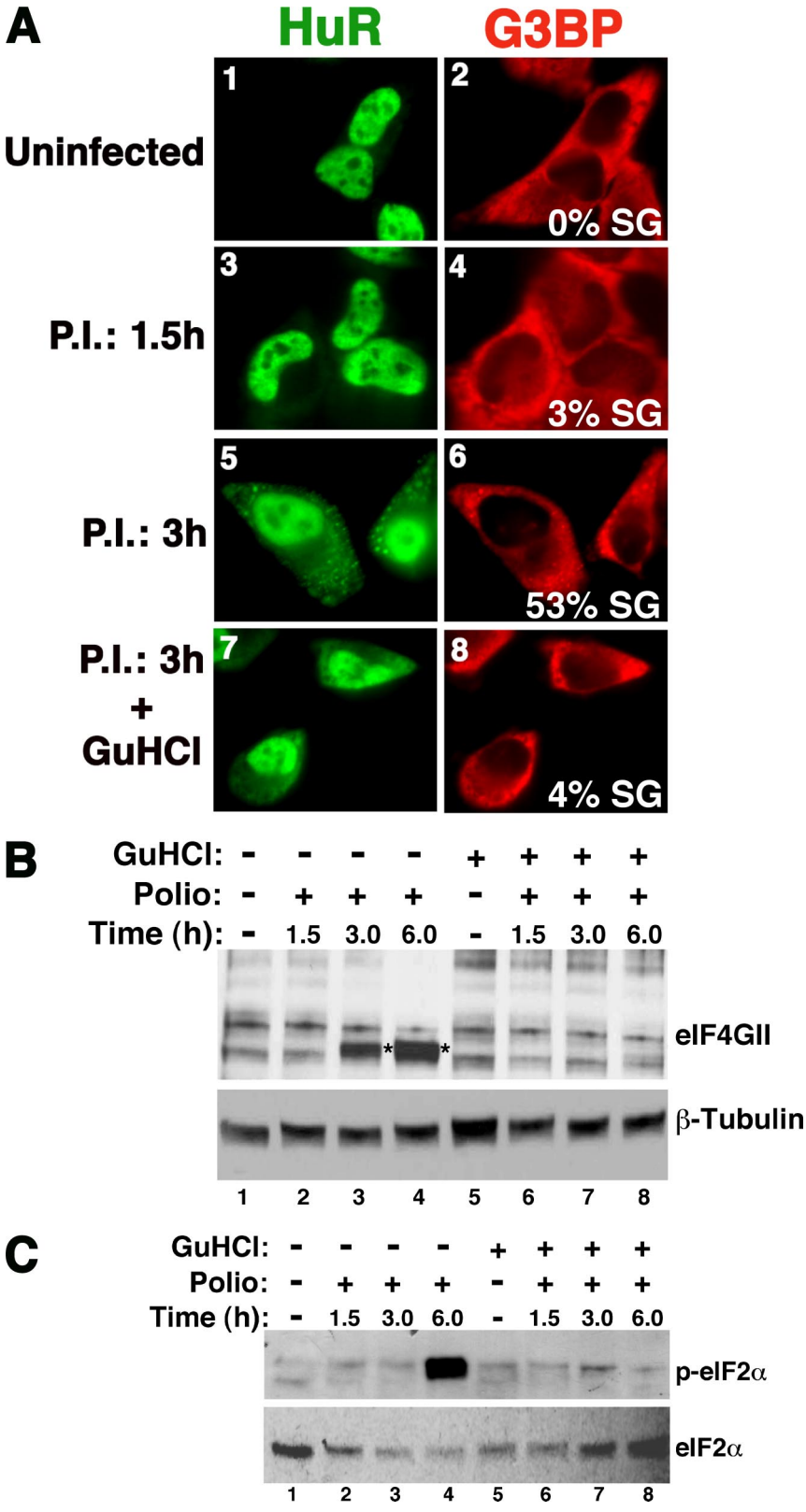

Figure 4. Inhibition of cap-dependent translation initiation by poliovirus is sufficient to induce cytoplasmic granule formation. (A) Cellular localization of HuR and G3BP was determined in control, uninfected cells (1 and 2) and in cells 1.5 h PI (3 and 4) and 3 h PI (5–8). Infection with poliovirus was performed in the absence (1–6) or presence (7 and 8) of guanidine hydrochloride (GuHCl). The percentage of cells harboring SGs is indicated to the bottom right. (B) Western blot analysis of eIF4GII integrity during poliovirus infection. Cell extracts were prepared from uninfected cells (lanes 1 and 5) or cells 1.5 h PI (lanes 2 and 6) 3 h PI (lanes 3 and 7), or 6 h PI (lanes 4 and 8). Poliovirus infections were performed in the absence (lanes 2–4) or presence (lanes 6–8) of GuHCl. The antibodies used to probe the blot are indicated to the right. Note that full-length eIF4GII was not detected well in this experiment, and only the cleavage product is clearly apparent (indicated by an asterisk). (C) Western blot analysis of eIF2α phosphorylation status during poliovirus infection. The same blot as in B was probed for eIF2α phosphorylation.

The granules formed by cellular exposure to hippuristanol and pateamine also were found to contain TIA-1 (Figure 2A, compare 2 and 3 with 1), FMRP (Figure 2A, compare panels 5 and 6 with 4), and HuR (Figures 2B and S2, compare panels 5 and 9 with 1). In HeLa cells, these proteins are present in SGs (Kedersha *et al*., 1999; Gallouzi *et al*., 2000; Mazroui *et al*., 2003). In contrast, neither hippuristanol nor pateamine significantly affected the appearance of PBs, as

shown by immunofluorescence of DCP1α, a known PB marker (Figures 2B and S2, compare panels 2, 6, and 10) (Sheth and Parker, 2003), although we cannot rule out subtle effects not detectable in our assay. Together, our data suggest that the cytoplasmic granules induced by hippuristanol and pateamine are similar in composition to SGs.

We considered the possibility that hippuristanol- or pateamine-induced SG formation was an indirect consequence of eIF2α phosphorylation. To assess this possibility, total extracts were prepared from HeLa cells treated for 1 h with arsenite, pateamine, or hippuristanol, and the extracts were used for Western blot analysis. We observed that eIF2α phosphorylation was observed only upon arsenite treatment and not when cells were exposed to pateamine or hippuristanol (Figure 3A). These observations indicate that pateamine- and hippuristanol-induced SG formation does not correlate with eIF2α phosphorylation. This conclusion was further supported by the fact that hippuristanol induced SG formation in MEFs expressing the nonphosphorylatable eIF2α mutant (eIF2$\alpha^{S51A/S51A}$), obtained from knockin mice (Scheuner *et al*., 2001) (Figure 3B, compare 9 and 10 with 7 and 8). In contrast, arsenite exposure did not induce SG assembly in eIF2$\alpha^{S51A/S51A}$-derived MEFs (Figure 3B, compare 11 and 12 with 7 and 8), as documented previously (McEwen *et al*., 2005). Both compounds induced SG formation in wt MEFs (Figure 3B, 1–6). (Note that in these experiments, staining of G3BP was not restricted to the cytoplasm, unlike what was observed for HeLa cells [e.g., Figure 1].) Nuclear localization of G3BP has previously been documented in quiescent fibroblasts (Tourriere *et al*., 2001). We therefore conclude that formation of SGs can occur via eIF2α phosphorylation-independent mechanisms. To assess whether the effect of our two compounds was dominant over stimuli that induced eIF2α phosphorylation, we pretreated cells with hippuristanol or pateamine, followed by exposure to arsenite. Probing for the phosphorylation status of eIF2α showed that arsenite is still capable of inducing eIF2α phosphorylation in hippuristanol- and pateamine-treated cells (Figure 3C, compare lanes 5 and 6 with 2 and 3), indicating that eIF4A-mediated SG formation occurs before eIF2α phosphorylation or resides in an independent pathway. These results demonstrate that perturbing eIF4A activity, in the absence of eIF2α phosphorylation, is sufficient to induce SG formation.

SGs could represent cytoplasmic entities where the cell assembles all components of the translation initiation machinery before commencing protein synthesis. Therefore, that these entities are highly dynamic could be one reason why they are not detected under normal cellular homeostasis and only become visible when translation initiation is blocked (Anderson and Kedersha, 2006). We have analyzed a number of additional translation inhibitors for their ability to induce SG formation. We tested compounds expected to stabilized polysomes (cepaeline, bouvardin, didemnin B, verrucarin A, and borrelidin) as well as two compounds that allow ribosomes to run off mRNA templates but block subsequent cycles of elongation by impairing the activity of newly initiated ribosomes (bruceantin and homoharringtonine) (Pelletier and Peltz, 2006). Although exposure of HeLa cells to concentrations of these elongation inhibitors was sufficient to inhibit protein synthesis (our unpublished data), they failed to induce SG formation (Supplemental Table 1).

We next determined whether other stimuli that inhibit translation initiation could also induce SG formation. For this purpose, we used poliovirus, because infected cells show inhibition of cap-dependent protein synthesis as a consequence of eIF4GI and eIF4GII subunit cleavage (Gradi *et al*., 1998). At 3 h postinfection (PI), we noted the appearance of SGs that were restricted to poliovirus-infected cells (Figure 4A, compare 5 and 6 with 1 and 2). At this time point, cleavage of eIF4GII was apparent (Figure 4B, compare lane 3 with lanes 1 and 2). SGs were not observed in poliovirus-infected cells that had been incubated with guanidine HCl, an inhibitor of poliovirus replication (Figure 4A, compare 7 and 8 with 5 and 6). Guanidine-HCl inhibits the function of poliovirus 2C protein, a protease that plays an essential role in viral replication (Pincus and Wimmer, 1986). As a consequence, synthesis of the poliovirus protease $2A^{pro}$ is diminished, and eIF4GII cleavage is delayed (Gradi *et al*., 1998) (Figure 4B, compare lanes 5–8 with lanes 1–4). Although phosphorylation of eIF2α has been reported after infection with poliovirus (Black *et al*., 1989; O'Neill and Racaniello, 1989), this is a late event that occurs after eIF4G cleavage. Indeed, Western blot analysis of extracts from infected cells indicated that phospho-eIF2α is detectable only at 6 h PI (Figure 4C, compare lane 4 with lanes 1–3). We note that large cytoplasmic aggregates after poliovirus infection have been reported previously in poliovirus-infected Hep-2 cells and shown to contain both positive- and negative-strand viral RNA (Bolten *et al*., 1998). We have not further investigated whether the composition of these granules is similar to the ones we described herein. These results support our conclusion that impairment of the cap-dependent ribosome recruitment phase of translation initiation, independent of eIF2α phosphorylation, is sufficient to induce SG formation.

To establish a link between exposure to hippuristanol and pateamine and the cell stress response, we monitored the expression of the heat shock protein heat-shock protein (HSP)70 in wt and eIF2$\alpha^{S51A/S51A}$ MEF cells. It is well established that the expression of HSP70 protein is induced during cell recovery from stress (Pelham, 1984). Therefore, we treated wt and eIF2$\alpha^{S51A/S51A}$ MEF cells with heat shock, arsenite, pateamine, or hippuristanol for 30 min and allowed them to recover 6 h at 37°C (Supplemental Figure 3). Western blot analysis in which cellular extracts were probed with an anti-HSP70 antibody revealed that HSP70 expression is induced upon heat-shock and arsenite treatment in both cell lines, but not in cells exposed to pateamine or hippuristanol (Supplemental Figure 3, compare lanes 4 and 5 with lanes 2 and 3). Furthermore, we noted that eIF2α phosphorylation is not required for HSP70 induction by arsenite (compare lane 8 with lane 3) but may be involved in the heat-shock response (compare lane 7 with lane 2). These data suggest that pateamine and hippuristanol may induce SG assembly by activating a cellular pathway that is independent of the well-known stress response process.

DISCUSSION

Herein, we demonstrate that perturbing eIF4A and/or inhibiting eIF4F activity induces SG assembly without the need to phosphorylate eIF2α. All of the stimuli we have used are expected to affect ribosome recruitment to mRNAs during translation initiation. Hippuristanol inhibits the RNA binding activity of both $eIF4A_f$ and $eIF4A_c$ (Bordeleau *et al*., 2006), whereas pateamine increases the RNA binding activity of $eIF4A_f$ removing it from the pool that is available for recycling through the eIF4F complex (Bordeleau *et al*., 2005; Low *et al*., 2005). In contrast, infection of cells by poliovirus cleaves the eIF4GI and eIF4GII subunits of eIF4F, preventing the recruitment of eIF4A to mRNA 5′ cap structures (Gradi *et al*., 1998). These results are consistent with the fact that SGs lack 60S ribosomes and agree with previous suggestions that this component of the translation apparatus may be

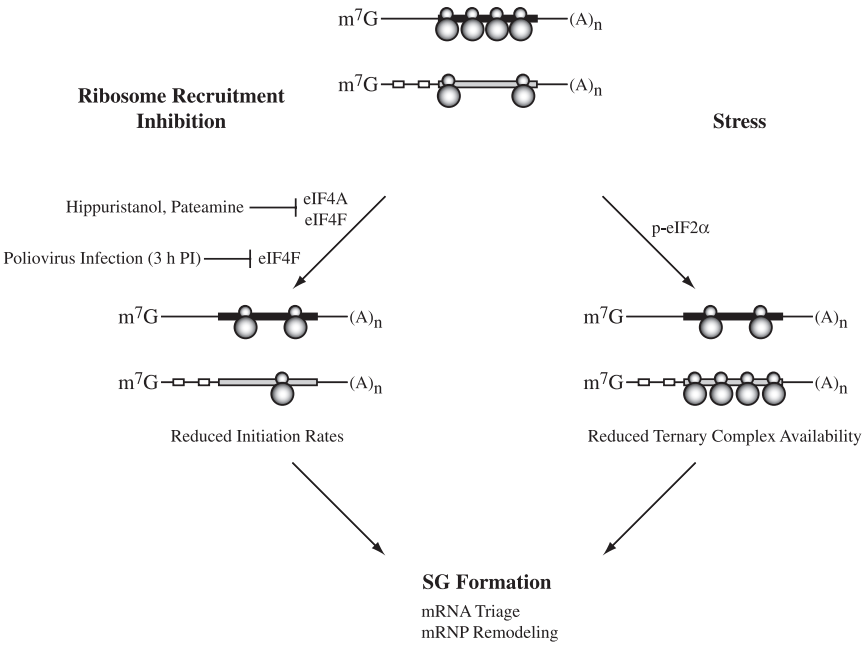

Figure 5. Schematic representation of the relationship between the ribosome recruitment phase of translation, eIF2α phosphorylation, and SG formation. The class of mRNA whose translation is increased as a consequence of eIF2α phosphorylation is depicted with 2 uORFs (small white boxes) and a larger coding region (gray box).

antagonistic to SG formation or is the feature that removes the mRNA (and associated factors) out of SGs (Kedersha *et al*., 2000). To better define the molecular mechanisms by which SGs are formed, an extended approach to the one described here could be applied in which each step of the initiation phase leading to 80S complex formation at the AUG codon could be targeted for inhibition, defining the critical boundary required for SG formation.

Our study defines a new mechanism by which SGs can form (Figure 5). Previously published data demonstrated that SG assembly upon inhibition of translation is triggered by stimuli that induce eIF2α phosphorylation (Anderson and Kedersha, 2006). Phosphorylation of eIF2α converts eIF2 from a substrate to a competitive inhibitor of eIF2B, preventing guanine nucleotide exchange and reducing ternary complex availability (for review, see Dever, 2002). Although this results in a severe block of general translation, the translation of a special class of mRNAs (e.g., ATF4), containing upstream ORFs is up-regulated (Figure 5) (Dever, 2002). This is a consequence of reduced ternary complex levels enabling newly initiated ribosomes to bypass some of the inhibitory upstream ORFs (uORFs) and commence protein synthesis more frequently at the appropriate downstream initiation codon (Dever, 2002). Our findings allow us to eliminate a potential role of these uORF-containing mRNAs in SG formation, because inhibiting the ribosome recruitment phase of translation initiation will also decrease translation of these mRNAs and yet is sufficient to induce SG formation (Figure 5). In addition, it is likely that it is reduced ternary complex availability (and its subsequent effects on global protein synthesis), and not phosphorylation of eIF2α per se, that is responsible for SG formation, because the latter is not necessary for SG formation (Figure 3).

It has not escaped our attention that other cellular processes that inhibit translation, such as some microRNA-mediated suppression may cause targeting of repressed mRNAs to SGs, because some of these are triaged for degradation to PBs. Along these lines, let-7 targeted and translationally inhibited mRNAs localize to PBs (Pillai *et al*., 2005), structures closely related in location to SGs (Anderson and Kedersha, 2006). As well, argonautes, proteins that play essential roles in RNA silencing, have been reported to localize to SGs (Anderson and Kedersha, 2006). Our study is consistent with a functional role of SGs in mRNA repression and expands our understanding of the stimuli capable of inducing SG formation.

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