Stable Formation of Compositionally Unique Stress Granules in Virus-Infected Cells

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Stress granules are sites of mRNA storage formed in response to a variety of stresses, including viral infections. Here, the mechanisms and consequences of stress granule formation during poliovirus infection were examined. The results indicate that stress granules containing T-cell-restricted intracellular antigen 1 (TIA-1) and mRNA are stably constituted in infected cells despite lacking intact RasGAP SH3-domain binding protein 1 (G3BP) and eukaryotic initiation factor 4G. Fluorescent in situ hybridization revealed that stress granules in infected cells do not contain significant amounts of viral positive-strand RNA. Infection does not prevent stress granule formation in response to heat shock, indicating that poliovirus does not block de novo stress granule formation. A mutant TIA-1 protein that prevents stress granule formation during oxidative stress also prevents formation in infected cells. However, stress granule formation during infection is more dependent upon ongoing transcription than is formation during oxidative stress or heat shock. Furthermore, Sam68 is recruited to stress granules in infected cells but not to stress granules formed in response to oxidative stress or heat shock. These results demonstrate that stress granule formation in poliovirus-infected cells utilizes a transcription-dependent pathway that results in the appearance of stable, compositionally unique stress granules.

Following exposure to a variety of environmental stress conditions, mammalian cells rapidly inhibit translation and shuttle a subset of cellular mRNAs and proteins to cytoplasmic bodies called stress granules (SGs) (reviewed in reference 22). SGs are nonmembranous structures that are 50 to 200 nm in diameter, are visible by phase-contrast microscopy, and are distinct from processing bodies (20, 25, 26). Two RNA binding proteins that appear to be critical for SG formation and that are themselves recruited to SGs are T-cell-restricted intracellular antigen 1 (TIA-1) and the RasGAP SH3-domain binding protein 1 (G3BP) (17, 48). The presence of translation initiation factors, such as eukaryotic initiation factor 3 (eIF3), eIF4E, and eIF4G, along with poly(A)+ binding protein 1 (PABP) and the 40S ribosomal subunit, has led to the suggestion that SGs may be sites where mRNAs are stored until the stress passes and translation can resume (23, 26). Alternatively, the finding that oxidative stress induces formation of processing bodies (PBs) along with SGs, which are often seen in close association with each other, suggests that mRNAs in SGs may be sent to PBs for degradation (25). Although SGs formed in response to diverse stimuli share many of the same components, certain factors appear to be recruited in a stress-specific fashion. For example, Hsp27 is found in SGs in heat-shocked cells but not in cells undergoing oxidative stress (26). Thus, it appears that SG composition and perhaps ultimately function may be fine-tuned in response to different environmental cues.

Stress granule formation occurs when translation initiation is inhibited and polysomes are disassembled (24). In contrast, compounds that stabilize mRNAs on polysomes, such as cycloheximide and emetine, inhibit SG formation and foster their disassembly (24). Inhibition of translation by oxidative stress is brought about by the phosphorylation of eIF2α and the resulting depletion of an eIF2GTP:RNA{sup*} ternary complex (12, 31). Heat shock treatment also induces eIF2α phosphorylation that contributes to translational inhibition (34). However, studies have indicated that eIF2α-independent mechanisms can also contribute to inhibition of translation (11). For example, in heat-shocked cells the canonical translation initiation factor eIF4G is bound by Hsp27 and recruited to SGs, thus inactivating the cap binding complex, eIF4F, and inhibiting translation initiation (9). More recently, hippuristanol and pateamine A, two compounds that inhibit translation initiation via interaction with eIF4A, another eIF4F component, were found to promote the formation of SGs (5, 6, 27, 29). Notably, SG formation by pateamine A does not require phosphorylation of eIF2α (10, 29). Similarly, poliovirus, which inhibits host protein synthesis via cleavage of eIF4G and PABP, also induces SG formation without significant eIF2α phosphorylation (29). Thus, it appears that SG formation can be caused by a variety of mechanisms that impair translation initiation.

Poliovirus is a small positive-strand RNA virus that is the causative agent of paralytic poliomyelitis. The ~7,500-nucleotide (nt) genome consists of a 5'-untranslated region (UTR), a single large open reading frame, and a 3'-UTR. Translation of the viral RNA results in the synthesis of a single large polypro-
tein that is co- and posttranslationally processed by the virus-encoded proteases 2A\textsubscript{pro} and 3C\textsubscript{pro} to produce the individual viral gene products (43). Translation of the viral genome is driven by an internal ribosomal entry sequence (IRES) located in the 5′-UTR. The poliovirus IRES allows for translation in the absence of functional eIF4F which, as mentioned above, is inactivated due to cleavage of eIF4G in infected cells (40, 45). In addition to reducing translation of cellular mRNAs, cleavage of host factors by 2A\textsubscript{pro} and 3C\textsubscript{pro} is also responsible for disrupting a number of cellular processes. For example, cleavage of nuclear pore complex proteins by 2A\textsubscript{pro} is thought to be responsible for inhibition of nucleo-cytoplasmic transport (2, 38), while cleavage of TFIIC and TBP by 3C\textsubscript{pro} contributes to the inhibition of transcription that occurs in infected cells (7, 8, 51). Numerous other targets for these proteases have been identified, including PABP (21), poly(rC) binding proteins 1 and 2 (41), the La autoantigen (46), the p65 subunit of NF-κB (35), and polyprymidine tract binding protein (1). Cleavage of these targets and the resulting inhibition or modification of their activities presumably provide the virus with an environment suitable for efficient replication.

Poliovirus was recently found to induce SG formation and can be added to the growing list of viruses that appear to activate and in some cases modify this cellular response (14, 19, 32, 33, 42, 47). In the case of poliovirus this was initially shown by the recruitment of HuR and G3BP to cytoplasmic foci within 3 h after infection (29). Subsequently White et al. found that eIF4G and PABP are also recruited to SGs at early times in poliovirus-infected cells (50). Examination of cells at later times, however, revealed that eIF4G, G3BP, and PABP are no longer found in SGs, indicating that poliovirus might cause the dispersal of these structures (50). In addition, exposure of infected cells to arsenite at 4 or 6 h postinfection failed to result in recruitment of eIF4G, PABP, or G3BP to SGs, indicating that poliovirus may actively interfere with SG formation at later times. The discovery that G3BP was cleaved by 3C\textsubscript{pro} and that this coincided with disassembly of SGs in infected cells provided a possible explanation for these findings (50). Indeed, mutation of the 3C\textsubscript{pro} cleavage site in G3BP resulted in a modest increase in eIF4G and G3BP-positive SGs in infected cells and restored the ability of these cells to recruit TIAR to SGs in response to arsenite (50). These results led White et al. to suggest a model whereby poliovirus induces SG formation but later, due to cleavage of G3BP, causes the disassembly of existing SGs and prevents de novo SG formation in response to external stress (50). However, as most of the SG markers used in the study by White et al. are cleaved by viral proteases, it is unclear if other SG components are also released from SGs.

Here we have further examined SG formation in poliovirus-infected cells. The results show that infection induces the formation of SGs containing host mRNA and TIA-1 and that these SGs do not disassemble at late times in infected cells. We also find that poliovirus-induced SGs can be distinguished from arsenite- or heat shock-induced SGs due to the presence of a unique marker, Sam68. SG formation in infected cells could be prevented by the addition of actinomycin D, while formation in arsenite-treated or heat-shocked cells was unaffected. Expression of a TIA-1 truncation mutant showed previously to inhibit SG formation during oxidative stress also prevented SG formation in poliovirus-infected cells. Using Hsp27 as a unique marker for heat shock-induced SGs, we show that poliovirus infection does not block de novo SG formation in response to heat shock. Cumulatively, these results indicate that poliovirus induces the stable formation of SGs that are compositionally distinct and dependent upon ongoing host mRNA synthesis for their formation.
fixed in 4% formaldehyde solution, dehydrated in ethanol, and stored at –20°C until needed. Cells were rehydrated, permeabilized, and hybridized as recommended, except the protease digestion step was omitted to facilitate the TIA-1 IFA (described above). Confocal microscopy and image acquisition were performed on an LSM 710 with the Zeiss Axio Observer Z1 inverted microscope using a 63× objective and the ZEN 2008 software (Carl Zeiss MicroImaging).

Colocalization analysis. Colocalization of Sam68 and poliovirus positive-strand RNA with TIA-1 was assessed by identifying all TIA-1-positive stress granules in a minimum of five randomly selected fields in each of three separate experiments (750 SGs). SGs were then scored for the presence/absence of Sam68 or poliovirus positive-strand RNA. Results determined in this manner were confirmed using the colocalization feature of the ZEN 2008 software (Carl Zeiss MicroImaging) with threshold determinations performed on a per field basis. The gating threshold intensities for TIA-1 and Sam68 reflected the mean intensity present in foci, while the gate for poliovirus positive-strand RNA was set low so as to ensure that any colocalization with TIA-1 was detected.

Immunoblot analysis. Forty-eight hours after plating in 35-mm wells, cells were infected or exposed to heat shock and lysates were prepared in Tdysis buffer as previously described (36). Protein was quantified using the Bradford assay, and equal amounts of protein were loaded on an 8% SDS-PAGE gel, electrophoresed, and transferred to an Immobilon-P membrane (Millipore Corporation). Total and phospho-eIF2α were detected using rabbit antibodies from Santa Cruz Biotechnology Inc. (sc-11386) and Cell Signaling Technology (9721), respectively. Antibody-antigen complexes were detected using secondary antibody conjugated to horseradish peroxidase (NA934; GE Healthcare) and chemiluminescence.

RESULTS

Stress granules in poliovirus-infected cells contain TIA-1 and mRNA. Cellular mRNA and the RNA binding protein TIA-1 are two major components found in stress granules that form in response to either oxidative stress or heat shock (26, 36). While previous examinations of poliovirus-infected cells revealed that eIF4G, PABP, G3BP, and HuR are all recruited to cytoplasmic foci that resemble SGs (29, 50), the status of mRNA and TIA-1 had not been examined. Consequently, HeLa cells were infected with poliovirus for 4 h and processed for fluorescent in situ hybridization and indirect immunofluorescence to visualize mRNA and TIA-1, respectively. The patterns of mRNA and TIA-1 staining in mock-infected cells were similar to those reported previously (26); mRNA was localized to predominant foci in the nucleus as well as diffusely staining both the nucleoplasm and cytoplasm, while TIA-1 staining was concentrated in the nucleus with weaker diffuse staining in the cytoplasm (Fig. 1). As expected, when oxidative stress was induced by exposing cells to 0.5 mM sodium arsenite, both mRNA and TIA-1 underwent a redistribution and localized to cytoplasmic foci that were visible by light microscopy and thus likely corresponded to SGs (Fig. 1 and data not shown). In cells infected with poliovirus for 4 h, mRNA and TIA-1 exhibited staining patterns that were very similar, if not identical to, that seen in cells undergoing oxidative stress. These results demonstrate that poliovirus infection, like oxidative stress and heat shock, induces SGs that contain both mRNA and TIA-1.
Stress granules do not disperse during the course of infection. Analysis of eIF4G, PABP, and G3BP revealed that in most cell lines poliovirus induced peak SG formation by 2 h postinfection, with cells exhibiting progressively fewer SGs 4 and 6 h after infection (50). These results suggested that poliovirus induced SG formation at early times during infection, followed subsequently by their disassembly. The results in Fig. 1, however, indicated that TIA-1 and mRNA were still present in SGs 4 h after infection and raised the possibility that poliovirus might not induce the complete disassembly of SGs. To examine this possibility in more detail the distributions of TIA-1 and eIF4G were examined in cells infected with poliovirus for increasing amounts of time. In agreement with previous reports, eIF4G was mostly cytosolic in uninfected cells (Fig. 2A) (26, 50). Two hours after infection 17% of cells exhibited foci that stained positive for both TIA-1 and eIF4G (Fig. 2A and B); this percentage is very similar to that reported by White et al. who used G3BP-1 as an SG marker (50). These foci were smaller than the SGs typically seen during oxidative stress and may thus reflect an early stage in their formation (compare Fig. 1 and 2). The number of cells exhibiting brightly staining foci that contain TIA-1 increased to greater than 90% by 4 h postinfection (hpi) and remained at this level even 6 h after infection (Fig. 2A and B). Similar results were obtained when mRNA was examined in infected cells (data not shown). As reported by White et al., eIF4G was not efficiently recruited to these foci, with only 22% and 18% of cells exhibiting eIF4G in SGs at 4 and 6 hpi, respectively (Fig. 2A and B) (50). These results indicate that upon poliovirus infection stress granules, as defined by the presence of TIA-1 and mRNA, are stably constituted within cells despite significant alterations in their composition.

mRNA incorporated into SGs is derived from the host. The results in Fig. 1 indicated that polyadenylated RNA was recruited to SGs in poliovirus-infected cells. Previous work, using in situ hybridization with strand-specific probes, found that both positive- and negative-strand viral RNA localized to small foci in the cytoplasm of infected cells (4, 13). Because, the positive strand of poliovirus RNA contains a poly(A) tail (52), it was possible that the SGs found in infected cells contain viral and/or host mRNA. To determine if poliovirus RNA was a component of SGs, infected cells were examined by fluorescent in situ hybridization using probes that hybridize to the poliovirus positive-sense RNA. Analysis of cells infected for
4 h revealed poliovirus positive-sense RNA was distributed throughout the cytoplasm of infected cells and often localized to numerous distinct structures (Fig. 3A), in agreement with previously published work (13). Costaining with antibodies to TIA-1 identified stress granules in infected cells and revealed that these were generally distinct from the structures containing poliovirus positive-strand RNA (Fig. 3A, merge). Quantitative analysis of these experiments revealed that only 5% (±1.8% [standard deviation]) of TIA-1-positive SGs also exhibited staining for poliovirus RNA (Fig. 3B). Interestingly, SGs were often found to be adjacent to structures containing poliovirus RNA, although the significance of this observation is unknown. When mock-infected cells were examined with this probe set, very little fluorescent signal was observed, confirming the specificity of the probe set. The results indicate that SGs in infected cells are largely devoid of positive-sense poliovirus RNA.

To examine the contribution of cellular mRNA to SG formation, infected cells were examined in the presence of the antibiotic ActD, which inhibits synthesis of cellular mRNA without affecting poliovirus RNA synthesis or yield (44). Treatment of mock-infected cells with ActD for 4 h resulted in a marked redistribution of both mRNA and TIA-1 compared to untreated cells, although no SGs were formed (Fig. 4). Cellular mRNA was concentrated into numerous brightly staining nuclear foci, while the majority of TIA-1 relocalized to the cytoplasm. Interestingly, redistribution of TIA-1 to the cytoplasm did not lead to SG formation, indicating that in the absence of cellular transcription, cytoplasmic accumulation alone is not sufficient to induce SG formation. Figure 4 shows that the addition of ActD to cells infected with poliovirus prevented formation of SGs containing mRNA or TIA-1. Closer inspection of the staining pattern in infected cells, however, revealed distinct foci that were smaller and less distinct than typical SGs (Fig. 4, arrows) and may represent abortive or impaired SG formation under these conditions. These results indicate that SG formation in infected cells is dependent upon ongoing cellular mRNA synthesis and suggest that host mRNA is a major component of these SGs.

Stress granules exhibit differential requirements for ongoing transcription. The finding that ActD prevented SG formation in poliovirus-infected cells prompted us to determine if SGs formed by other stress stimuli were also dependent upon ongoing host mRNA synthesis. As expected, cells exposed to oxidative stress or heat shock in the absence of ActD accumulated mRNA and TIA-1 in SGs (Fig. 4, left panels). To examine the role of transcription in SG formation during these stress responses, ActD was added to cells coincident with heat shock or oxidative stress and cells were incubated for an additional 4 h prior to analysis. In contrast to the results seen with poliovirus, the addition of ActD did not have as dramatic an effect on SG formation during oxidative stress or heat shock (Fig. 4). ActD treatment did, however, result in an overall decrease in mRNA staining intensity, and there were generally fewer SGs present in each cell (Fig. 4). Despite these changes, and unlike what was seen in poliovirus-infected cells, TIA-1 was still readily detectable in cytoplasmic foci following heat shock or oxidative stress. Similar results were obtained following shorter exposures to ActD (data not shown). These data indicate that poliovirus-induced stress granules are readily distinguishable from those found in arsenite-treated or heat-shocked cells due to their greater sensitivity to inhibitors of mRNA synthesis. These findings also suggest that there are mechanistic differences between how SGs are formed in po-
poliovirus-infected cells and how they are formed during oxidative stress or heat shock.

In light of the results with ActD described above, it was of interest to determine if cycloheximide (Cx) had differential effects upon SG formation in poliovirus-infected, arsenite-treated, or heat-shocked cells. Previous work showed that Cx prevents SG formation in response to oxidative stress, presumably by trapping mRNAs on polysomes (24). Indeed, we found that the addition of Cx to cells at the onset of infection, oxidative stress, or heat shock completely prevented the appearance of SGs (data not shown). Thus, SG formation in response to poliovirus infection, oxidative stress, or heat shock is sensitive to agents that promote polysome stability.

Role for TIA-1 in poliovirus-induced stress granule formation.

The finding that transcription was required for SG formation in infected cells prompted us to examine the role of other factors known to play a role in conventional SG formation. TIA-1 is one such factor and is essential for SG formation during oxidative stress and heat shock (17). TIA-1 contains three RNA recognition motifs in its N terminus and a glutamine-rich prion-related domain (PRD) in its C terminus. Expression of the C-terminal region of TIA-1 containing the PRD acts as a dominant-negative inhibitor of SG formation following oxidative stress (26). This may be explained by the ability of the PRD to associate with and trap endogenous TIA-1 in cytoplasmic microaggregates, thus decreasing its availability for stress granule formation (17). To better understand the role of TIA-1 in SG formation in response to poliovirus infection, Cos-7 cells were transiently transfected with HA-tagged constructs expressing either the C-terminal PRD (HA-PRD) or full-length TIA-1 (HA-TIA-1). Following transfection, cells were infected with poliovirus and then processed for IFA to detect the HA-tagged fusion protein in transfected cells and FISH to determine if mRNA was recruited to SGs. Expression of full-length TIA-1 had very little effect on SG formation in poliovirus-infected cells; 79% of HA-TIA-1-positive cells exhibited redistribution of both HA-TIA-1 and mRNA to stress granules (Fig. 5A and B). Conversely, expression of HA-PRD severely inhibited SG formation in infected cells, with only 8% of transfected cells displaying obvious SG formation (Fig. 5A and B). In both cases a low percentage of cells showed atypical cytoplasmic foci, which were dispersed throughout the cell and did not resemble typical SGs. These findings reveal that although poliovirus-induced SGs are distinguishable from oxidative stress-induced SGs, due to their dependence upon ongoing transcription, SG formation under both conditions utilizes pathways involving TIA-1.
Stress granules induced by poliovirus are compositionally distinct structures. While SGs formed in response to different stimuli share many common factors, some components are unique to the particular stress. For example, Hsp27 is recruited to SGs during heat shock but not oxidative stress or UV irradiation (23, 26). In addition, the results of White et al. demonstrated that at late times in poliovirus-infected cells SGs lack eIF4G, PABP, and G3BP, which are core constituents of SGs formed under most circumstances (50). This difference between poliovirus-infected cells and cells undergoing oxidative stress or heat shock is shown directly in Fig. 6A, with eIF4G used as an example. Cells subjected to heat shock or oxidative stress showed strong eIF4G staining that colocalized with that for TIA-1, while infected cells showed much less eIF4G staining of SGs despite robust TIA-1 staining. These results demonstrate that poliovirus-induced SGs can be distinguished from those formed during oxidative stress or heat shock by differences in their composition.

Previous work showed that Sam68 localizes to cytoplasmic foci reminiscent of SGs in poliovirus-infected cells (30). Sam68 is an RNA binding protein in the signal transduction and activation of RNA family of proteins that has been shown to interact with the poliovirus RNA-dependent RNA polymerase (28, 30). To further delineate the features of SGs in poliovirus-infected cells and to determine if the Sam68 cytoplasmic foci were indeed SGs, cells were costained for Sam68 and mRNA.

FIG. 5. A dominant-negative form of TIA-1 blocks stress granule formation in poliovirus-infected cells. (A) Cos-7 cells were transfected with vectors encoding the HA tag fused to either the C-terminal PRD of TIA-1 (HA-PRD) or full-length TIA-1 (HA-TIA-1) and 48 h later infected with poliovirus. Four hours after infection cells were costained for mRNA (green fluorescence) using FISH and for the HA tag (red fluorescence) using IFA. Arrows indicate transfected cells. (B) Quantitation of the results shown in panel A. The percentages of cells staining positive for either HA-PRD or HA-TIA-1 that exhibited stress granules, no stress granules, or atypical foci are indicated. Quantitation was done by analyzing more than 100 HA-positive cells in multiple fields for each experiment, with the average results from three independent experiments shown.
Following infection Sam68 redistributed to the cytoplasm and concentrated in distinct foci that colocalized with TIA-1 (Fig. 6B). Quantitation of these data indicated that 94% (±2%) of TIA-1-positive SGs also stained positive for Sam68. These results have been confirmed by showing that Sam68 also colocalizes with mRNA in infected cells (data not shown). These findings demonstrate that Sam68 is recruited to SGs in poliovirus-infected cells.

To better characterize the similarities and differences between poliovirus-induced SGs and those induced by heat shock and oxidative stress, SGs were examined for the presence of Hsp27 and Sam68 (Fig. 6C). As mentioned above, Hsp27 is found in heat shock-induced SGs but not those formed during oxidative stress, and its status in poliovirus-infected cells was unknown. As shown in Fig. 6B, Sam68 is recruited to SGs in infected cells, but it was not clear if this also occurred during oxidative stress or heat shock. In mock-infected cells Sam68 localized to the nucleus, while Hsp27 was predominantly cyto-

**FIG. 6.** Poliovirus-induced stress granules are compositionally distinct from those formed during oxidative stress or heat shock. (A) HeLa cells were mock infected or infected with poliovirus for 4 h, heat shocked, or exposed to arsenite (oxidative stress) and then processed for costaining with antibodies to TIA-1 (red fluorescence) and eIF4G (green fluorescence). (B) Mock-infected or poliovirus-infected cells as shown in panel A were costained for DNA (Hoechst; blue fluorescence), TIA-1 (green fluorescence), and Sam68 (orange fluorescence) using IFA. The merge images represent an overlay of the corresponding Hoechst, TIA-1, and Sam68 images. (C) Cells prepared as for panel A were costained for Hsp27 (green fluorescence) and Sam68 (red fluorescence) by using IFA.
Figure 6C shows that while heat shock induced a marked accumulation of Hsp27 in SGs, Sam68 remained predominantly nuclear with very little accumulating in SGs. In contrast, poliovirus infection caused the formation of Sam68-positive SGs that lacked significant amounts of Hsp27. Oxidative stress did not induce SGs that contained significant amounts of Sam68 or Hsp27. These data indicate that Sam68 is a unique component for poliovirus-induced SGs, while Hsp27 is unique to SGs induced by heat shock, and the data demonstrate the compositional differences between SGs formed in response to different stimuli.

Poliovirus does not block de novo stress granule formation in response to heat shock. Using eIF4G, PABP, and G3BP as SG markers, it has been shown that poliovirus prevents de novo stress granule formation in response to oxidative stress (50). However, as these components are all cleaved in poliovirus-infected cells, it was possible that their recruitment to stress granules could be impaired without affecting overall SG formation. This hypothesis seems to be supported by the results presented above showing that TIA-1, mRNA, and Sam68 remain associated with SGs throughout infection. To examine this possibility further we took advantage of the finding that Sam68 is a unique component of poliovirus-induced SGs, and Hsp27 is almost exclusively observed in heat shock-induced SGs (Fig. 6C). To determine if poliovirus prevents heat shock-induced recruitment of Hsp27 to SGs, HeLa cells were infected with poliovirus for 4 h and then subjected to heat shock for 40 min. Analysis of Sam68 and Hsp27 revealed that both of these factors were recruited to SGs under these conditions (Fig. 7A). As poliovirus-infected cells contain numerous SGs 4 h after infection and all SGs were observed to stain positive for both Sam68 and Hsp27, these results indicate that poliovirus infection does not prevent Hsp27 recruitment to preexisting SGs. To determine if poliovirus prevents de novo SG formation, the effect of heat shock on cells infected in the presence of ActD was examined. As shown earlier, ActD prevents the formation of SGs in infected cells but has no effect on SG formation following heat shock (Fig. 4). Figure 7B again shows that in the presence of ActD neither TIA-1 nor mRNA is recruited to SGs in infected cells. In contrast, when infected cells were exposed to ActD for 4 h followed by heat shock, both mRNA and TIA-1 were readily detectable in SGs (Fig. 7B).
agreement with the results of White et al., analysis of lysates from infected cells confirmed that eIF4G was cleaved by 2 h postinfection, while G3BP1 was targeted later, with cleavage essentially complete by 4 h postinfection (Fig. 7C) (50). Significantly, heat shock did not impede cleavage of G3BP1 (Fig. 7C, lanes 4 and 5), indicating that intact G3BP1 was not necessary for heat-shock-induced SG formation. These results demonstrate that poliovirus does not block the de novo formation of SGs in response to heat shock and that formation can occur in the absence of intact G3BP and eIF4G.

Heat shock causes inhibition of protein synthesis that is caused, at least in part, by the phosphorylation of eIF2α (34). In contrast, poliovirus inhibits translation via cleavage of eIF4G and PABP, with phosphorylation of eIF2α not occurring until later times after infection (18, 21, 29, 37). To further examine the heat shock response in poliovirus-infected cells, the status of eIF2α phosphorylation was examined. Figure 7D shows that very little phosphorylated eIF2α was detected in mock-infected cells or cells that had been infected for 2 h and that levels increased modestly by 4 hpi (lanes 1, 2, and 4, respectively). Following heat shock the total amount of eIF2α in the cells was essentially unchanged, while the levels of phosphorylated eIF2α increased substantially (Fig. 7D, compare lanes 1 and 6). Heat shock of cells infected with poliovirus for 2 or 4 h resulted in phosphorylation of eIF2α similar to that seen when uninfected cells were exposed to heat shock (Fig. 7D, compare lanes 3, 5, and 6). Cumulatively these results indicate that poliovirus does not interfere with the signaling pathways leading to eIF2α phosphorylation and SG formation following heat shock.

DISCUSSION

These results provide important insights into our understanding of SG formation in poliovirus-infected cells. SGs formed in infected cells were found to contain host mRNA as well as TIA-1 and other classical markers of SGs but to lack poliovirus RNA. In addition, once recruited to SGs these components remained associated with SGs at least up to 6 h postinfection. As was the case with oxidative stress, SG formation in infected cells was prevented by expression of a dominant-negative TIA-1 construct, suggesting that TIA-1 has a critical role in SG formation under both conditions. Despite these similarities, distinct differences were also apparent between SGs formed in response to different stresses, for example, the recruitment of Sam68 to poliovirus-induced SGs, but not those found in oxidative stress or heat shock. Similarly, SG formation in infected cells was severely inhibited when host mRNA synthesis was blocked, while SG formation following heat shock and arsenite treatment was not. Notably, despite the proteolysis of several key SG components, including G3BP-1, eIF4G, and PABP, SG formation in response to heat shock was not inhibited in poliovirus-infected cells.

Previously, White et al. reported that G3BP, eIF4G, and PABP were recruited to SGs at early times in poliovirus-infected cells but at later times were dispersed throughout the cytoplasm (50). Their disappearance from SGs was taken as an indication that poliovirus induced disassembly of SGs at later times of infection. The finding that SGs containing mRNA, TIA-1, and Sam68 are readily detected at late times after infection indicates that poliovirus does not induce their disassembly. Rather, infection by poliovirus appears to alter the composition of SGs by fostering the release of specific components from these structures. As G3BP, eIF4G, and PABP are all cleaved by viral proteases, one possibility is that proteolysis triggers their release from SGs. Alternatively, it is possible that release is triggered by as-yet-unidentified mechanisms, followed soon thereafter by cleavage. Interestingly, PCBP-2, an RNA binding protein that plays an important role in translation and replication of the viral RNA genome, was recently shown to be recruited to SGs during heat shock and oxidative stress (3, 15, 16, 39, 49). As PCBP-2 is also cleaved by the viral 3C protease (41), it will be interesting to see if it also is initially recruited and then released from poliovirus-induced SGs.

Using Hsp27 as a unique marker of heat shock-induced SGs, we found that poliovirus does not prevent Hsp27 recruitment to preexisting SGs or de novo SG formation following heat shock. In contrast, White et al., using PABP, eIF4G, G3BP, and TIAR as markers, reported that poliovirus blocks SG formation in response to oxidative stress (50). Given that PABP, eIF4G, and G3BP are cleaved in infected cells, it seems possible that their failure to be recruited to SGs at late times might be a consequence of their cleavage and not indicative of a general block in SG formation by poliovirus. The finding that TIAR, a protein closely related to TIA-1 and that is not cleaved during infection, was not efficiently recruited to SGs in infected cells that had been exposed to arsenite strongly suggested that poliovirus inhibited arsenite-induced SG formation (50). However, our finding that infected cells already harbor numerous SGs containing TIA-1, Sam68, and mRNA suggests that poliovirus prevents the arsenite-induced recruitment of TIAR to SGs rather than SG formation per se. These findings also suggest that the lack of TIAR may represent another distinguishing feature of poliovirus-induced SGs.

The analysis of SG formation in poliovirus-infected cells where eIF4G, PABP, and G3BP are proteolyzed provides several intriguing observations regarding the roles of these factors in SG formation and maintenance. First, the finding that SGs in infected cells lack these factors indicates that intact eIF4G, PABP, and G3BP are not required for maintenance of SGs, at least in the context of infected cells. Second, when infected cells are exposed to heat shock, Hsp27 is efficiently recruited to SGs. Since Hsp27 colocalizes with Sam68 in all instances, this indicates that poliovirus-induced and heat shock-induced SGs are not physically distinct structures. Third, the ability of heat shock to induce relocation of Hsp27 to SGs at 4 hpi, when eIF4G, PABP, and G3BP are cleaved, indicates that these factors are not required for Hsp27 recruitment to SGs. Finally, the observation that heat shock induced efficient SG formation in infected cells treated with ActD suggests that intact forms of eIF4G, G3BP, and PABP are not necessary for de novo SG formation in response to heat shock. Currently it is not known if proteolysis of these host factors results in their complete removal from SGs or if portions, perhaps undetectable with the antibodies used, remain associated with SGs or could participate in the formation of SGs.

The prior demonstration that heat shock-induced SGs contain Hsp27, while those formed in response to oxidative stress do not, indicated that SGs can differ compositionally depending upon the type of stress responsible for their induction (26).
The results presented here extend this analysis to poliovirus-induced SGs and also discern some noticeable stress-sensitive differences in the role of transcription in SG formation. Like oxidative stress-induced SGs, those found in poliovirus-infected cells lack Hsp27. Due to the presence of Sam68, however, poliovirus-induced SGs can be distinguished from those formed in response to oxidative stress or heat shock. Poliovirus-induced SG formation is also distinguished by its increased dependence upon ongoing host cell transcription. Currently, it is not clear if this is due to a requirement for a protein product whose transcript is induced following infection or if newly synthesized mRNA itself has a role in SG formation in infected cells. The finding that ActD resulted in only a modest decrease in mRNA staining in heat shock-induced SGs suggests that under these conditions the majority of mRNA in SGs is derived from mRNAs that are associated with polysomes. In contrast, the inability to detect SGs in infected cells treated with ActD suggests that a major component of SGs in infected cells is newly synthesized mRNA. This may extend to SGs formed during oxidative stress, as ActD also reduced mRNA staining in oxidative stress-induced SGs. These results not only suggest that distinct pathways exist for the recruitment of newly synthesized and preexisting mRNAs but also raise the possibility that different mRNAs may be recruited to SGs in response to different stresses.

Currently the significance of SG formation in infected cells is not known. Overexpression of wild-type or cleavage-resistant forms of G3BP, which favor SG formation, reduces viral titer by nearly 1 log (50), suggesting that SGs may negatively impact viral replication. If this were the case, however, one would expect elimination of SGs to increase virus yield. Previous work showing that ActD does not reduce viral yields (44), combined with the work presented here showing that SGs do not form in ActD-treated cells, indicates that this may not be the case. Perhaps targeting of newly synthesized mRNAs to SGs in infected cells augments translation inhibition mediated by eIF4G cleavage. If this were the case then eliminating newly synthesized mRNA by the addition of ActD would also obviate SG formation and no decrease in viral yield would be expected. Alternatively, SG formation may contribute to aspects of the virus life cycle not apparent in tissue culture, for example, by sequestering newly synthesized transcripts encoding antiviral functions or proteins with antiviral or immune-modulatory activities. Ultimately, determining the significance of SGs during poliovirus infection will require the analysis of viral replication kinetics and pathogenesis in systems incapable of SG formation.

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