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# Inhibition of Cytoplasmic mRNA Stress Granule Formation by a Viral Proteinase

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## SUMMARY

**Mammalian cells form dynamic cytoplasmic mRNA stress granules (SGs) in response to environmental stresses including viral infections. SGs are involved in regulating host mRNA function and metabolism, although their precise role during viral infection is unknown. SGs are thought to assemble based on functions of the RNA-binding proteins TIA-1/TIAR or Ras-GAP SH3 domain-binding protein (G3BP). Here, we investigated the relationship between a prototypical plus-strand RNA virus and SGs. Early during poliovirus infection, SG formation is induced, but as infection proceeds this ability is lost, and SGs disperse. Infection resulted in cleavage of G3BP, but not TIA-1 or TIAR, by poliovirus 3C proteinase. Expression of a cleavage-resistant G3BP restored SG formation during poliovirus infection and significantly inhibited virus replication. These results elucidate a mechanism for viral interference with mRNP metabolism and gene regulation and support a critical role of G3BP in SG formation and restriction of virus replication.**

## INTRODUCTION

Infection of cells by poliovirus (PV) results in rapid viral disruption of cellular gene regulation at several levels. Many of these processes are mediated by the two viral proteinases, $2A^{pro}$ and $3C^{pro}$, which have evolved to inactivate key cellular targets to enable the virus to rapidly take over cell functions and subvert innate host defenses. PV induces rapid inhibition of translation of capped cellular mRNAs through cleavage of host initiation factors eIF4GI, eIF4GII, and PABP, which interact with the 5′ cap and 3′ poly(A) tail, respectively (Bovee et al., 1998a; Gradi et al., 1998; Joachims et al., 1999; Kuyumcu-Martinez et al., 2002; Liebig et al., 1993; Lloyd, 2006). In addition, viral proteinases cleave factors involved in Pol I, Pol II, and Pol III transcription (Weidman et al., 2003); disrupt nucleocytoplasmic traffic via cleavage of nuclear pore proteins (Gustin and Sarnow, 2001); and interfere with U snRNP assembly (Almstead and Sarnow, 2007). These multiple cleavage events together contribute to viral pathogenesis and interfere with the cell's ability to activate antiviral programs such as interferon secretion.

Mammalian cells form cytoplasmic granular RNA structures in response to many types of environmental stress. Stress granules (SG) are postulated to play a critical role in regulating mRNA metabolism during stress, and their formation may inhibit translation of many housekeeping mRNAs to favor translation of critical stress response proteins such as chaperones (Nover et al., 1989). SGs are thought to organize around stalled 48S preinitiation complexes, thus they contain heterogenous mRNAs and many translation initiation factors, including eIF4G, eIF4E, eIF3, eIF2, and PABP (Anderson and Kedersha, 2002a, 2006; Kedersha et al., 2005). In addition, several other RNA-binding proteins become concentrated in SGs, including tristetraprolin (TTP), fragile X mental retardation protein (FMRP), AU-rich element-binding protein (HuR), TIA-1, TIAR, and Ras-GAP SH3 domain-binding protein (G3BP-1) (Anderson and Kedersha, 2006). SGs are reported to assemble based on functions of TIA-1 and TIAR, which contain postulated prion-like domains in their C termini (Anderson and Kedersha, 2002b). In addition, G3BP-1 has been reported to be key for nucleation of SGs and is activated in SG formation by dephosphorylation (Tourriere et al., 2003). Overexpression of G3BP-1 or TIA-1 can lead to SG formation in cells (Kedersha et al., 2005; Tourriere et al., 2003), and both are considered to be marker proteins for SGs. However, the relative importance of G3BP-1 or TIA-1 in SG formation is unclear.

In addition to application of oxidative, heat, or nutritional stress to cells, several factors that interrupt protein translation can induce SG formation. Interestingly, compounds that promote disassembly of polysomes (e.g., puromycin) or block translation initiation (e.g., hippuristanol) also promote SG formation, whereas compounds that stabilize polysomes (e.g., cycloheximide) inhibit SG formation (Kedersha et al., 2000; Mazroui et al., 2006). It has been proposed that SGs can be induced by stalling ribosome preinitiation complexes via two pathways. The first is phosphorylation of eIF2α, which reduces concentrations of ternary complex eIF2-GTP-$tRNA^{i}$met, and the second pathway requires inhibition of eIF4A helicase function. Treatment of cells with many stressors is known to result in eIF2α phosphorylation, including arsenite stress, heat shock, and factors that induce the unfolded protein response (Anderson and Kedersha, 2006; Holcik and Sonenberg, 2005). Interference with eIF4A helicase function with

hippuristanol or pateamine A and application of mitochondrial inhibitors can also induce SG formation; thus, the exact trigger mechanism(s) of SG formation is unclear and may be multifaceted (Kedersha et al., 2002; Mazroui et al., 2006).

SGs interact with cytoplasmic RNA processing bodies (PBs) in cells. Processing bodies contain enzymes for decapping and degradation of mRNAs as well as argonaut proteins required for shRNA- and microRNA-mediated repression of gene expression (Anderson and Kedersha, 2006). However, degradative enzymes are not confined to the PBs, since SGs have also been reported to contain Ago2 and Xrn1 (Leung et al., 2006). Further, some RNA-binding proteins that stimulate mRNA decay, such as TTP, localize in both SGs and PBs, and overexpression of TTP prolongs physical interaction between SGs and PBs in cells (Kedersha et al., 2005). Taken together, an attractive hypothesis has been formulated that SGs comprise a dynamic intermediate compartment for storage and remodeling of mRNPs where mRNAs are protected from degradation but may be passed either back to polysome pools or on to PBs for degradation, depending on cellular conditions (Kedersha et al., 2005). Likely, SGs represent an intermediary in the close linkage between the mRNA translation and degradation that is required to protect the cell from insults and promote recovery after stress.

Viral infection can certainly be viewed as an alternate source of stress for cells, and SGs have been reported in some virus-infected cells. Alphavirus inhibits SG formation via an unknown mechanism (McInerney et al., 2005), and flavivirus inhibits SG formation via binding TIAR/TIA1 to viral RNA and NS3 (Emara and Brinton, 2007). Sendai virus-induced SGs in some infected cells and viruses expressing trailer RNAs that bind TIAR were found to antagonize SG formation (Iseni et al., 2002). Another report indicated that PV was able to induce SG formation by 3 hr postinfection, but studies were not carried through a complete infection cycle (Mazroui et al., 2006). Thus, it is unclear if RNA viruses generally induce or repress SG formation in cells.

Here, we investigated the relationship between a prototypical plus-strand RNA virus and SGs. We show that PV infection of cells causes an initial induction of SGs, then disperses SGs sharply during the remainder of the virus replication cycle. Further, PV infection prevents the ability of cells to accumulate SGs in response to strong stress stimuli. We show that G3BP-1 was cleaved by viral 3C protease with kinetics that correlate with the loss of SG formation in cells, but TIA proteins were stable. SG formation was inhibited by G3BP-1 cleavage and could be rescued in cells by expression of cleavage-resistant G3BP-1. RNAi knockdown of G3BP-1 also diminished the ability of cells to produce SGs. We report a virus attacking G3BP-1 function and elucidate a unique mechanism to block the ability of cells to reorganize mRNPs into SGs. The data show that intact G3BP-1 is required for SG formation and that G3BP-1 functions to restrict virus replication in cells.

## RESULTS

### PV Infection Activates Then Inhibits mRNA SG Formation

Since some plus-strand RNA viruses reportedly induce SGs, while others block their formation (Mazroui et al., 2006; McInerney et al., 2005), we wished to clarify the role of PV infection in SG formation. First we examined PV infections in several different cell types for extent and kinetics of SG formation. No additional stress agents were applied to cells to induce SG formation. G3BP-1 is an accepted marker for SG, thus immunofluorescence analysis of G3BP with a monoclonal antibody was performed to monitor SG formation. HeLa cells stained only for G3BP-1 show strong cytoplasmic distribution of G3BP-1 and intense formation of G3BP-containing granules after arsenite treatment (Figures 1 and 2). We observed that PV infection was able to induce the formation of SG or SG-like granules containing G3BP-1 within 2 hr of infection in HeLa, 293T, MCF7, and Vero cells; however, the intensity of SGs and the size of the granules was significantly less than in cells induced to form SGs with arsenite treatment (Figure 1). The type and extent of SG response also varied by cell type. In HeLa and Vero cells, only ~25% of cells formed large SGs, and many cells did not. 293T cells and MCF7 cells formed numerous small granules in the majority of cells within 2 hr of infection. The kinetics of SG formation was delayed in Vero cells, consistent with a slower viral replication cycle. Interestingly, SGs were observed to sharply diminish in all cell types after 3–4 hr of infection, correlating with the phase of the infection cycle where virus-dependent translation and RNA replication increase dramatically (data not shown). In each instance, granules were nearly absent in cells at 6 hr postinfection, suggesting that SGs were dispersed later in infection.

To distinguish if the loss of SGs in midphase of the replication cycle was due to dissolution of granules or a diminished ability to form SGs, we performed a series of infections and treated all infected cells with arsenite before fixation and preparation for immunofluorescence analysis. Examination of mock-infected cells before and after stress revealed rapid condensation of eIF4GI, PABP, and G3BP-1 into numerous large cytoplasmic SGs in all cells (Figure 2). Cells infected with PV for 2 hr revealed near normal ability to form SGs. In contrast, by 4 hr postinfection, cells displayed severely diminished capacity to form SGs, and by 6 hr postinfection, the majority of cells could not form SGs in response to arsenite. Uptake and metabolism of MTT in infected cells were nearly unchanged at 4–6 hr postinfection compared to controls (Figure S1 in the Supplemental Data available with this article online); thus, the decline in SG formation at these time points was not likely due to cell death or general metabolic toxicity. Therefore, PV infection results in an initial induction of SG formation, but continued infection results in blockage of the pathway that leads to SG formation in response to arsenite treatment.

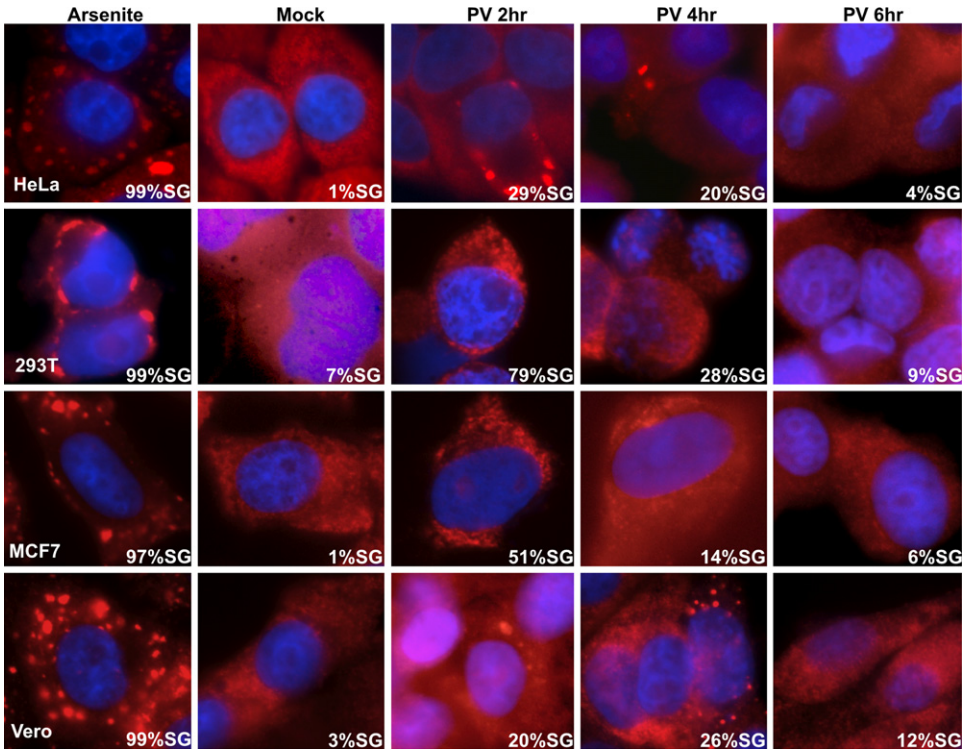


**Figure 1. PV Infection Initially Induces SG Formation Then Diminishes SGs**

Four series of panels show HeLa, 293T, MCF7, and Vero cells subjected to treatments and stained for G3BP (red). Panels are costained with Dapi (blue) to mark nuclei. Control cells were treated with arsenite or mock treated. Cells infected with PV (MOI = 10) for 2, 4, or 6 hr as indicated were not treated with NaArs. The percentages of cells containing SGs are indicated in the lower right of each panel (counting 150 cells in at least two repeat experiments).

## PV Infection Results in Cleavage of G3BP but Not TIA-1

PV interferes with translation by cleavage of eIF4G and PABP with viral 2A proteinase and 3C proteinases. To determine if the inhibition of SG formation was linked to viral proteases, we performed immunoblot analysis of G3BP-1 and TIA-1 in infected cells, both of which are reported to be required for SG formation (Anderson and Kedersha, 2002b; Tourriere et al., 2003). Figure 3A shows that PV induced cleavage of G3BP-1 that started after 2 hr postinfection and a putative cleavage product migrating near 55 kDa apparent mobility. This cleavage product was variably detected in immunoblots performed with a monoclonal antibody (data not shown), possibly due to destruction of the epitope or instability of G3BP-1 cleavage fragments in cells (note loss of signal at 6 hr, Figure 3A). The cleavage was apparently complete by 6 hr postinfection (hpi) in most experiments but not all (data not shown). In contrast to the observed G3BP-1 cleavage, levels of TIA-1 and TIAR were stable in cells through 5–6 hpi, which is after the replication phase of PV infection is mostly finished (Figure 3B). To further analyze the cleavage of G3BP-1, we expressed a fusion protein in cells containing GFP downstream of the G3BP coding region. PV infection resulted in cleavage of 100 kDa fusion protein, and a 40 kDa GFP-G3BP cleavage fragment appeared; however, extent of fusion protein cleavage was diminished compared to endogenous G3BP-1 (Figure S2A). The 40 kDa migration of the cleavage product containing a C-terminal GFP suggested that cleavage of G3BP-1 was occurring closer to the C terminus than the N terminus of the G3BP open reading frame.

PV produces two cysteine proteases (2A$^{pro}$ and 3C$^{pro}$) that cleave some cellular proteins as well as the viral polyprotein. To determine if either protease cleaved G3BP, we incubated $^{35}$S-radiolabeled G3BP-1 in vitro with purified 2A$^{pro}$ or 3C$^{pro}$. Examination of proteins by SDS-PAGE autoradiography revealed that incubation with 2A$^{pro}$ had no effect on G3BP (Figure 3C). This protease was highly active and cleaved all eIF4GI in similar lysates within 5 min (data not shown) (Kuyumcu-Martinez et al., 2004b). In contrast, 3C$^{pro}$ caused complete cleavage of G3BP-1 (Figure 3C). Only two cleavage products were observed, migrating with apparent mobilities of 55 kDa and 17 kDa, suggesting that a single cleavage site was present within the G3BP-1 sequence.

To further establish which cleavage fragment derived from N and C termini, we expressed G3BP-1 bearing an N-terminal His-tag in cells and incubated cell lysates with 3C$^{pro}$. Examination of His-specific immunoblots revealed full-length G3BP and 55 kDa bands, indicating that the large cleavage product contained the N-terminus

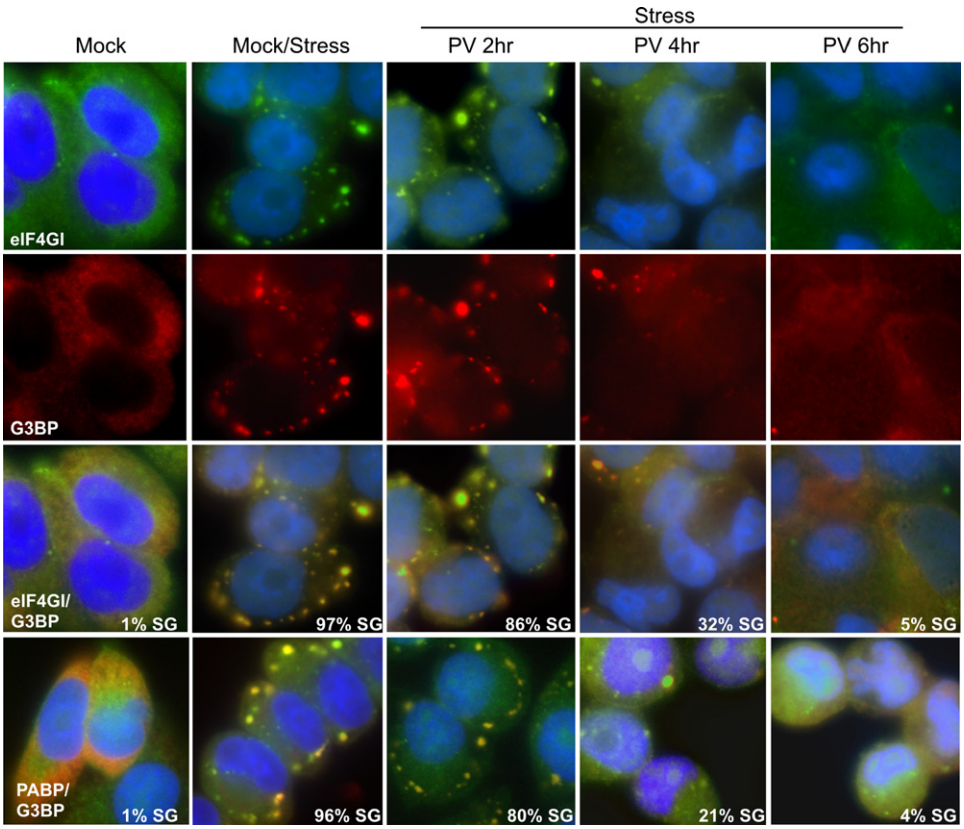


**Figure 2. PV Infection Blocks the Ability of Cells to Form SGs in Response to Arsenite Stress**

Mock-infected HeLa cells were untreated or stressed with arsenite for 30 min before immediate fixation and preparation for IFA. Infected cells were similarly treated with arsenite for 30 min beginning at the indicated time points. Cells were stained for eIF4GI and Dapi (top panels) or G3BP (second row panels). Merged images are shown on the third row of panels. Cells were also dual stained for PABP plus G3BP; merged images are shown in bottom panels. FITC-secondary antibody reveals eIF4GI or PABP, Texas red secondary antibody labels G3BP, and Dapi stain images nuclei.

(Figure S2B). Purified His-G3BP from bacteria was digested with 3C$^{pro}$, and the 17 kDa C-terminal cleavage product band (Figures S2C and S1D) was sequenced by Edman degradation and revealed a sequence consistent with cleavage of G3BP between amino acids 326 and 327. Cleavage site recognition by 3C$^{pro}$ entails both sequence requirements in the P4-P1′ positions (A/VxxQ/G) and conformational constraints (Blair and Semler, 1991; Dewalt et al., 1989; Ypma-Wong and Semler, 1987). All sequence requirements are met in the G3BP sequence (Figure S2D), similar to other known 3C$^{pro}$ cleavage sites in the viral polyprotein and in PABP. Significantly, the mapped 3C$^{pro}$ cleavage site in G3BP-1 lies immediately upstream of the RNA-binding RRM motif (Figure 3D) and results in separation of the NTF2-like protein-interaction domain from the RRM.

## Inhibition of SG Formation Requires Viral Replication

PV RNA replication can be blocked by treatment of cells with 2 mM guanidine-HCl, which inhibits the function of viral 2C ATPase (Pincus et al., 1987; Tolskaya et al., 1994). Guanidine treatment does not block eIF4GI cleavage, which is caused by low levels of 2A$^{pro}$ and activated cellular proteinases (Bonneau and Sonenberg, 1987; Bovee et al., 1998b). Cap-dependent translation is inhibited only 2-fold in guanidine-treated infected cells due to incomplete cleavage of eIF4GII by 2A$^{pro}$ and no cleavage of PABP by 3C$^{pro}$ (Gradi et al., 1998; Joachims et al., 1999). When infected cells were treated with guanidine and then stressed with arsenite, the ability to form SGs was not blocked, even through 6 hpi (Figure 4A). Immunoblot analysis of guanidine-treated infected cells showed that eIF4GI was cleaved with normal kinetics as expected, yet cleavage of G3BP-1 was completely abrogated (Figure 4B). This was consistent with the inhibition of 3C$^{pro}$ expression in guanidine-treated cells and suggested that high-level expression of 3C$^{pro}$ was required to block SG formation. Since guanidine-treated infected cells only contain cleaved eIF4GI (Figure 4B), the results also demonstrate that intact eIF4GI is not required to form SGs in cells and that the cleaved N terminus of eIF4GI can enter SGs.

## Cleavage-Resistant G3BP Restores SG Formation during Infection

To assess the importance of G3BP-1 cleavage in the mechanism of SG formation, we introduced a point mutation into the G3BP-1 coding sequence to block cleavage

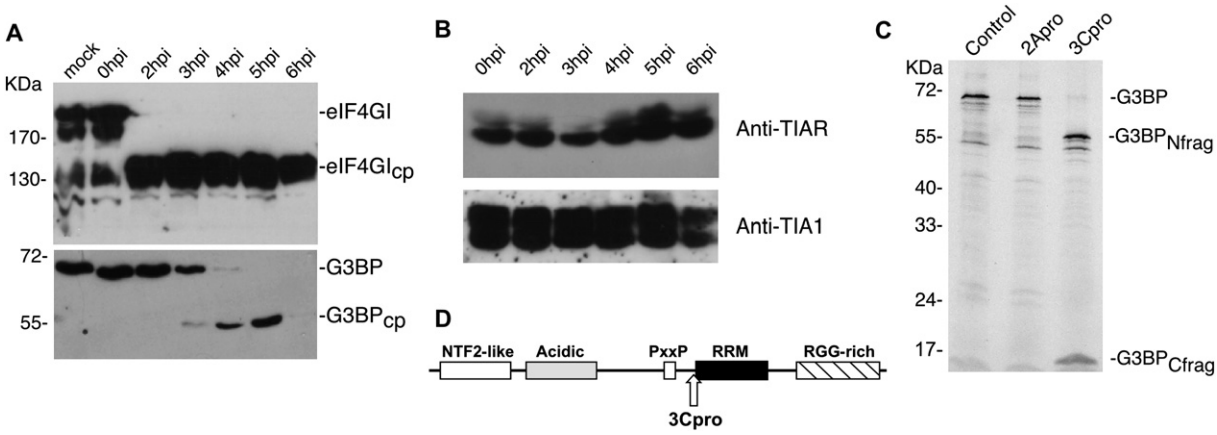


**Figure 3. PV Infection Results in Cleavage of G3BP by 3C Proteinase**

(A) Immunoblots showing kinetics of eIF4GI and G3BP cleavage in PV-infected HeLa cells and appearance of cleavage products (cp). Migration of molecular mass standards is shown on the left.

(B) Immunoblots show lack of significant cleavage of TIA-1 and TIAR during PV infection in HeLa cells. Apparent late degradation of TIA-1 shown here was only variably reproducible.

(C) G3BP is cleaved by poliovirus 3C$^{pro}$. Autoradiograph of His-G3BP radiolabeled by translation in vitro that was incubated with 0.5 μg of 3C$^{pro}$ or 2A$^{pro}$. N-terminal and C-terminal cleavage fragments are identified.

(D) Cartoon showing location of 3C$^{pro}$ cleavage site and protein domains of G3BP-1.

by 3C$^{pro}$. The mutation changed the scissile Q/G amino acid pair to E/G. First, we validated that the mutation blocked 3C$^{pro}$-mediated cleavage. G3BP$^{Q326E}$ was radiolabeled by translation in reticulocyte lysates and then incubated with increasing concentrations of recombinant 3C$^{pro}$. At the highest protease concentration, the mutant G3BP-1 was not cleaved at all, whereas the wild-type G3BP-1 was readily cleaved (Figure 5A). We also expressed His-G3BP or His-tagged G3BP$^{Q326E}$ in 293T cells that were subsequently infected with PV. Immunoblot analysis shows that ectopic G3BP expression was equivalent to endogenous G3BP in 293T cells (Figure 5B). During infection, the transgenic G3BP$^{Q326E}$ was not cleaved, whereas endogenous and transgenic G3BP were both cleaved, producing discernable cleavage products due to His tag and linker amino acids.

To determine if expression of cleavage-resistant G3BP-1 altered SG formation during infection, cells were transfected with His-G3BP or His-G3BP$^{Q326E}$ 36 hr before viral infection was initiated. At the beginning of the infection, the cells transfected with either transgene were equally able to form SGs (Figure 6A). As infection progressed, there was a decline in average SGs in both cells; however, cell cultures expressing G3BP produced 2- to 3-fold fewer average SGs containing eIF4GI and G3BP-1 than cells expressing G3BP$^{Q326E}$ (Figure 6C). Due to incomplete transfection efficiency, some cells were not able to form SGs by 6 hpi; however, a large fraction cells retained a robust SG response at 6 hpi. Importantly, expression of cleavage-resistant G3BP-1 restored the ability of cells to form SGs containing both eIF4GI and G3BP. To confirm the observed rescue of SG formation, cells were mock transfected ("No DNA") or transfected with plasmids expressing His-PCNA (as a control), His-G3BP, or His-G3BP$^{Q326E}$. These cells were similarly infected, Ars-stressed at 2 or 6 hpi, and stained with anti-TIAR and anti-His to directly visualize SGs in those cells expressing His-G3BP transgene (Figure 6B). With TIAR as a SG marker, untransfected cells and cells expressing PCNA quickly lost the ability to form SGs, whereas cells expressing HisG3BP or cleavage-resistant His-G3BP$^{Q326E}$ formed significant numbers of SGs per cell. In many cells, the intensity of His-G3BP signal correlated with the size or number of SGs (Figure 6B). There was a wide range of numbers of rescued SGs/cell (Figure 6D), and a few cells that expressed His-G3BP but did not form SGs. However, these results taken together suggest that intact G3BP-1 is required for SG formation and that cleavage by 3C$^{pro}$ is sufficient to block SG assembly in vivo.

To confirm that reduction in G3BP-1 levels in cells was sufficient to inhibit SG formation, we employed siRNAs to knock down G3BP-1 protein levels in cells. Application of control noncoding or GAPDH-specific siRNA had no effect on G3BP-1 levels or SG formation (Figure S2). Average G3BP-1 levels were reduced modestly (~3-fold) by G3BP-specific siRNA, and cells displaying G3BP knockdown had decreased number, size, and intensity of SGs that formed (Figure S2). In many G3BP knockdown cells, granules contained reduced G3BP and eIF4GI levels, as expected (Figure 7). However, other knockdown cells contained only very faint G3BP-staining granules but displayed strong eIF4GI SG foci, nearly unchanged from adjacent nonknockdown cells. Thus, the data showed that G3BP-1 knockdown by a noncleavage mechanism could reduce SG formation in cells, but overall this was less effective than virus-induced cleavage of G3BP-1.

We wanted to determine whether cleavage of G3BP-1 resulted in any benefit for virus replication in cells. To do this, we expressed G3BP or G3BP$^{Q326E}$ in MCF7 cells before infection. MCF7 cells were chosen because the viral

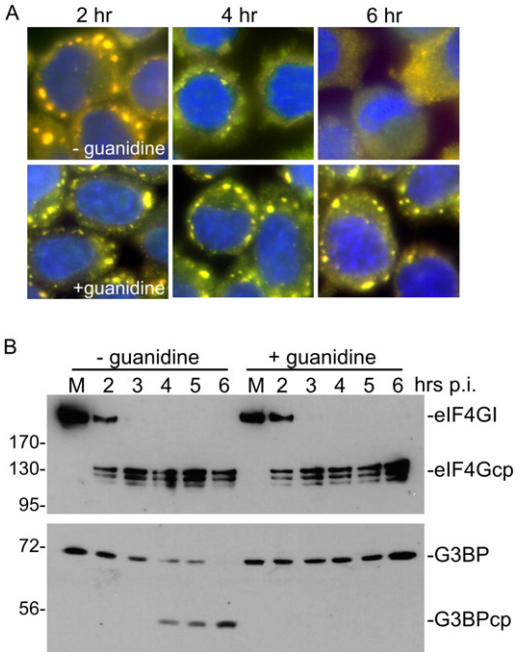


**Figure 4. PV Replication Is Required for Inhibition of SG Formation**

(A) PV-infected HeLa cells were treated with (+) or without (−) 2 mM guanidine as indicated, and all cells were stressed with Ars for 30 min before fixation and processing with G3BP and eIF4GI antibody for immunofluorescence.

(B) Immunoblot analysis of mock-infected (M) or PV-infected HeLa cell lysates collected at indicated time points postinfection with anti-eIF4GI and polyclonal anti-G3BP antibodies. Intact proteins and cleavage products (cp) are indicated on the right, and migration of molecular mass standards (kDa) is shown on the left.

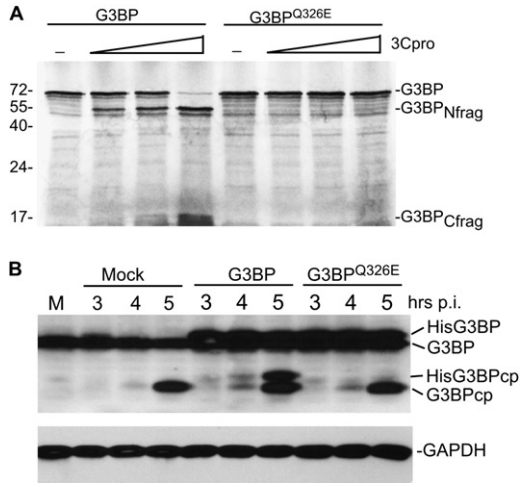


**Figure 5. G3BP-1 Bearing Q326E Mutation Is Resistant to 3C$^{pro}$ Cleavage**

(A) WT or mutant G3BP was translated in vitro in rabbit reticulocyte lysates containing $^{35}$S-Trans label. Translation products were incubated with increasing doses of 3C$^{pro}$ (0, 0.1, 0.3, 1.0 μg) for 2 hr at 37°C before proteins were analyzed by SDS-PAGE/autoradiography. Migration of intact G3BP and N-terminal and C-terminal cleavage fragments are shown on the right, and molecular mass standards are shown on the left.

(B) His-G3BP or His-G3BP$^{Q326E}$ was transiently expressed in 293T cells that were infected with PV 40 hr posttransfection. At the indicated time points postinfection, cells were harvested and cytoplasmic lysates were analyzed by immunoblot analysis with anti-G3BP antibody.

replication cycle is less robust and slower than in HeLa cells. Cells were cotransfected with low levels of control GFP expression plasmid, and cells were FACS sorted at 48 hr posttransfection to select transfected cells. The sorted transfected-cell populations were then infected with PV, and viral titers were determined after 24 hr replication. Figure 6E shows that cells overexpressing His-G3BP produced less virus than mock-transfected cells. Further, cells expressing cleavage-resistant His-G3BP$^{Q326E}$ produced 8-fold less PV than the controls. These data show that overexpression of G3BP-1, and especially cleavage-resistant G3BP-1, can interfere with viral replication significantly.

## DISCUSSION

Here we have shown that PV infection first induces SG formation to variable extents in cells and then later reverses the phenotype, and SGs do not form but are dispersed. After 3 hr infection the capacity of cells to form SGs is rapidly inhibited and is absent late in infection. The kinetics and degree of inhibition of SG formation correlated closely with the expression of 3C$^{pro}$ in the cell, which also correlated closely with cleavage of G3BP-1 in cells. We were able to rescue formation of SGs by expression of a cleavage-resistant form of G3BP-1, suggesting that intact G3BP-1 is required for formation of SGs in cells. We describe a virus controlling SGs by proteolytic cleavage of a host factor and a virus targeting G3BP-1 function instead of TIA-1.

Previously it was shown that PV could induce SG formation, but curiously, IFA analysis of SGs was not reported later than 3 hpi. Thus, the abrupt reversal of SG formation reported here was not elucidated or discussed (Mazroui et al., 2006). Semliki Forest virus was also observed to inhibit SG formation; however, a mechanism was not described. The data herein unite and extend the previous reports, since we show that a plus-strand RNA virus can both induce and block SG formation, and we provide a mechanism for viral inhibition of SG formation. The results also suggest that regulation of SG formation may be a broadly common theme of many RNA virus families during replication.

The mechanism of SG formation was initially linked to formation of abortive initiation complexes due to inhibition of translation by eIF2α phosphorylation. Indeed, all the stress treatments that induced SGs, such as arsenite, ER stress, and heat shock, result in increased eIF2α phosphorylation, and the four eIF2α kinases, PKR, GCN2, PERK, and HRI, can be viewed as sensors of various types of cellular stress (Kaufman, 2004). Further, drugs that destabilize polysomes (e.g., puromycin) promote SG assembly, whereas drugs that stabilize polysomes (e.g., cycloheximide, emetine) promote SG dispersion (Kedersha

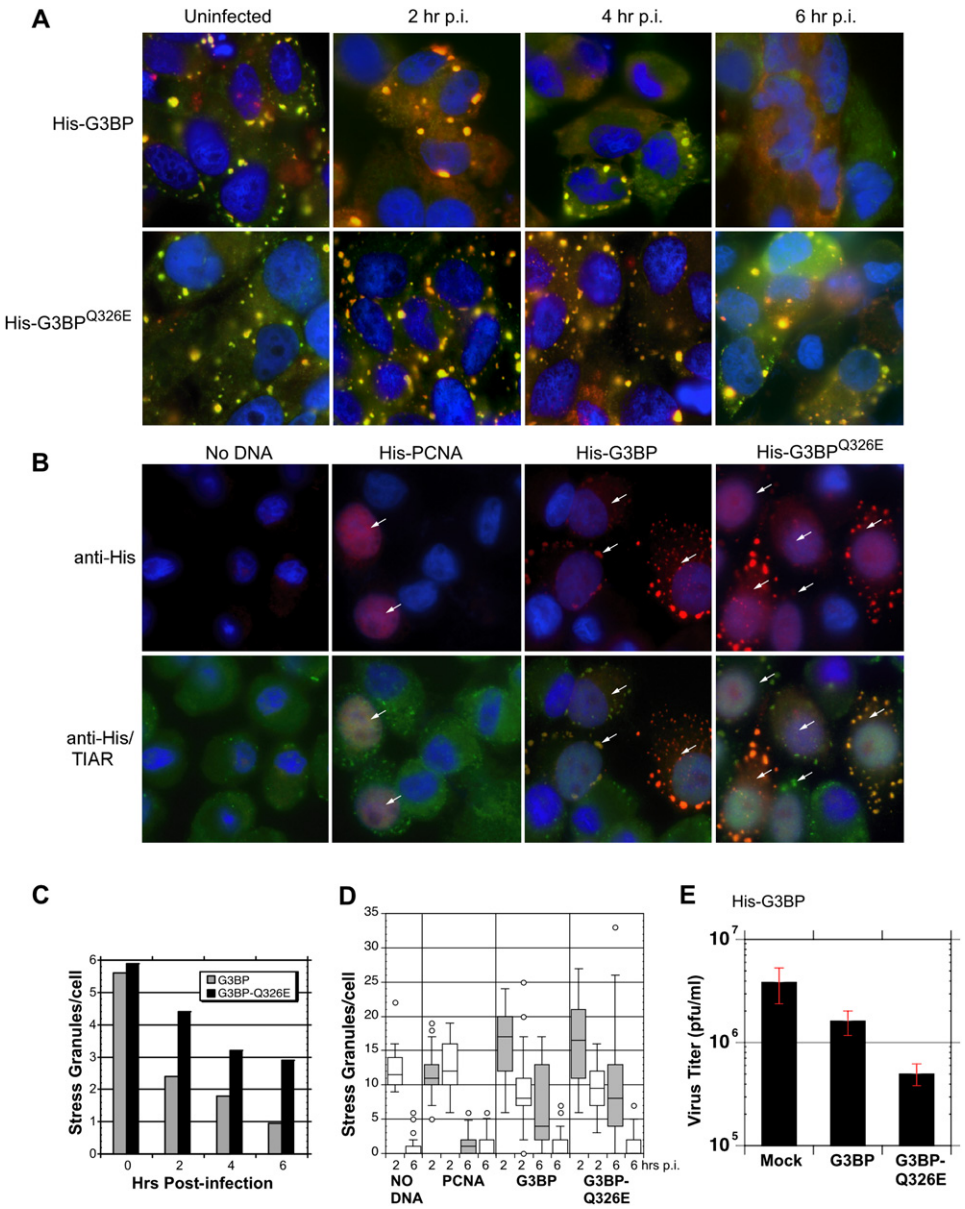


**Figure 6. Rescue of SG Formation in HeLa Cells Expressing Cleavage-Resistant G3BP-1**

(A) Cells were transfected with constructs expressing G3BP or His-G3BP$^{Q326E}$ for 24 hr and then infected with PV, MOI = 10. At time points, cells were stressed with arsenite to test SG formation capacity, then fixed and immunostained for G3BP-1 (TxRed) and eIF4GI (FITC). Merged images are shown.

(B) Mock-transfected (No DNA) or plasmid-transfected cells were infected at 36 hr posttransfection, arsenite treated at 2 or 6 hpi, then fixed and coimmunostained with anti-His and anti-TIAR antibodies. Expression of His-PCNA (proliferating cell nuclear antigen) was included as a control. All panels show cells at 6 hpi. Upper panels show cells stained for His-tagged proteins with TxRed plus Dapi, and lower panels show merged images for TxRed, TIAR (FITC), and Dapi. Arrows indicate cells expressing His-tagged proteins.

(C) Quantitation of SG formation in total cells from the experiment in (A); 120 cells were counted per time point.

(D) Distribution of SG formation in cells expressing His-tagged G3BP or G3BP$^{Q326E}$ represented by box graphs. Gray bars indicate SGs/cell in cells expressing His-tagged proteins in micrographs; white bars indicate SGs/cell in nontransfected or nonexpressing cells. Boxed regions indicate distribution of middle 50% of values, horizontal lines represent medians, capped vertical lines represent range of upper or lower quartile values, and circles represent outlier values. Over 120 cells were scored for each time point/condition.

(E) Expression of G3BP$^{Q326E}$ inhibits PV replication. MCF7 cells were mock transfected or cotransfected with either 1 μg pcDNA-HisG3BP or 1 μg pcDNA-HisG3BP plus 0.1 μg pcDNA-GFP. Forty-eight hours posttransfection, EGFP-expressing cells were selected by FACS, and then equal numbers of cells were infected with PV at MOI = 0.1. Viral titers were determined in cell supernates taken 24 hpi.

Error bars indicate standard deviation of the mean.

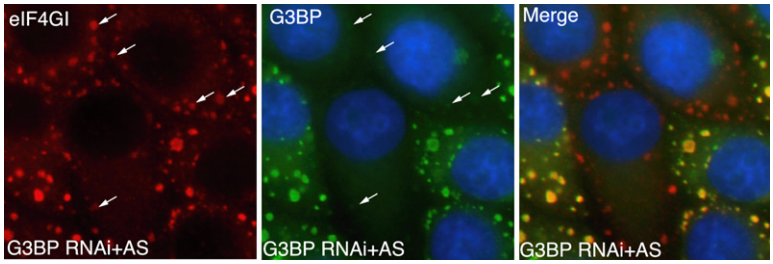


**Figure 7. Differential Reduction in eIF4G and G3BP Granules by G3BP siRNA**
Cells transfected with G3BP-specific siRNA were fixed and stained with eIF4GI- or G3BP-specific antibodies. Arrows denote granules containing abundant eIF4G but only trace G3BP.

et al., 2000). Finally, newly formed SGs contain most of the translation initiation factors, and a phosphomimetic eIF2α (S51D) mutant induces SG formation when expressed in cells. The cytoplasmic/nuclear shuttling proteins TIA-1 and TIAR rapidly redistribute from mostly nuclear to cytoplasmic localization upon stress and enter SGs as they form. Expression of truncated TIA-1 (lacking RRMs) that sequester endogenous TIA-1 was able to reduce SG formation (Kedersha et al., 1999, 2000). Thus, TIA-1 was proposed to bind specific transcripts in the 3′ UTR and assemble SGs around these transcripts based on its auto-assembly prion-like domain (Anderson and Kedersha, 2002a; Gilks et al., 2004).

Similar to TIA-1, G3BP-1 was shown by Tourriere et al. to localize to SGs, and overexpression of a central fragment of G3BP-1 that binds RasGAP and contains the S149 phosphorylation target was able to reduce SG formation in cells. Further, expression of a larger G3BP fragment that resembles the N-terminal fragment released by 3C$^{pro}$ cleavage was also able to reduce SG formation in cells (Tourriere et al., 2003). Similarly to TIA-1, G3BP can auto-associate into dimers and trimers (Tourriere et al., 2003). Thus, both G3BP-1 and TIA-1 have been branded effector molecules that promote SG assembly based on their ability to bind mRNA and auto-assemble into larger complexes, but it is unclear if both proteins are required or if they function independently in this process.

Several RNA-binding proteins located in SGs were found to induce SG formation upon overexpression, including TIA-1, G3BP-1, fragile X mental retardation protein, TTP, survival of motor neurons protein, and caprin-1 (Gilks et al., 2004; Hua and Zhou, 2004; Mazroui et al., 2002; Solomon et al., 2007; Tourriere et al., 2003). Interestingly, a caprin/RNA complex was suggested to directly trigger eIF2α phosphorylation, possibly by activating PKR (Solomon et al., 2007), but curiously, caprin-1 was also found associated with active translating polysomes. However, the observation that overexpression of a component of SGs drives SG formation does not demonstrate that component is required for SGs to form. Only mutants or fragments of G3BP-1 and TIA-1 have been shown to inhibit SG formation in cells, and we report a virus attacking G3BP-1 and blocking SG function. These observations show that G3BP-1 is critical for general formation of heterogeneous SGs and that scission of the G3BP-1 RRM domain from the known protein-interaction motif destroys this function.

We show that reduction of G3BP-1 via two means, cleavage or RNAi silencing, inhibits SG formation. Only viral cleavage was able to totally block formation of granules containing eIF4GI, suggesting that this mechanism is more potent, possibly from formation of dominant-negative cleavage products. RNAi silencing caused two phenotypes in cells displaying significant G3BP knockdown. In many cells abundant large SGs formed containing eIF4G but only trace foci of G3BP (Figure 7, arrows). Other cells showed a reduction in the number and size of SGs containing eIF4G. These results suggest that G3BP may be required for SG formation in a nonstoichiometric sense or that cells can compensate with other processes to sustain SG formation when G3BP is reduced but not totally eliminated.

SGs can also form through inhibition of eIF4A function, possibly by stalling scanning 48S ribosome complexes. Presumably, inhibition of translation per se is not sufficient to trigger SG formation, since treatment of cells with compounds that stabilize polysomes or block ribosome runoff of templates do not induce SGs (Mazroui et al., 2006). But how does PV induce SG formation? Currently, the mechanism is unclear. PV rapidly causes cleavage of eIF4GI and eIF4GII in cells, blocking cap-dependent translation initiation and dissociating the eIF4F complex. eIF4G cleavage may directly induce SG formation but will not form stalled 48S ribosome initiation complexes; rather, it will prevent their formation through inhibition of mRNA binding to 40S ribosomes. PV does not cleave eIF4A or directly inhibit its function. PV blocks induction of eIF2α phosphorylation until very late in infection, partly due to degradation of PKR (e.g., 5–6 hr) (Black et al., 1993; Mazroui et al., 2006; O'Neill and Racaniello, 1989); thus, there is no correlation between kinetics of SGs and eIF2α-PO$_4$ as seen in arsenite-stressed cells. Our guanidine experiments (Figure 5) did not test which viral gene products are required for SG induction. However, they did demonstrate that SGs do not require intact eIF4G to form, despite the fact that the majority of mRNPs that entered these SGs did contain capped mRNA. Our anti-eIF4GI antibody is N-terminal specific, demonstrating that the N terminus of eIF4G enters SGs. This is interesting, since cleavage of eIF4G is thought to result in dissociation of cellular ribosomes and mRNA. We do not yet know if SGs in PV-infected cells are deficient in 40S subunits or in the C-terminal ribosome-interacting domain of eIF4GI.

What is the advantage to PV from blocking SG formation? We have shown that G3BP-1 cleavage results in higher yields of virus during the replication cycle. There are several possibilities to explain this result. First, virus

infection induces a stress response that leads to activation of SGs and to potential limitation of translation factors that may be available for viral mRNA use. It is possible that large-scale SG formation may somehow sequester PV mRNA polysomes and repress viral translation. Another intriguing possibility is that the microRNA-mediated translation silencing functions through G3BP and micro-SGs. Indeed, reporter mRNAs that were translationally silenced by Let-7 miRNA were found to localize in a micro-granule immediately adjacent to PBs (Pillai et al., 2005) and enter SGs in a miRNA-dependent fashion (Leung et al., 2006). Thus, it is possible that translationally silenced mRNPs contain G3BP and are contained in foci that might be viewed as "micro-SGs." PV may attack G3BP to limit miRNA regulation of gene expression.

Other potential roles of G3BP in virus replication are possible. G3BP binds RasGAP and functions in RasGAP signaling to promote cell proliferation and survival downstream of Ras. G3BP-1 is constitutively phosphorylated in quiescent cells, but G3BP in any phosphorylation state can associate with RasGAP. However, the RasGAP-G3BP interaction is stimulated in proliferating cells (Gallouzi et al., 1998). G3BP phosphorylation, particularly at residue Ser149, may affect downstream functions such as SG assembly or reported endoribonuclease activity (Tourriere et al., 2001, 2003). Further, G3BP can bind mRNAs for Cdk7 and Cdk9, but this association increased cdk7 protein and reduced cdk9 protein levels (Lypowy et al., 2005). Therefore, G3BP has not been viewed exclusively as a negative regulator of translation. Thus, cleavage of G3BP may have additional undiscovered benefits for virus replication not investigated in this work.

It is important to point out that there are two G3BP genes. G3BP-1 was examined in this and all other SG-related publications, whereas G3BP-2, which is related by 44% homology, has not been previously reported to enter SGs and was not examined here. The scissile Q-G bond in G3BP-1 is not conserved in G3BP-2, and a potential 3C$^{pro}$ cleavage site was not detected elsewhere by examination of the protein sequence; thus, G3BP-2 is unlikely to be a substrate of 3C$^{pro}$. Further, since SG formation could be destroyed by G3BP-1 cleavage and rescued by expression of G3BP-1, we conclude that G3BP-2 has minimal or no functional role in SG formation.

PV is able to interfere with cellular mRNA expression at several levels via expression of its two proteinases. Transcription by Pol I, Pol II, and Pol III is inhibited by cleavage of key factors, including Tata-binding protein (Clark et al., 1991, 1993; Yalamanchili et al., 1997a, 1997b). Translation is regulated by cleavage of translation initiation factors eIF4G and PABP (Lloyd, 2006). Recently, Almstead and Sarnow showed that PV inhibited U snRNP assembly via cleavage of Gemin 3 by 2A$^{pro}$ (Almstead and Sarnow, 2007), and 2A$^{pro}$ has been shown to stabilize PV RNA in lysates (Jurgens et al., 2006). G3BP-1 cleavage by 3C$^{pro}$, reported in this work, extends viral regulation of mRNA metabolism to new levels that invoke indirect translation control. It will be interesting to determine if G3BP cleavage represents a mechanism for viral regulation of miRNA-mediated translation silencing or other forms of innate immunity.

## EXPERIMENTAL PROCEDURES

### Cells, Virus, and Stress Treatments

HeLa, 293T, MCF7, and Vero cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Cellgro) supplemented with 10% fetal calf serum and maintained at 37°C with 5% $CO_2$. Cells were infected with DMEM containing 2% FCS and PV type 1 (Mahoney strain) with a multiplicity of infection of 10 or as indicated in figure legends. Mock-treated controls received 2% FCS-DMEM without virus. PV was prepared and purified on CsCl gradients as previously described (Jones and Ehrenfeld, 1983). PV was titrated by plaque assay on HeLa cell monolayers overlaid with 2% FCS-DMEM containing 1% methylcellulose. To induce SGs, at various time points cells were stressed by addition of (0.5–1.0 mM final concentration) sodium arsenite (NaArs) to growth medium and incubation for 30 min before fixation and preparation for microscopy. MTT toxicity assays were performed as described by the manufacturer (Sigma) with 5 × $10^5$ infected 293T cells per time point. Absorbances were read at 570 nm, and viability was expressed as percent absorbance of mock-infected cells.

### Plasmids

The plasmid pcDNA3-HisG3BP was obtained from R. Kumar at the M.D. Anderson Cancer Center. It contains a BglII-EcoRI fragment containing the human G3BP1 open reading frame cloned into pcDNA3.1-HisC (Invitrogen) (Barnes et al., 2002). The plasmid pEGFP-G3BP was constructed by PCR amplification of the G3BP open reading frame from pcDNA3.1His-G3BP with primers that introduce EcoRI 5′ and BamHI 3′ restriction sites. Primer sequences were 5′-CCGGTGGATCCCCTGCCGTGGCGCAACCCC-3′ and 5′-GCAGAATTCACTGCCGTGGCGCAA-3′ for the forward and reverse directions. The PCR fragment was digested with EcoRI and BamHI and inserted into the same sites of the pEGFP-N2 vector (Clontech). The resultant plasmid produces G3BP-EGFP fusion proteins in cells.

#### *G3BP-1 Mutation*

The plasmids pEGFP-G3BP$^{Q326E}$ and pcDNA3-HisG3BP$^{Q326E}$ were made by quick-change PCR-based mutagenesis using the primers G3BPmut3Ca and G3BPmut3Cb with sequences 5′-CCAATCCGTGAGGCTGGTGAGGAAGGTGACATTGAACCC-3′ and 5′-GGGTTCAATGTCACCTTCCTCACCAGCCTCACGGATTGG-3′, respectively. PCR reactions were subjected to 95°C for 30 s, 55°C for 1 min, and 68°C for 14 min for a total of 16 cycles using Pfu DNA polymerase (Stratagene). The presence of the Q326E mutation in the resulting plasmid was confirmed by DNA sequence analysis.

### Transfections

Plasmids were introduced into cells using dual transfections with Lipofectamine (Invitrogen) and Fugene (Roche). Cells were transfected with 1 μg plasmid DNA plus 3 μl Lipofectamine per well in serum-free media for 3 hr at 37°C. Controls were mock transfected with 1 μg sheared salmon sperm DNA. After 3 hr, the Lipofectamine was removed, and the cells were supplemented with fresh media containing 2% serum for at least 1 hr. The cells were then transfected a second time with 1 μg/μl plasmid DNA plus 3 μl Fugene reagent per well overnight at 37°C. The following morning, the Fugene reagent was removed, and the cells were supplemented with fresh media containing 10% serum. Alternatively, cells were transfected once with Lipofectamine 2000. Transfected 293T cells were infected with PV 36 hr posttransfection. For some experiments 1 × $10^7$ MCF7 cells were cotransfected with 1 μg pcDNA3-HisG3BP (or pcDNA3-HisG3BP$^{Q326E}$) plus 0.1 μg pcDNA-eGFP. Forty-eight hours posttransfection, GFP-expressing cells were selected by sorting at the BCM Flow Cytometry Core, and cells were immediately infected with PV. For RNAi knockdown, 293T or HeLa cells were transfected with four pooled G3BP-specific siRNAs

(On-Target Plus, Dharmacon) in Lipofectamine 2000 (InVitrogen) according to the manufacturer's directions.

### Immunofluorescence

Cells grown on coverslips were fixed in 4% paraformaldehyde for 30 min on ice, treated with 1 mg/ml sodium borohydride (Sigma) for 5 min to quench autofluorescence, permeabilized for 30 min in PEM buffer (80 mM PIPES [pH 6.8], 5 mM EGTA, 2 mM $MgCl_2$) plus 0.5% Triton X-100, and blocked overnight at 4°C in TBS containing 5% powdered milk and 1% Tween 20. Primary anti-G3BP monoclonal (BD Transduction Labs), anti-TIAR (Santa Cruz), anti-His (BD Transduction Labs), or anti-eIF4GI antibodies (Byrd et al., 2005) were diluted to final concentration of 1:1000 in blocking buffer and incubated with the cells for 2 hr at room temperature. Following the primary antibody incubation, the cells were washed three times with blocking buffer before the addition of Texas red-labeled goat anti-mouse (Invitrogen) and FITC-labeled goat anti-rabbit or rabbit anti-goat (Zymogen) secondary antibodies at a final concentration of 1:600 in blocking buffer. The cells were fixed with 4% formaldehyde for 30 min and quenched with 1 mg/ml sodium borohydride for 5 min before being mounted on microscope slides and stored at 4°C.

### Immunoblots

Cell pellets from 1–5 × $10^6$ cells were lysed in 1% NP40 or freeze-thawed three times to release cytoplasmic components. Lysates were clarified by centrifugation for 10 min at 10,000 rpm. Aliquots were separated through 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Whatman). The following primary antibodies were used in immunoblots: anti-G3BP monoclonal (BD Transduction Labs), anti-TIA-1 (Santa Cruz), anti-TIAR (Santa Cruz), anti-eIF4GI (Byrd et al., 2005), anti-PABP (Kuyumcu-Martinez et al., 2004a), anti-His (BD Transduction Labs), and anti-EGFP (Clontech). Alternatively, anti-G3BP antibody was raised against a synthetic peptide sequence (EERQQTEPVVPDDSGTFYDQAC) (aa 157–177) coupled to keyhole limpet hemocyanin using an Imject maleimide-activated mcKLH kit (Pierce). Rabbits were immunized and sera were collected by ProSci (San Diego). Secondary peroxidase-coupled anti-mouse, anti-rabbit, or anti-goat antibodies (Santa Cruz) were used at 1:000–1:4000 dilutions. Immunoblots were developed using Enhanced Chemiluminescence (Pierce) according to the manufacturer's directions.

### In Vitro Translation

RNAs synthesized by in vitro transcription were translated in vitro in rabbit reticulocyte lysates (Promega) containing 0.5 μCi $^{35}$S-Trans label (MP Biomedicals) according to the manufacturer's directions.

### Supplemental Data

The Supplemental Data include three supplemental figures and can be found with this article online at http://www.cellhostandmicrobe.com/cgi/content/full/2/5/295/DC1/.

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