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Junín virus infection impairs stress-granule formation in Vero cells treated with arsenite via inhibition of eIF2α phosphorylation

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Stress granules (SGs) are ephemeral cytoplasmic aggregates containing stalled translation preinitiation complexes involved in mRNA storage and triage during the cellular stress response. SG formation is triggered by the phosphorylation of the alpha subunit of eIF2 (eIF2α), which provokes a dramatic blockage of protein translation. Our results demonstrate that acute infection of Vero cells with the arenavirus Junín (JUNV), aetiological agent of Argentine haemorrhagic fever, does not induce the formation of SGs. Moreover, JUNV negatively modulates SG formation in infected cells stressed with arsenite, and this inhibition correlates with low levels of eIF2α phosphorylation. Transient expression of JUNV nucleoprotein (N) or the glycoprotein precursor (GPC), but not of the matrix protein (Z), inhibits SG formation in a similar manner, comparable to infectious virus. Expression of N and GPC also impaired eIF2α phosphorylation triggered by arsenite. A moderate inhibition of SG formation was also observed when DTT and thapsigargin were employed as stress inducers. In contrast, no inhibition was observed when infected cells were treated with hippuristanol, a translational inhibitor and inducer of SGs that bypasses the requirement for eIF2α phosphorylation. Finally, we analysed SG formation in persistently JUNV-infected cells, where N and GPC are virtually absent and truncated N products are expressed abundantly. We found that persistently infected cells show a quite normal response to arsenite, with SG formation comparable to that of uninfected cells. This suggests that the presence of GPC and/or N is crucial to control the stress response upon JUNV infection of Vero cells.

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INTRODUCTION

Cell response to stress, e.g. excess heat, oxidation, UV irradiation and virus infection, comprises a series of changes in cellular metabolism that enable the cell to repair stress-induced damage and survive adverse environmental conditions (Anderson & Kedersha, 2008; Thomas *et al.*, 2011). Translation inhibition is accomplished by inactivation of the alpha subunit of eIF2 (eIF2α) by specific kinases that respond to distinct stress stimuli. As a consequence of global translational arrest, the appearance of conspicuous structures in the cytoplasm of stressed cells, designated stress granules (SGs), becomes evident (Anderson & Kedersha, 2002). SGs vary slightly in composition according to the cell stressor employed and the time course of induction, but always contain polyadenylated mRNA and a number of translation initiation factors, comprising the non-canonical 48S pre-initiation complex (eIF3; eIF4E and eIF4G, small ribosomal subunits) and RNA-binding proteins such as T-cell-restricted intracellular antigen 1 (TIA-1), the TIA-1 related protein (TIAR) and the poly(A)-binding protein (PABP) (Anderson & Kedersha, 2008; Buchan & Parker, 2009; Thomas *et al.*, 2011). Members of a second class of RNA granules related closely to SGs are known as processing bodies (PBs), which contain the microRNA-dependent silencing protein GW182 as well as components of the 5′–3′ mRNA-degradation pathway, such as the decapping enzymes DCP1a, DCP2 and Hedls (human enhancer of decapping), and exclude ribosome subunits and PABP (reviewed by Anderson & Kedersha, 2008; Buchan & Parker, 2009; Thomas *et al.*, 2011). However, SGs and PBs differ in several ways: PBs are constitutive and are observed in actively growing, unstressed cells. SG assembly, but not PB formation, usually requires the inactivation of eIF2α by stress-induced phosphorylation at Ser51. This post-translational modification is accomplished by any of the four cellular kinases activated by different stresses: dsRNA-dependent protein kinase (PKR), haem-regulated inhibitor (HRI), general control non-derepressible-2 (GCN2) and PKR-like endoplasmic reticulum kinase (PERK) (Sonenberg & Hinnebusch, 2009). Whether the

oxidative stress caused by arsenite activates HRI (de Haro *et al.*, 1996; McEwen *et al.*, 2005) or PKR (Brostrom *et al.*, 1996; Daher *et al.*, 2009) remains controversial.

In some cases, virus infection triggers SG formation transiently by activating eIF2α kinases and thus inhibiting translation initiation. SGs may function to limit infection, and poliovirus, West Nile virus, dengue virus and Semliki Forest virus can inhibit SG formation (reviewed by Beckham & Parker, 2008; Buchan & Parker, 2009; Thomas *et al.*, 2011). In this regard, the virus-production rates of vesicular stomatitis virus, Sindbis virus and herpes simplex virus increase in mouse embryo fibroblasts knocked out for TIA-1 (Li *et al.*, 2002). However, RNA-binding proteins present in SGs can have a positive impact on some virus infections: the binding of TIAR to the 3′ stem–loop structure in the negative strand of West Nile virus promotes the synthesis of the positive strand (Beckham & Parker, 2008; Schütz & Sarnow, 2007).

Junín virus (JUNV), aetiological agent of Argentine haemorrhagic fever, belongs to the family *Arenaviridae*, a group of enveloped viruses with genomes composed of two negative-sense ssRNA segments (L and S) that encode the viral proteins N (nucleoprotein), L (viral RNA-dependent RNA polymerase), GPC (glycoprotein precursor; G1 and G2) and Z (matrix protein), using an ambisense coding strategy (Buchmeier, 2002; Meyer *et al.*, 2002). JUNV internalization into Vero cells triggers the activation of the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) signalling pathway (Linero & Scolaro, 2009), which is regulated by both DNA and RNA viruses by either activating or inactivating some aspects of it, in order to achieve an efficient replication process (Cooray, 2004). Phosphorylated Akt promotes cell survival by inhibition of a number of pro-apoptotic proteins, and activates its main downstream effector Raptor/mTOR, which is a major controller of cap-dependent translational initiation (Ikenoue *et al.*, 2009). In view of these facts, we hypothesized that SGs and PBs may affect JUNV multiplication. In the present study, we found that acute JUNV infection was able to inhibit SG formation induced by several stressors, impairing normal eIF2α phosphorylation when stress was induced by arsenite treatment. In addition, SG formation induced by translational inhibitors that bypass the requirement for eIF2α phosphorylation was not affected by JUNV infection. Finally, we found that SG formation in persistently JUNV-infected cells is comparable to that in uninfected cells. These observations suggest that virus components that are expressed early during infection are able to affect the cellular response to stress. Supporting this notion, we observed that transfected full-length N or GPC, but not Z, blocked SG formation.

RESULTS

JUNV infection of Vero cells does not induce SG formation

In order to assess whether JUNV infection induces SG formation, Vero cells were infected with JUNV and the presence of SGs was investigated by indirect immunofluorescence assay (IFA) for the SG markers TIA-1 or PABP-1 at several time points after infection. Infected cultures, characterized by the presence of cells positive for viral N and GPC [Fig. 1a(i–iv)], did not show the presence of SGs at either 24 or 48 h post-infection (p.i.). At 24 h p.i., the proportions of cells expressing GPC or N were 15 ± 5 and 25 ± 8 %, respectively. At 48 h p.i., these values increased to 57 ± 8 and 72 ± 7 %, respectively. In all cases, the subcellular localization of TIA-1 and PABP-1 was quite normal, and these molecules were detected in the nucleus or the cytoplasm, respectively [Fig. 1a(i–iv)]. The lack of cellular stress response to JUNV infection, visualized as SG formation, was not due to the inability of Vero cells to respond to stress; as can be seen in Fig. 1(b), treatment of cells with sodium arsenite readily induced SG formation in a dose-dependent manner, with a maximum of 90 % of cells bearing SGs after treatment with 500 μM sodium arsenite, an oxidative-stress inducer. As expected, arsenite was able to induce eIF2α phosphorylation in these cells, whereas JUNV infection did not modify the phosphorylation level of this factor in comparison to non-stressed uninfected cells (Fig. 1c).

The presence of PBs was analysed in parallel experiments, using eIF4E and Hedls as markers. We found no significant differences in the number of cells bearing PBs, and differences in abundance, size and subcellular distribution were not observed between infected and mock-infected controls [Fig. 1(v–vi)]. Collectively, these results suggest that JUNV cytopathic effect in Vero cells is not preceded by a stress response characterized by SG formation or alteration of PB dynamics.

JUNV inhibits SG formation upon arsenite-induced stress

It has been shown that several viruses prevent SG assembly when infected cells are exposed to stress conditions (Emara & Brinton, 2007; Montero *et al.*, 2008; Thomas *et al.*, 2011). To investigate whether JUNV infection interferes with SG formation in response to cellular stress, Vero cells were mock-infected or infected with JUNV and exposed to oxidative stress by treatment with 0.5 mM sodium arsenite for 60 min at 24 or 48 h p.i. Infection progression was evident by the increment in the number of cells expressing the viral proteins N and GPC [Fig. 2(i–ii)]. SGs were visualized by either TIA-1 or PABP staining and, remarkably, a reduction in SG formation was observed accompanying infection progression [Fig. 2(iii–iv)]. One day after infection, the percentage of cells showing SGs was 80 % relative to control cultures, and was reduced further to 40 % at 48 h p.i. [Fig. 2(iii–iv)]. The effect was observed for the two SG markers TIA-1 and PABP, which were analysed in three independent experiments.

In addition to SG formation, cellular stress enhances the presence of PBs, and thus we analysed their distribution by

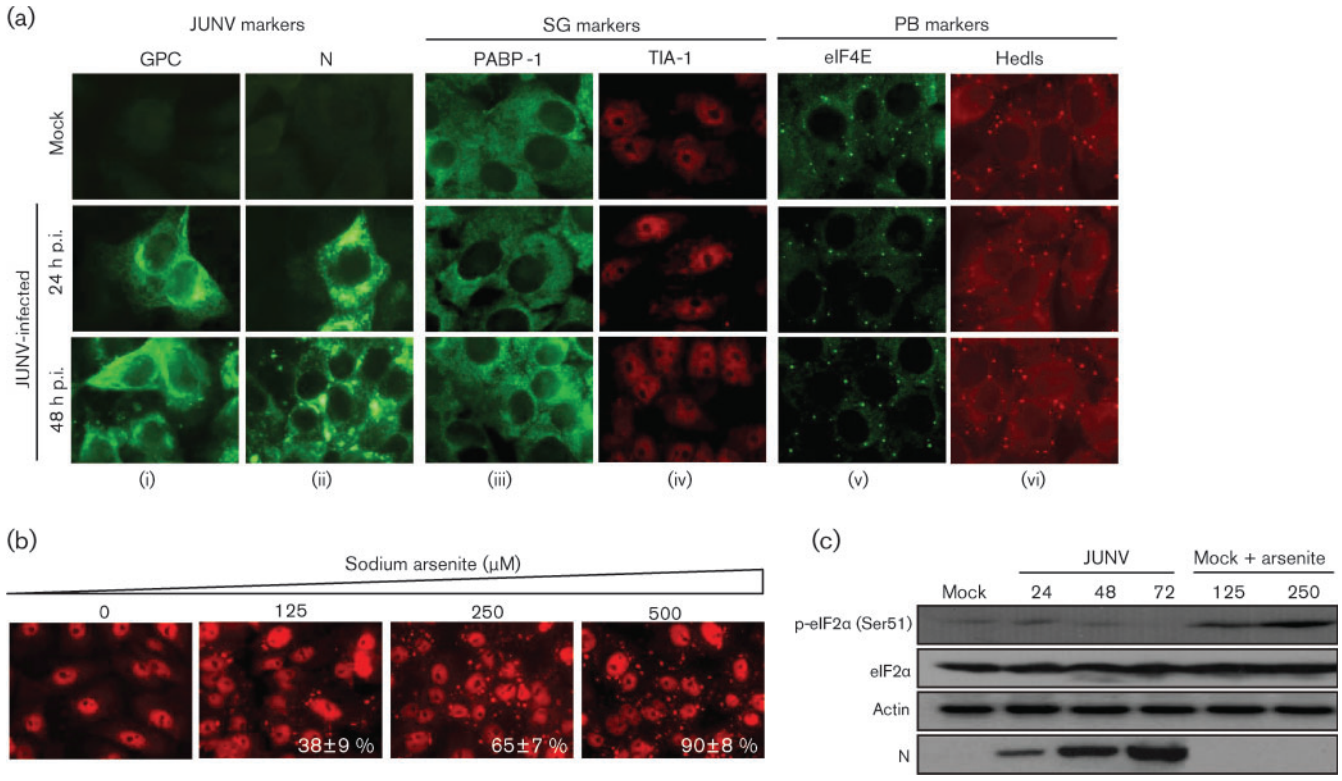

Fig. 1. Presence of SGs and PBs in JUNV-infected cells. (a) Vero cells were mock-infected (upper row) or infected with JUNV at an m.o.i. of 1 and, at 24 h p.i. (middle row) and 48 h p.i. (lower row), cells were fixed and processed for IFA. Cells were stained for viral antigens with anti-GPC and anti-N antibodies [(i) and (ii), respectively], for SGs with anti-PABP and anti-TIA-1 antibodies [(iii) and (iv), respectively] and for PBs with anti-eIF4E and anti-Hedls antibodies [(v) and (vi), respectively]. (b) Vero cells were treated with sodium arsenite at 125–500 μM for 1 h before processing for IFA. Numbers indicate the percentage of cells with SGs stained for TIA-1, from a total of 150 counted cells. (c) Vero cells were mock-infected or infected with JUNV at an m.o.i. of 1. At 24, 48 and 72 h p.i., JUNV-infected cells were processed for Western blotting to detect phospho-eIF2α (Ser51), total eIF2α, N and actin. Alternatively, mock-infected cells were untreated or treated for 1 h with 125 or 250 μM sodium arsenite before processing for Western blotting.

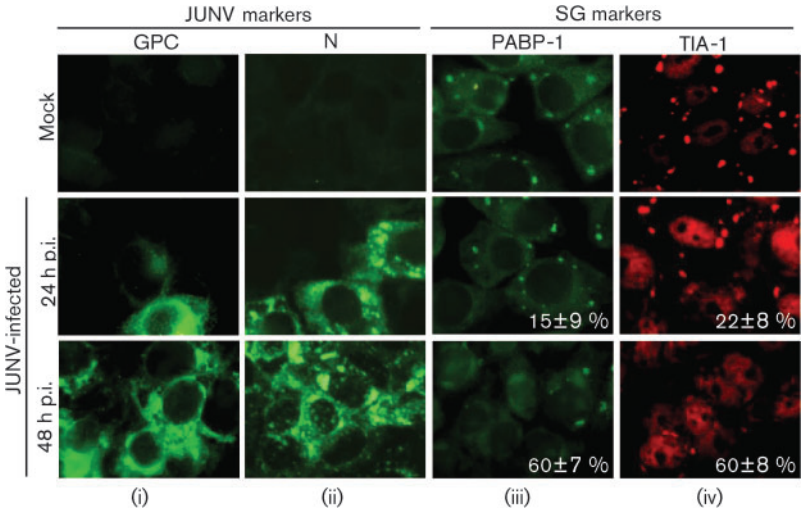

Fig. 2. SG formation induced by arsenite treatment in JUNV-infected cells. Vero cells were mock-infected (upper row) or infected with JUNV at an m.o.i. of 1 and, at 24 h p.i. (middle row) or 48 h p.i. (lower row), cultures were treated for 1 h with 500 μM sodium arsenite before processing for IFA. Cells were stained for viral antigens with anti-GPC and anti-N antibodies [(i) and (ii), respectively] and for SGs with anti-PABP-1 and anti-TIA-1 antibodies [(iii) and (iv), respectively]. Numbers in images indicate the percentage of inhibition of cells with SGs, from a total of 150 counted cells, for each stress marker. Values represent means ± SD of three independent experiments.

staining Hedls. We found that arsenite increased PB size in all cases, and that the abundance of PBs in JUNV-infected cells did not differ significantly from that in mock-infected cells at either 24 or 48 h p.i. (data not shown). Thus, JUNV infection specifically affects stress-induced SGs, without significantly affecting the response of closely related silencing foci, namely PBs.

Next, to analyse whether the effect on SGs is connected directly with the expression of JUNV proteins, we stained N and TIA-1 simultaneously with specific antibodies. As before, SGs were absent from unstressed cells, either N-positive or N-negative (Fig. 3a). Following exposure to arsenite, SG formation was observed, but the presence of SGs was significantly lower in N-positive cells. We found that <20 % of N-positive cells contained SGs, whereas >90 % of neighbouring N-negative cells harboured SGs upon stress induction performed 2 days p.i. (Fig. 3b).

Arsenite induces mostly an oxidative-stress response. To assess whether the inhibitory effect similarly affects endoplasmic reticulum (ER)-stress models, we investigated the response of JUNV-infected cells to DTT or thapsigargin, two known inducers of ER stress that indirectly activate PERK (Loschi *et al.*, 2009; McEwen *et al.*, 2005; Thomas *et al.*, 2011). We found that SG formation upon either thapsigargin or DTT treatment was hampered in N-expressing cells, in comparison with neighbouring N-negative cells or with non-infected cultures (Fig. 3c, d). DTT-induced SG formation was reduced from 94 to 55 %, whereas thapsigargin-induced SG formation was reduced from 60 to 39 % (Fig. 3f). Collectively, these observations indicate that JUNV impairs SG formation induced by either oxidative or ER stress. In both cases, SG formation is mediated by eIF2α phosphorylation. Then, we investigated whether JUNV similarly affects eIF2α phosphorylation-independent SGs. We used hippuristanol, an eIF4A inhibitor that induces SG assembly by blocking translation initiation directly (Mazroui *et al.*, 2006; Thomas *et al.*, 2009). Remarkably, we found that JUNV did not affect SG induction by this drug (Fig. 3e). In both infected and non-infected cultures, hippuristanol-induced SGs were observed in 100 % of cells, whereas arsenite-induced SGs were reduced from 92 to 17 % (Fig. 3f). Hippuristanol-induced SGs are sensitive to polysome-stabilizing factors (Thomas *et al.*, 2009) and thus our results indicate that the virus does not affect polysome integrity or any other step downstream of eIF2α phosphorylation.

In view of these findings, we hypothesized that formation of SGs in Vero cells may be detrimental for JUNV multiplication. To test this hypothesis, virus yield obtained in infected cells that had been stressed for 1 h prior to infection was monitored by a plaque assay of cell supernatants at 24 h p.i. Pre-treatment with arsenite, thapsigargin or hippuristanol, at concentrations that induced SG formation in >90 % of cells, inhibited virus yield by 84, 80 and 92 %, respectively, suggesting that SG formation previous to infection impaired virus multiplication.

JUNV infection impairs eIF2α phosphorylation

The first step governing SG formation is the phosphorylation of eIF2α by different kinases that sense specific stress factors. Thus, we sought to compare eIF2α phosphorylation levels in JUNV-infected and mock-infected cells by Western blot analysis. As expected, basal phosphorylation was relatively low in control or JUNV-infected cells analysed at 48 h p.i. (Fig. 4). Upon treatment with arsenite, DTT or thapsigargin, non-infected cultures showed a strong phospho-eIF2α signal, which increased with increasing concentrations of stressors. In contrast, in the case of arsenite, eIF2α phosphorylation was much lower in JUNV-infected cells, which responded weakly to increasing arsenite concentrations (Fig. 4a). This was not the case for DTT- and thapsigargin-treated JUNV-infected cultures, which showed phospho-eIF2α levels similar to those in stressed uninfected cells (Fig. 4b, c). This result suggested that JUNV infection might abrogate SG formation induced by arsenite by blocking eIF2α phosphorylation. Reduced phospho-eIF2α levels were not a consequence of a blockage of total eIF2α expression by JUNV. As can be seen in Fig. 1(c), total eIF2α levels in JUNV-infected cells at 24, 48 and 72 h p.i. were similar to those in uninfected controls. To test this further, we performed additional studies using salubrinal, an inhibitor of eIF2α dephosphorylation (Boyce *et al.*, 2005). If defective eIF2α phosphorylation causes defective SG formation, a reversion is expected in the presence of the eIF2α phosphatase inhibitor. We found that salubrinal partially compensates the effect of JUNV infection in arsenite-treated Vero cells. SGs were induced in 25.4 ± 6.2 % of JUNV-infected cells treated with arsenite, and this value increased to 46.8 ± 8.6 % when cells were treated with arsenite in the presence of salubrinal. A more moderate increase in SG formation was observed in mock-infected cells when stress was induced in the presence of salubrinal. SGs were induced in 62.6 ± 9.7 % of control cells treated with arsenite, and this value increased to 88.3 ± 10.5 % when these cells were treated with arsenite in the presence of salubrinal. Thus, SG inhibition by JUNV infection was partially reversed by an eIF2α phosphatase inhibitor, indicating that defective eIF2α phosphorylation is implicated directly, without excluding additional factors.

To test which viral proteins are involved in the resistance to SG formation, we transfected Vero cells with plasmids expressing full-length N, GPC, Z fused to eGFP (Z–eGFP) or eGFP as a control. We found that, in the absence of JUNV infection, the expression of N or GPC was sufficient to provoke a strong inhibition of arsenite-induced SG formation (Fig. 5): N expression allowed SG formation in only 3.6 ± 2 % of cells and, similarly, SGs were observed in only 3.7 ± 2 % of GPC-expressing cells. In contrast, cultures expressing Z–eGFP or eGFP showed SGs in 29 ± 9 and 40 ± 10 % of cells, respectively (Fig. 5). These observations indicate that both N and GPC are important in determining the resistance to SG formation.

In accordance with these results, transient transfection of N or GPC reduced the number of cells with detectable levels

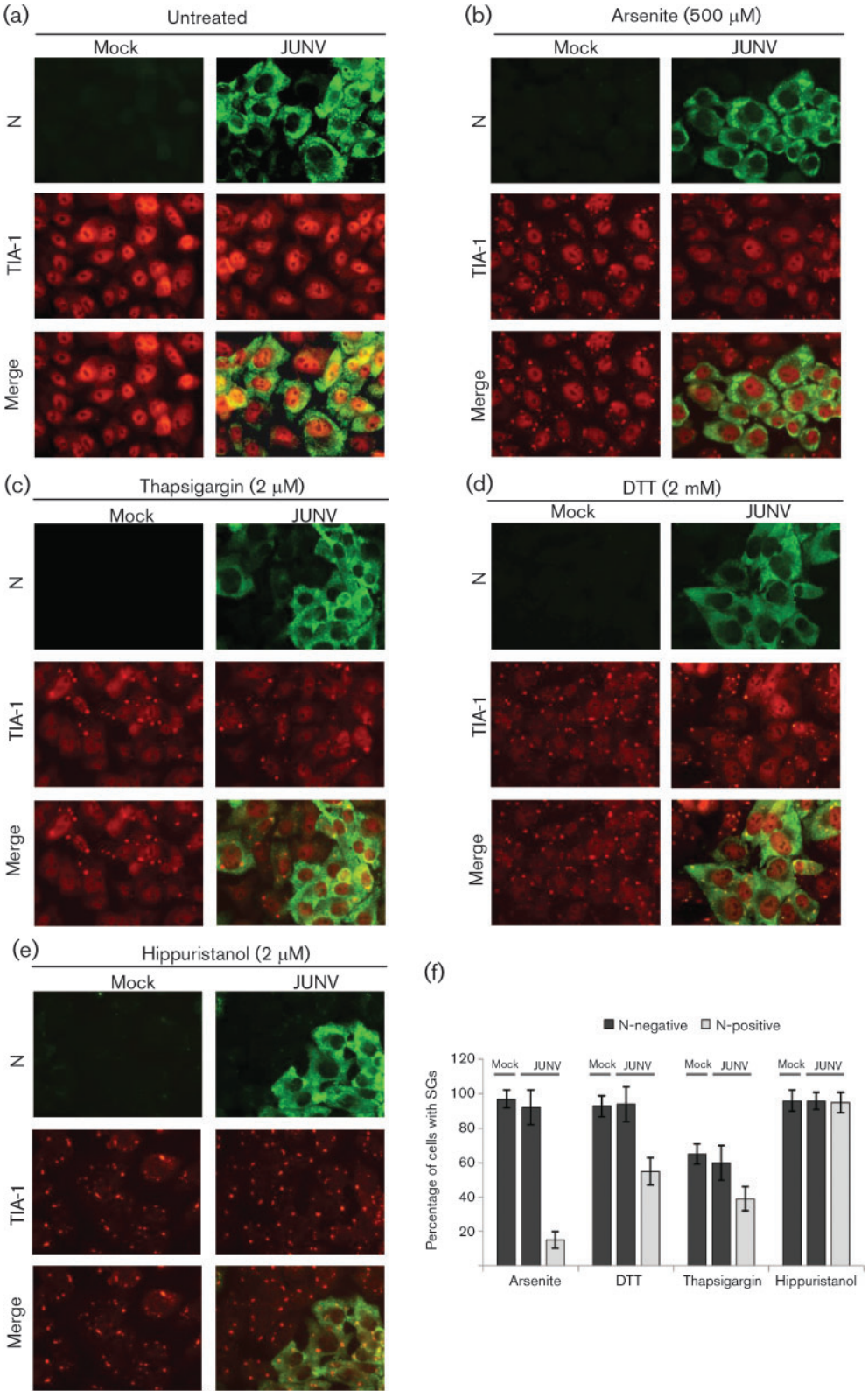

Fig. 3. SG formation induced by thapsigargin, hippuristanol and DTT in JUNV-infected cells. Vero cells were mock-infected or infected with JUNV at an m.o.i. of 1 and, at 48 h p.i., cells were untreated (a) or treated for 1 h with 500 μM sodium arsenite (b), 2 μM thapsigargin (c), 2 mM DTT (d) or 2 μM hippuristanol (e) before processing for IFA. N-positive cells exhibiting SGs were detected by using anti-N and anti-TIA-1 antibodies. The graph (f) indicates the percentage of N-positive and N-negative cells exhibiting SGs in mock-infected or JUNV-infected cells treated with sodium arsenite, DTT, thapsigargin or hippuristanol.

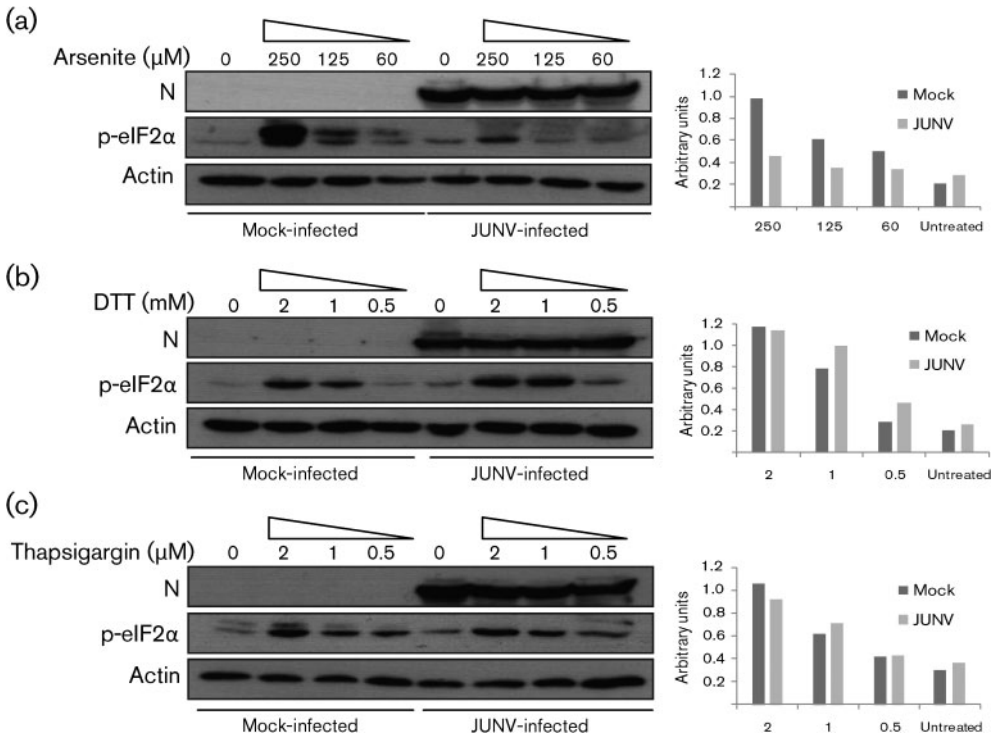

Fig. 4. Phosphorylation of eIF2α induced by different stress inducers in JUNV-infected cells. Vero cells were mock-infected or infected with JUNV at an m.o.i. of 1 and, at 48 h p.i., cells were untreated or treated for 1 h with different concentrations of sodium arsenite (a), DTT (b) or thapsigargin (c), and then processed for Western blot assay to detect N, phospho-eIF2α (Ser51) and actin. The graphs on the right indicate fold change of eIF2α phosphorylation expressed as densitometric units (Scion Image software) of bands normalized to the actin level relative to the untreated, mock-infected control from each treatment.

of phospho-eIF2α upon treatment with arsenite (Fig. 6). The immunofluorescence signal for phosphorylated eIF2α was low in non-treated cells and was detected in a dispersed pattern in cells either expressing or not expressing N, GPC or eGFP. Stressed cultures transfected with eGFP showed higher levels of phospho-eIF2α in both non-expressing and expressing cells (>2-fold). In contrast, in stressed N- or GPC-transfected cells, only non-expressing cells showed a similar increase in fluorescence, whereas N- and GPC-expressing cells maintained the level of phospho-eIF2α found in non-stressed cells (Fig. 6b).

Persistent JUNV infection does not interfere with SG formation

Persistent infection, a salient biological feature of arenaviruses, is established readily by JUNV in Vero cells once the initial acute stage of infection is overcome and the surviving cells repopulate the monolayer. In contrast to the acute stage of infection, persistence is characterized by a scarce or null level of infectivity, high levels of truncated N products and low levels of full-length N, greatly reduced levels of GPC and a marked resistance to superinfection by homologous and heterologous antigenically related viruses (Ellenberg *et al.*, 2002).

To assess whether SG formation is hampered similarly by persistent JUNV infection, we used a persistently infected cell line, termed V3, at 6 months p.i. (see Methods). Under basal conditions, V3 cells did not show the presence of SGs at either 3 or 12 months p.i. (data not shown). Strikingly, we found that, when V3 cells were exposed to stress by treatment with arsenite, thapsigargin or DTT, SG formation occurred in a fashion similar to that seen in mock-infected cells (Fig. 7a). In contrast to acutely infected cells, most N-positive V3 cells showed SGs, independent of the levels of N expression. It should be emphasized that the anti-N antibody employed for IFA does not distinguish between N and its truncated products, which are predominant in persistently JUNV-infected cells. Most N-expressing cells in V3 cultures, which showed fluorescence in coarse granules, formed SGs upon stress induction.

Next, we monitored eIF2α phosphorylation in V3 cells after treatment with arsenite or DTT. Taking into account that acute JUNV infection of Vero cells leads to an impairment of SG formation by reducing eIF2α phosphorylation, we analysed the effect on SG formation in V3 cells superinfected with JUNV. We found that arsenite or DTT treatment induced eIF2α phosphorylation in both cultures (Fig. 7b, c), suggesting that persistently JUNV-infected cells, either superinfected or not, were unable to maintain

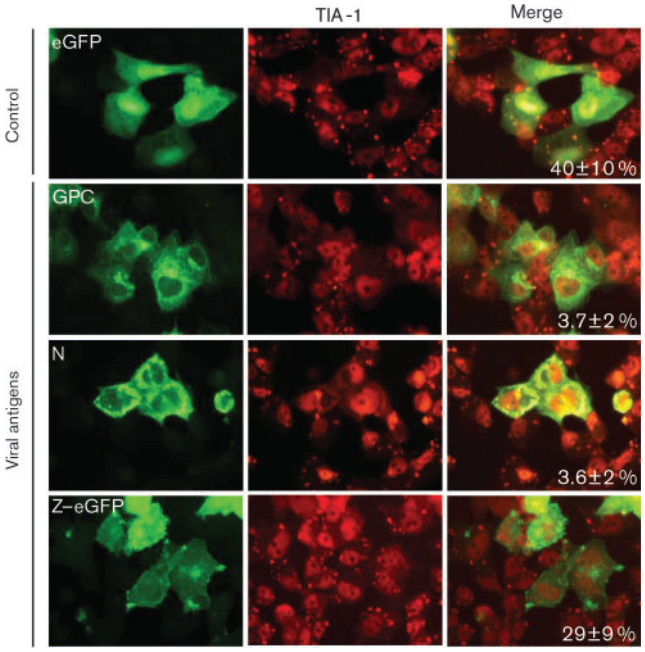

Fig. 5. SG formation in N-, GPC- and Z-transfected cells. Vero cells were transfected with pcDNA3.1 expression plasmids of the viral proteins N, GPC or Z–eGFP); eGFP was used as a control. At 36 h post-transfection, cells were stressed for 1 h with 500 μM sodium arsenite and then processed for IFA, to evaluate the presence of SGs stained for TIA-1. Numbers in images indicate the percentage of transfected cells with SGs, from a total of 250 counted cells. Values represent means ± SD of three independent experiments.

low levels of eIF2α phosphorylation after arsenite treatment, as described above for Vero cells acutely infected with JUNV. V3 cells showed phosphorylation levels of eIF2α similar to those in uninfected Vero cells (data not shown). Of note, superinfection of V3 cells with JUNV did not modify the level of expression of virus antigens in these cells, and only very scarce amounts of infectivity could be detected in supernatants (data not shown; Ellenberg *et al.*, 2004). Resistance to superinfection of V3 cells would explain the fact that JUNV was not able to modulate SG formation in these cells.

DISCUSSION

In this study, we found that JUNV infection of Vero cells does not induce SG formation. Moreover, JUNV abolishes SG formation upon treatment of cells with arsenite, thapsigargin and DTT, suggesting the capability of this virus to prevent a stress response that would prevent protein translation, reducing virus yield. This modulation of the cellular stress response was associated, particularly in the case of arsenite, with an inhibition of eIF2α phosphorylation associated with the presence of N and GPC viral proteins. On the other hand, PB dynamics were similar to those of

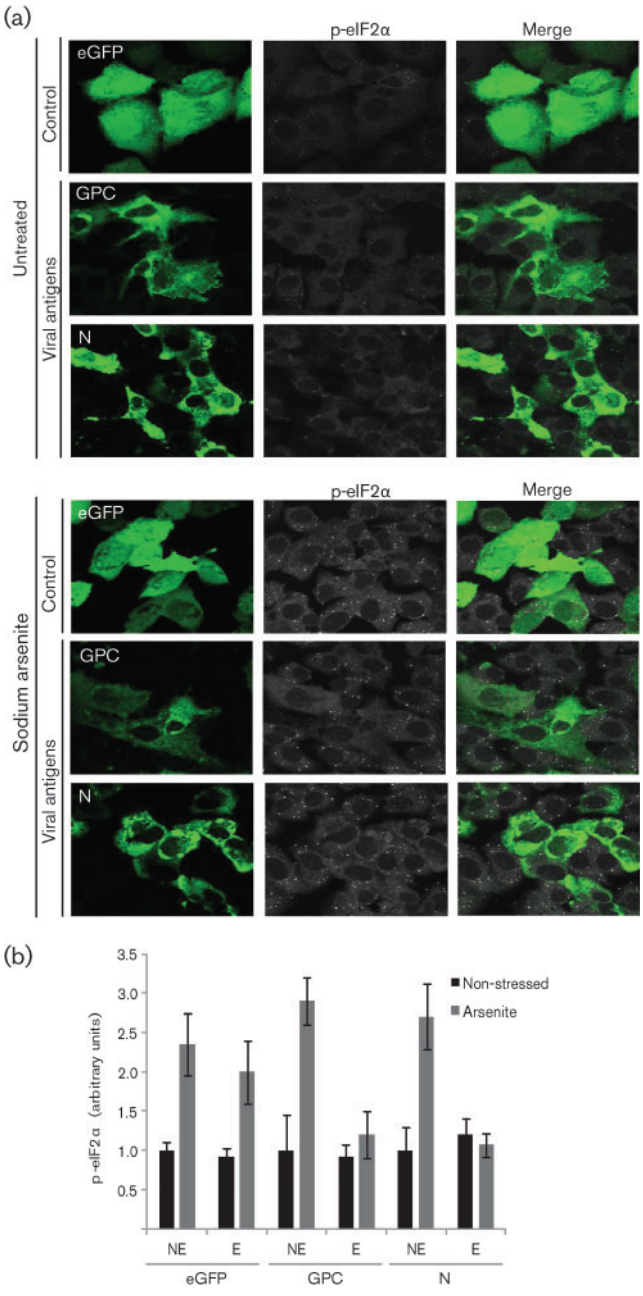

Fig. 6. Phosphorylation of eIF2α in N- and GPC-transfected cells. Vero cells were transfected with pcDNA3.1 expression plasmids of the viral proteins N and GPC, or eGFP (green) used as control. At 36 h post-transfection, cells were stressed for 1 h with 500 μM sodium arsenite and then processed for IFA, to evaluate the phosphorylation of eIF2α at Ser51 (grey). (b) Levels of phosphorylated eIF2α were determined by immunofluorescence in single cells expressing eGFP, GPC or N under non-stress conditions (black bars; $n=20$) or stressed by arsenite exposure (grey bars; $n=35$) and in neighbouring non-expressing cells (NE) under non-stress ($n=20$) or stress ($n=30$) conditions, using Image-J software.

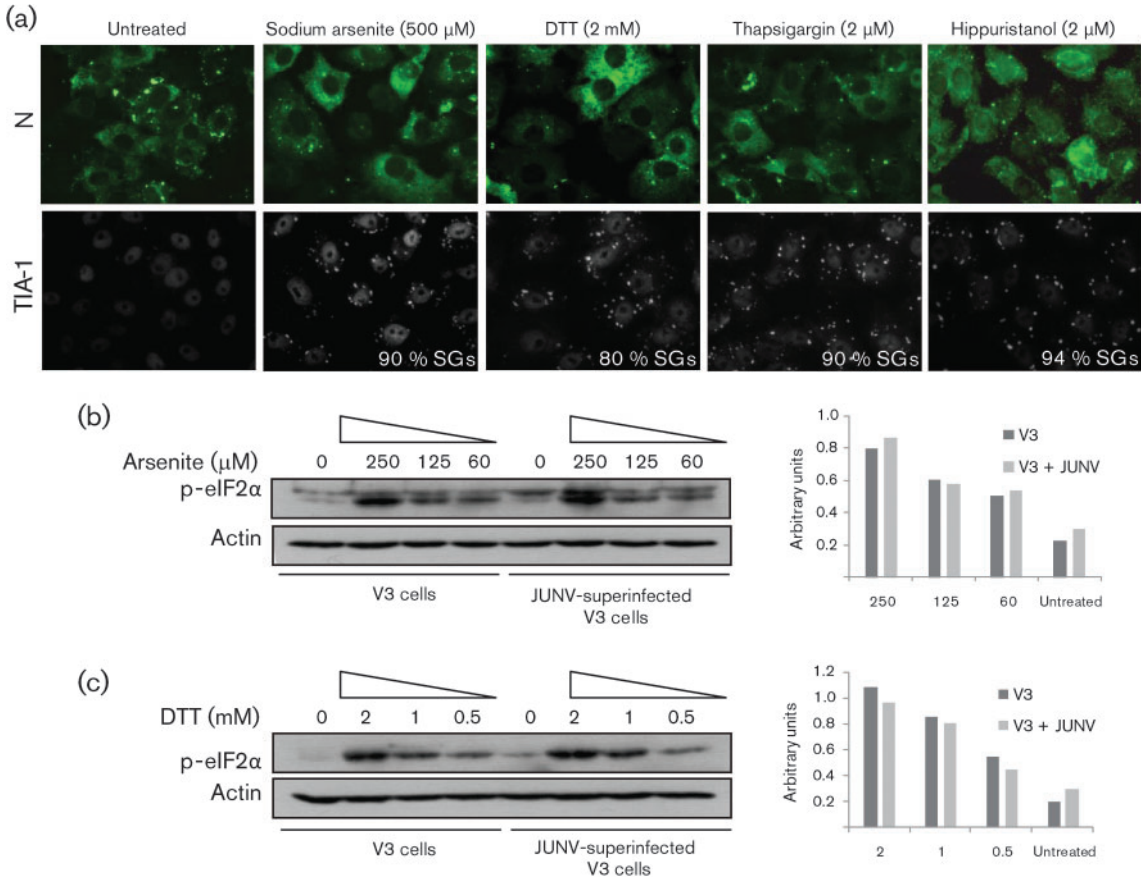

Fig. 7. SG formation in persistently JUNV-infected cells. (a) Persistently JUNV-infected Vero cells (V3) at 200 days p.i. were untreated or treated during for 1 h with 500 μM sodium arsenite, 2 mM DTT, 2 μM thapsigargin or 2 μM hippuristanol and then processed for IFA using anti-N and anti-TIA-1 antibodies. Numbers in images indicate the percentage of cells with SGs, from a total of 150 counted cells for each stress treatment. V3 cells and JUNV-superinfected V3 cells (m.o.i. of 1) were treated at 48 h p.i. with different concentrations of sodium arsenite (b) or DTT (c) for 1 h and then processed for Western blot assay to detect phospho-eIF2α (Ser51) and actin. The graphs on the right indicate fold change of eIF2α phosphorylation expressed as densitometric units (Scion Image software) of bands normalized to the actin level relative to the untreated, mock-infected control for each treatment.

uninfected controls. This is in agreement with the notion that PBs contain mRNAs repressed by eIF2α phosphorylation-independent mechanisms, and further suggests that stress-induced eIF2α-independent silencing is not affected by JUNV infection. Oxidative stress induced by arsenite leads to eIF2α phosphorylation by PKR (Brostrom *et al.*, 1996; Daher *et al.*, 2009). Control of PKR activation has been developed by several viruses, particularly those that manipulate the interferon response, where PKR plays a key role (Gale *et al.*, 2000; Schneider & Mohr, 2003). The N protein of the prototypic arenavirus lymphocytic choriomeningitis virus (LCMV) inhibits the type 1 interferon response through a mechanism that involves interferon regulatory factor 3 (IRF-3) (Martínez-Sobrido *et al.*, 2006). Our results are compatible with JUNV affecting eIF2α phosphorylation levels by blocking either specific kinases or the cognate phosphatase, or both. This is supported by the observation that salubrinal partially reverses the effect of JUNV on SGs induced by arsenite, suggesting an enhanced PP1-dephosphorylation activity by JUNV, as described for human cytomegalovirus (Hakki & Geballe, 2008) and herpes simplex virus (Zhang *et al.*, 2008). It remains to be elucidated whether it is N or GPC that binds PP1, as reported for the DPL71 protein from African swine fever virus, which reduces phospho-eIF2α levels in transfected P15 cells (Zhang *et al.*, 2010).

In addition to deregulated eIF2α phosphorylation, the mechanism underlying abrogation of SG formation during acute JUNV infection may involve the unavailability of key SG components, as reported for other viruses. Rotavirus inhibits SG formation early after infection, despite eIF2α becoming phosphorylated and remaining in this state throughout the virus replication cycle, leading to inhibition of cellular protein synthesis without affecting translation of viral mRNAs significantly. Rotavirus relocates PABP from

cytoplasm to nucleus, thus making this protein unavailable for SG assembly (Montero *et al.*, 2008). We found that PABP remained in the cytoplasm of JUNV-infected cells, suggesting that PABP translocation is not a strategy employed by JUNV, thus highlighting a diversity of mechanisms evolved by viruses to interfere with SG formation. Moreover, the fact that JUNV infection reduced phospho-eIF2α levels in the case of arsenite, but not DTT or thapsigargin, suggests a parallel mechanism, other than eIF2α phosphorylation, that is exerted by this virus to control SG formation. This is in accordance with the observation that salubrinal only partially reversed the inhibition of SG formation induced by infection. Inhibition may reflect the necessity of JUNV for SG components for replication. Indeed, virus yield was diminished greatly when SG formation previous to infection was induced by arsenite, thapsigargin or hippuristanol. Similarly, human immmunodeficiency virus (HIV) inhibits SGs and PBs to assemble specific ribonucleoparticles, termed Staufen 1 HIV-1-dependent RNA transport ribonucleoproteins (SHRNPs), which serve as a scaffold for efficient packaging of the viral RNA (Abrahamyan *et al.*, 2010). On a similar basis, West Nile virus infection dramatically increases the resistance to SG induction by arsenite and use of TIA-1 and/or TIAR as a viral transcription factor, and promotes the relocalization of TIAR and TIA-1 to the cytoplasm, thus improving viral RNA synthesis, inhibiting SG formation and preventing the shut-off of host translation (Emara & Brinton, 2007).

We also found that a persistently JUNV-infected cell line, namely V3, did not form SGs spontaneously, as in the case of acutely infected cells, and both acutely and persistently infected cells exhibited the presence of PBs in a manner similar to mock-infected controls. The fact that, during persistence, a marked downregulation in the expression of JUNV GPC and N was observed, together with the presence of N-derived truncated products (Ellenberg *et al.*, 2002, 2004), agrees with our observation that GPC and N abolish SG formation by modulating eIF2α phosphorylation. Recently, it has been demonstrated that acute LCMV infection of Huh7 cells selectively induces the activating transcription factor 6 (ATF6) branch of the cellular stress response, known as the unfolded-protein response (UPR), without activating the PERK and IRE1 branches. Expression of individual LCMV proteins revealed that the viral GPC, but not other viral proteins, is responsible for the induction of ATF6. Rapid downregulation of the viral GPC during transition from acute to persistent LCMV infection restored basal levels of UPR signalling (Pasqual *et al.*, 2011). This may allow the persistent virus to 'merge' into the normal background level of ER stress of the host, and this may be crucial for the establishment of a long-term asymptomatic chronic infection. In persistently JUNV-infected cells, the lack of GPC and the scarce amount of full-length N would be responsible for the restoration of the arsenite-induced stress response.

METHODS

Cells, drugs and virus. Vero cells were grown in minimum essential medium (MEM) containing 5 % FBS (Invitrogen) and 50 μg gentamicin ml^{-1}. Cells were subcultured weekly and maintained in MEM/1.5 % FBS after infection. A stock of JUNV strain XJCl3 was prepared by infecting BHK-21 cells at an m.o.i. of 0.5 and harvesting supernatant at 4 days p.i. Virus infectivity was quantified by a p.f.u. assay on Vero cells. Establishment of persistently JUNV-infected Vero cells, named V3 cells, was accomplished by infecting confluent Vero cell monolayers with JUNV at an m.o.i. of 0.1. Cells were maintained with MEM/1.5 % FBS until complete recovery of the monolayer at around 25–30 days p.i. Thereafter, V3 cells were cultured in the same way as uninfected-control Vero cultures (Ellenberg *et al.*, 2002).

Infection of Vero or V3 cells grown in 24-well microtitre plates was performed by inoculation with JUNV (m.o.i. of 1). After 1 h adsorption, supernatant was removed and MEM/1.5 % FBS was added to the plates. For stress treatments, sodium arsenite (Sigma), thapsigargin (Calbiochem), DTT (Invitrogen) and hippuristanol (kindly supplied by Dr J. Pelletier, McGill University, Montréal, Québec, Canada) were prepared at the concentration indicated for each assay, in MEM/1.5 % FBS, and added at indicated times p.i. 1 h before processing for Western blot or indirect immunofluorescence assays. When indicated, drugs (250 μM arsenite, 5 μM thapsigargin and 5 μM hippuristanol) were added 1 h before infection; supernatants were collected at 24 h p.i. and titrated by p.f.u. assay. Salubrinal (Calbiochem) was used at 25 mM.

Immunological assays. Western blot analyses were performed as described previously (Ellenberg *et al.*, 2004). Briefly, cell monolayers (10^5 cells) grown in 24-well microtitre plates were washed with PBS and lysed with 25 μl equal parts of PBS and SDS-PAGE loading buffer (Bio-Rad). Cell lysates were separated by SDS-PAGE (10 % polyacrylamide gel) and transferred to a PVDF membrane (Hybond P; Amersham Pharmacia) in a dry system (Multiphor II; LKB Instruments). Membranes were blocked with TBS containing 0.1 % Tween and 5 % non-fat dry milk at 4 °C overnight. Then, membranes were washed with 0.1 % Tween in TBS and incubated with the indicated primary antibody in blocking buffer for 1 h at 37 °C. After rinsing in 0.1 % Tween in TBS, secondary antibodies were diluted in blocking buffer and incubated with the cells for 1 h at 37 °C. Peroxidase-coupled secondary antibodies were visualized by a chemiluminescence-detection system (ECL; Amersham Pharmacia). The following primary antibodies were used: mouse monoclonal anti-JUNV N (NA05AG12; Sanchez *et al.*, 1989) at 1 : 600, rabbit polyclonal anti-eIF2α (total) and anti-phospho-eIF2α (Ser51) (Cell Signaling Technology) at 1 : 1000, and rabbit polyclonal anti-actin (Cell Signaling Technology) at 1 : 1000. Goat anti-mouse (Sigma) at 1 : 1000 and goat anti-rabbit (Amersham) at 1 : 1000 were used as secondary antibodies.

For IFA, cells grown on coverslips were fixed by incubation in 4 % paraformaldehyde in PBS for 15 min at room temperature, washed three times with PBS and finally permeabilized by incubation in 0.2 % Triton X-100 in PBS for 20 min at room temperature. Fixed cells were rinsed three times with PBS before incubation in blocking buffer (3 % BSA, 0.15 % Triton X-100 in PBS) for 1 h at 37 °C. Primary antibodies were diluted in blocking buffer and added for 1 h at 37 °C. After rinsing with PBS, cells were incubated with secondary antibodies for 1 h at 37 °C. Washed coverslips were then mounted on a 90 % glycerol solution in PBS containing 2.5 % 1.4-diazabicyclo (2.2.2) octane (DABCO). As primary antibodies for IFA, goat anti-TIA-1 (Santa Cruz Biotechnology) at 1 : 200, mouse anti-PABP1 (provided by Evita Mohr, University of Hamburg, Germany) at 1 : 200, rabbit anti-eIF4E (Cell Signaling Technology) at 1 : 50, rabbit anti-Hedls (Bethyl Laboratories) at 1 : 200, mouse monoclonal anti-JUNV N (SA02BG12; Sanchez *et al.*, 1989) at 1 : 300 and mouse monoclonal anti-JUNV glycoprotein 1 and

GPC (GB03BE08; Sanchez *et al.*, 1989) at 1 : 300 were used. The following secondary antibodies were used: donkey anti-mouse–Alexa 488 (Molecular Probes) at 1 : 400, donkey anti-goat–Cy3 (Molecular Probes) at 1 : 200, goat anti-rabbit–FITC (Sigma) at 1 : 100 and goat anti-rabbit–TRITC (Sigma) at 1 : 100.

Plasmids and transfection. For transfection assays, Vero cells grown on coverslips were transfected with 0.5 μg plasmid pcDNA3.1 encoding the viral antigens N, GPC or Z–eGFP (Artuso *et al.*, 2009) or eGFP (as a control), using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. At 36 h post-transfection, cells were treated with 0.5 mM sodium arsenite in MEM/1.5 % FBS for 1 h, and then were fixed for IFA. Transfected cells were evaluated for the capacity to induce SG formation in response to arsenite treatment, using TIA-1 as an SG marker and anti-N or anti-GPC antibodies (Sanchez *et al.*, 1989) as virus markers. Alternatively, transfected cells were evaluated for the capacity to phosphorylate eIF2α by using an anti-phospho-eIF2α antibody (CST), as described previously (Thomas *et al.*, 2009).

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