Rubella virus (RUBV) replicates slowly and to low titre in vertebrate cultured cells, with minimal cytopathology. To determine whether a cellular stress response is induced during such an infection, the formation of Ras-GAP-SH3 domain-binding protein (G3BP)-containing stress granules (SGs) in RUBV-infected cells was examined. Late in infection, accumulation of G3BP granules was detected, albeit in fewer than half of infected cells. Active virus RNA replication was required for induction of these granules, but they were found to differ from SGs induced by arsenite treatment both in composition (they did not uniformly contain other SG proteins, such as PABP and TIA-1) and in resistance to cycloheximide treatment. Thus, bona fide SGs do not appear to be induced during RUBV infection. The distribution of G3BP, either on its own or in granules, did not overlap with that of dsRNA-containing replication complexes, indicating that it played no role in virus RNA synthesis. However, G3BP did co-localize with viral ssRNAs in perinuclear clusters, suggesting an interaction that could possibly be important in a post-replicative role in virus replication, such as encapsidation.
for translation on ribosomes or degradation in processing bodies (P-bodies) (Anderson & Kedersha, 2002; Beckham & Parker, 2008; Kedersha & Anderson, 2002).

SGs are believed to play a defensive role against virus infection by downregulating the translation of viral or host mRNAs that encode proteins required for virus replication (Beckham & Parker, 2008); however, it has been suggested that some viruses may take advantage of SG formation as an aggregating point for RC assembly. Whilst numerous viruses, including members of the families Flaviviridae (Emara & Brinton, 2007), Togaviridae (alphaviruses) (McInerney et al., 2005) and Picornaviridae (Mazroui et al., 2006; White et al., 2007), all of which are positive-strand RNA viruses, have been shown to modulate stress proteins, little is actually known about the role of the cellular stress response during virus infection. Emerging evidence points to the involvement of G3BP in the replication of alphaviruses through interactions with the non-structural proteins (Cristea et al., 2010; Gorchakov et al., 2008), and thus it may also play a similar role in the replication of RUBV.

In contrast to the alphaviruses, which replicate rapidly, cytolically and to high titre in vertebrate cells, with shutdown of both host transcription and translation, RUBV replicates slowly and to low titre with minimal cytopathology (Frey, 1994). The purpose of this study was to determine whether RUBV induces a stress response in infected cells, as detected by the generation of G3BP-containing SGs. Concomitantly, we were interested in gaining insight into any role that G3BP SGs might play in RUBV replication. This study shows that, during late infection (48 h post-infection (p.i.)), RUBV induces the formation of G3BP granules, which were, however, distinct from bona fide SGs induced by arsenite treatment. Although these granules were formed concomitantly with active viral RNA synthesis and non-structural protein accumulation, the distributions of neither the granules nor G3BP on its own overlapped with the distribution of RCs, indicating that G3BP, in either form, was not involved directly in virus RNA synthesis. However, G3BP did occasionally co-localize with viral ssRNA and P150, suggesting a possible post-synthetic role in virus RNA replication.

RESULTS

RUBV induces the formation of G3BP granules compositionally distinct from SGs

The levels of the cellular stress protein G3BP were measured and compared between mock- and RUBV-infected cells during a time-course of 3–48 h p.i. Western blotting of cellular lysates revealed no differences in G3BP levels between mock-infected cells and cells infected with Robo502/P150-HA, a virus expressing a haemagglutinin (HA)-tagged P150 (m.o.i. = 3), at 3 or 24 h p.i. (Fig. 1a). However, compared with mock-infected cell lysates, those from Robo502/P150-HA-infected cells showed a small decrease in G3BP levels by 48 h p.i. (Fig. 1a). There were no differences in the subcellular location of G3BP between mock- and Robo502/P150-GFP-infected cells by 24 h p.i. (not shown). However, by 48 h p.i., the subcellular distribution of G3BP had changed from being a diffuse cytoplasmic pattern to accumulating in distinct round granules (blue arrows) or into elongated perinuclear clusters (yellow arrows), which sometimes contained P150 (Fig. 1b). Besides co-localizing with RCs in infected cells, P150 was previously reported to also accumulate in subcellular regions lacking RCs (Matthews et al., 2009), and these perinuclear clusters may be such sites. The subcellular location of G3BP was examined in >100 mock-infected or infected cells from two or more independent mock-infected or infected cultures (Fig. 1c) and this analysis revealed that, among the infected cells, the G3BP pattern was similar to the mock-infected pattern in approximately 40% of the cells. However, approximately 35% of the infected cells contained G3BP granules and the remaining approximately 20% contained G3BP perinuclear clusters (Fig. 1c).

The appearance of G3BP granules in the cytoplasm of infected cells was consistent with the hypothesis that SGs form during RUBV infection. PABP was used as a second marker for SGs. In mock-infected cells, PABP and G3BP were distributed in a diffuse cytoplasmic pattern (Fig. 2a, top panel). As expected, mock-infected cells treated with arsenite showed strict co-localization of PABP with G3BP in SGs (Fig. 2a, bottom panel). Surprisingly, however, in Robo502/P150-HA-infected cells, approximately 75% of RUBV-infected cells lacked PABP granules (Fig. 2b, c). Interestingly, in about 10% of infected cells, PABP had localized to the nucleus and, in another about 10% of infected cells, PABP was localized in both the nucleus and granules (Fig. 2c). Taken together, approximately 35% of the infected cells had changes in PABP localization, compared with untreated mock-infected cells. The contrast in the percentage of infected cells with G3BP granules (Fig. 1c) against those with PABP granules (Fig. 2c) was the first indication that the granules observed during RUBV infection are not the same as those produced as a result of arsenite treatment.

Thus, the composition of the G3BP granules formed during RUBV infection was investigated further by determining the percentage of co-localization of resident SG proteins in the virus-induced structures versus those formed by arsenite treatment. To this end, cells with G3BP granules were scored for the presence or absence of either PABP or TIA-1 in the granules, as determined by immunofluorescence staining (i.e. G3BP/PABP or G3BP/TIA-1 co-staining). In arsenite-treated cells, virtually all of the cells with G3BP-positive SGs exhibited PABP or TIA-1 co-localization in the SGs with G3BP (Fig. 3a, b, respectively). However, at 48 h p.i., in only about 55% of infected cells with G3BP granules did these granules also contain PABP (Fig. 3a), corresponding to roughly 20% of the total population of infected cells, and in <40% of infected cells with G3BP granules did the granules also contain TIA-1 (Fig. 3b), roughly 15% of total infected cells. Additionally, following treatment with cycloheximide, the arsenite-induced SGs disassembled, but the virus-induced
G3BP granules largely remained intact (Fig. 3c). Thus, whilst some granules in RUBV-infected cells appeared to be similar to arsenite-stimulated ones (these granules might be in the initial stages of RUBV-specific disruption), the remainder of the granules did not appear to function like arsenite-induced SGs.

**G3BP granules form concomitantly with virus RNA synthesis**

A permissive/non-permissive pair of continuous cell lines was used to determine whether the presence of the virus replicase proteins or the presence of the virus proteins and active RNA replication was required for G3BP granule induction. A RUBV replicon construct that lacks the structural protein ORF and expresses a GFP-tagged P150 that replicates only in the presence of CP was employed. Vero cells are non-permissive for this replicon, whereas C-Vero cells that are stably transfected with the CP can replicate it. In both cell lines, following transfection with the replicon, translation of P150 and P90 occurs, but only in C-Vero cells can RNA synthesis be detected. As can be seen in Fig. 4(a, bottom), GFP-tagged P150 was produced in both replicon-transfected Vero and C-Vero cells, but only the replicon-transfected C-Vero cells contained dsRNA (stained in red). Fig. 4(b) shows that the levels of P150 are approximately 1.5-fold higher in C-Vero cells than in similarly transfected Vero cells. Fig. 4(c) shows the percentages of GFP–P150–positive cells that contained G3BP granules at 48 h post-transfection. Roughly 45 % of such cells contained G3BP granules in the C-Vero culture, whilst the figure was approximately 10 % in the Vero cells. Thus, G3BP granules formed concomitantly with viral RNA replication.

**G3BP granules co-localize with ssRNA, but not dsRNA**

In order to determine whether G3BP granules were involved in viral RNA synthesis, infected Vero cells were co-stained for G3BP and dsRNA, a marker for RCs. In Robo502/P150-HA–infected cells at 48 h p.i., the distribution of G3BP granules did not overlap significantly with that of RCs (Fig. 5a, top panel), indicating no involvement. As G3BP is an RNA-binding protein, we subsequently used fluorescence in situ hybridization (FISH) to analyse whether viral ssRNA accumulated in the G3BP granules. Both nonsense (not shown) and RUBV-specific probes...
were used for analysis, with only the specific probes producing a signal in infected cells. No signal was detected in mock-infected cells. As shown in Fig. 5(a, middle row) and Fig. 5(b), whilst in a small percentage (approx. 10 %) of infected cells, viral ssRNA was observed in G3BP granules (Fig. 5a, bottom panel, blue arrows), in the majority of the infected cells (approx. 90 %), viral ssRNA did not co-localize with G3BP granules. However, viral ssRNA localized in perinuclear clusters in about 35 % of infected cells and G3BP was found in these structures 40–50 % of the time (corresponding to roughly 15–20 % of total infected cells). Therefore, G3BP granules and perinuclear clusters do not appear to be involved directly in viral RNA synthesis.

**DISCUSSION**

This study was initiated to determine whether a stress response is induced during RUBV infection. To this end, G3BP levels and subcellular distribution were analysed in RUBV-infected cells at different times p.i. Whilst RUBV did not dramatically alter the expression levels of G3BP, it did induce the formation of what initially seemed to be SGs in the late stages of infection (i.e. 48 h p.i.), concomitant with peak accumulation of viral macromolecules (Hemphill et al., 1988). However, induction was not uniform and accumulation of G3BP into the apparent SGs was detected in fewer than half of infected cells. In fact, granule formation tended to coincide with cells exhibiting the highest levels of the markers used to detect infection (GFP-tagged P150 and dsRNA) (data not shown). These granules required viral RNA replication for induction, but unexpectedly proved to be compositionally and functionally distinct from arsenite-induced SGs. The RUBV-induced granules often did not contain other known SG proteins such as PABP and TIA-1 and, furthermore, unlike arsenite-induced SGs, were resistant to dispersion by cycloheximide. Taken together, a stress response induced by RUBV infection appears to require a threshold level of virus replication (that is surpassed only in some cells late in infection) and then the formation of SGs is somehow countered.

Whilst RUBV and the alphaviruses share a common replication strategy, the interaction of these viruses with infected cells differs profoundly. Namely, whilst alphaviruses replicate robustly in vertebrate cells, inducing both complete transcriptional and translational shutdown of the host, RUBV replication occurs more slowly and to lower titres with minimal cytopathology (Frey, 1994). The results of the current study also demonstrate distinct differences between RUBV and alphaviruses in the induction and regulation of the stress response during infection. SFV induces the formation of SGs during the early phases of virus infection but, during the later stages, the SGs disappear in the vicinity of ongoing viral RNA synthesis (McInerney et al., 2005). As the composition of the granules formed during SFV infection was determined to be consistent with that found in functional SGs, SFV does not seem to alter the formation or function of these SGs. Instead, SFV has evolved translational enhancer sequences in the subgenomic RNA that allow for efficient translation, despite the host translational shutoff that occurs during infection (McInerney et al., 2005). In contrast, our study shows that a cellular stress response is initiated in a non-uniform manner only during the late stages of RUBV infection and that the G3BP granules formed are not like functional SGs induced by arsenite treatment. This might in part explain why there is only a modest decrease in total protein synthesis during the late stages of RUBV infection (Hemphill et al., 1988).
Recently, a possible role for G3BP in alphavirus RNA replication was suggested, as it was reported that G3BP interacts with the alphavirus replicase proteins nsP3 (Gorchakov et al., 2008) and nsP4 (Cristea et al., 2010). In contrast, data from this study provided no evidence that G3BP was associated with RUBV RCs. However, G3BP appeared to associate with the viral ssRNA in perinuclear clusters in 15–20% of RUBV-infected cells, which sometimes contained P150. The dynamic localization status of G3BP makes the significance of the G3BP/ssRNA co-localization more difficult to interpret, but our analysis may have only captured a smaller (or possibly larger) percentage of the co-localizing events. Nonetheless, the existence of an interaction between G3BP and RUBV ssRNAs would not be surprising, considering the known RNA-binding capacity of G3BP (Parker et al., 1996; Tourrière et al., 2001, 2003) and its general role in the stress response. It is possible that P150 in these structures is behaving as an intermediary for virus RNA transfer from RCs to the sites of virus RNA encapsidation. We hypothesize that G3BP accumulates around virus positive-strand RNAs that have reached high levels during the late stages of RUBV infection, but G3BP fails to transfer virus RNAs to SGs, as demonstrated by the lack (<10%) of RUBV genomes in the G3BP granules. Whilst the function of the co-localization between virus ssRNA and G3BP remains to be determined, we and others (Beatch & Hobman, 2000) have found that CP also accumulates in the perinuclear region, suggesting that these virus-specific perinuclear clusters may be where encapsidation is taking place.

Consistent with previous reports on PABP localization during stress (Kedersha et al., 2000), PABP localized in G3BP SGs during arsenite treatment. However, PABP showed a dramatically different redistribution in response to RUBV infection, in particular migration to the nucleus. Nuclear relocalization of PABP has been observed in rotavirus-infected cells following ejection from the small ribosomal initiation complex during the induction of SG formation (Harb et al., 2008). Herpes simplex virus also causes nuclear localization of PABP by activating JNK and p38 mitogen-activated protein kinases that are sensitive to oxidative stress. However, when oxidative stress was applied to uninfected Vero cells via arsenite treatment, we observed no nuclear PABP localization. Other studies have shown that PABP shuttles between the nucleus and cytoplasm and accumulates in the nucleus upon transcription shutoff (Afonina et al., 1998; Brune et al., 2005); however, RUBV is not known to induce transcriptional shutoff (Hemphill et al., 1988). We tried to simulate transcriptional shutoff by treating uninfected cells with various doses and exposures of actinomycin D, but observed no nuclear localization of PABP (data not shown). Finally, under plasmid-directed
expression, low-level expression of PABP resulted in a predominantly cytoplasmic distribution, whilst high-level expression led to nuclear localization (Afonina et al., 1998). Ilkow et al. (2008) reported higher levels of PABP expression in RUBV-infected cells. The nuclear localization of PABP that we observed probably reflects the higher protein levels that appeared to reside in RUBV-infected cells. In fact, many of the RUBV-infected cells with nuclear PABP exhibited a more intense signal than was observable in the cytoplasm of mock-infected cells. Collectively, these data support the notion that PABP nuclear localization is the result of higher levels of PABP expression during RUBV infection.

In conclusion, RUBV is capable of inducing a cellular stress response during the late stages of infection, albeit in a non-uniform manner, marked by subcellular redistribution of G3BP, PABP and TIA-1. However, the organization of this response seems to be disrupted in that functional SGs are not formed. Whilst some G3BP was found to co-localize with the virus-specific perinuclear clusters that contained multiple virus components, G3BP does not appear to play a direct role in the synthesis of RUBV RNA. However, G3BP may play a post-replicative role in RUBV infection, possibly as an intermediary in the processing of virus sRNA from RCs to the sites of encapsidation. The function of the virus-specific perinuclear clusters is currently under further investigation.

**METHODS**

**Cells, viruses and replicons.** The Vero African green monkey kidney cell line (obtained from ATCC) was used in this study along with a previously published cell line, C-Vero (Tzeng et al., 2006), that stably expresses the RUBV CP. Cells were maintained at 35 °C and 5% CO₂ in Dulbecco’s modified Eagle’s medium (Mediatech) with 5% FBS (Atlanta Biologicals) and infections were performed in 1% FBS/PBS as described previously (Tzeng et al., 2006). The viruses used in this study were produced from the infectious clones Robo502/P150-HA (Tzeng et al., 2006) and Robo502/P150-GFP (Matthews et al., 2010) as described by Pugachev et al. (2000). RUBrep/P150-GFP is a derivative of Robo502/P150-GFP that contains a CAT reporter gene in place of the structural protein ORF. In vitro transcripts of RUBrep/P150-GFP, synthesized as described previously (Tzeng et al., 2006) using linearized plasmid template, were transfected into Vero cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s directions. Accordingly, approximately 5 µg in vitro-transcribed RNA (estimated by gel electrophoresis and ethidium bromide staining) and 5 µl Lipofectamine 2000 per monolayer in 60 mm culture plates were used for transfection. Mock-transfected cells received only Lipofectamine 2000.

**Immunofluorescence.** At appropriate time points, Vero cells grown at low density (30–40% confluent) on glass coverslips and infected or transfected accordingly were simultaneously fixed and permeabilized with ice-cold methanol for 5 min. After washing and equilibration in PBS, the cells were stained with the following antibodies diluted in 2% BSA/PBS solution: rabbit or chicken anti-G3BP (recognizing G3BP1), Sigma, 1/500; mouse anti-PABP, Sigma, 1/200; mouse anti-dsRNA, Scientific Consultants, 1/1000. Rabbit anti-TIA-1 was obtained from Sigma. In some experiments, primary antibodies were detected by goat anti-mouse or anti-rabbit secondary antibodies with the desired conjugate for red (TRITC) or green (FITC) fluorescence (obtained from Sigma). In other experiments, primary antibodies were detected by donkey anti-chicken, -rabbit, or -mouse secondary antibodies conjugated with Alexa Fluor 488 or 595 (Invitrogen). Nuclei were stained with Hoechst 33342 (Invitrogen).

**FISH assay.** This assay was performed essentially as described by Jimenez-Garcia & Spector (1993) with some minor modifications. Briefly, cells harvested at the appropriate time point were fixed in 4%
formaldehyde in PBS before permeabilization by 0.5 % Triton X-100 for 10 min at room temperature. Coverslips were washed in PBS and then 2× SSC. Next, nick-translated probes labelled with Alexa Fluor 594–dUTP (Invitrogen) and purified from free nucleotides using Ambion NucAway columns as described in the manufacturer’s protocol were boiled for 10 min in 50 % formamide, 10 % dextran sulfate, 2× SSC and 1 mg E. coli tRNA ml−1, and stored on ice until being added to each coverslip and incubated overnight at 42 °C. Coverslips were washed in 50 % formamide/2× SSC for 15 min at 42 °C, briefly washed with 2× SSC at 42 °C and once with 1× SSC at room temperature before mounting or proceeding to immunofluorescence.

Microscopy. Images in Fig. 2 were acquired on a Zeiss Axioplan epifluorescence wide-field microscope with a 40× objective and processed with AxioVision software. The remaining images were acquired on a Zeiss LSM700 confocal microscope using a 63× objective with immersion oil and ZEN software. Images were processed with LSM Image Browser or LSM700 software.

Western blotting. Lysates from infected or transfected cells were prepared essentially as described previously (Tzeng et al., 2006). Briefly, cells in 60 mm plates were lysed in 500 μl lysis buffer [10 mM Tris (pH 8.0), 150 mM NaCl, 0.5 % Triton X-100, 0.5 % sodium deoxycholate, 0.1 % SDS and 1 × protease inhibitor cocktail (EDTA-free, Roche)] at the appropriate time point. After clearing insoluble debris by high-speed centrifugation (10 min at 16 000 g in an Eppendorf tabletop #5415D centrifuge), lysates were adjusted to 1× with Laemmli sample buffer, heat-denatured by boiling and 5 % of each lysate was loaded onto an SDS-PAGE gel, resolved and then transferred to nitrocellulose membranes for probing with the appropriate antibodies. P150–HA and P150–GFP were detected by mouse anti-HA, Roche, 1/1000, or rabbit anti-GFP, Clontech, 1/40, respectively. The other proteins were detected with the following: rabbit anti-G3BP, Sigma, 1/1000; rabbit anti-calnexin, Sigma, 1/5000. Each of the primary antibodies was visualized on the blot with an appropriate secondary antibody, i.e. anti-rabbit or -mouse that was conjugated to alkaline phosphatase (Promega, 1/5000) and subsequently reacted with NBT/BCIP (Roche) for colour development according to the manufacturer’s suggestions.

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