

# The C-Terminal Repeat Domains of nsP3 from the Old World Alphaviruses Bind Directly to G3BP

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**The Old World alphaviruses block stress granule assembly by sequestration of RasGAP SH3-domain binding protein (G3BP). Here, we show that the proline-rich sequences in the hypervariable domain of nonstructural protein 3 (nsP3) of both Semliki Forest virus and Chikungunya virus were dispensable for binding to G3BP. nsP3 variants with or without this domain colocalized with G3BP. Furthermore, we show that the C-terminal repeat motifs of nsP3 were sufficient for G3BP binding.**

Stress granules (SGs) are foci of accumulation of translationally silent mRNP complexes, which are induced during cellular stress (1). Their assembly is dependent on the proteins TIA-1/R (2) and G3BP (3). Many diverse virus infections employ mechanisms to restrict the formation of SGs (4, 5), which are induced by early events in the replication of many viruses, including the alphaviruses (6, 7). We recently showed that in cells infected with the alphavirus Semliki Forest virus (SFV), nonstructural protein 3 (nsP3) binds RasGAP SH3-domain binding proteins 1 and 2 (G3BP-1 and G3BP-2, respectively), recruits them to foci containing other viral proteins and often double-stranded RNA (dsRNA), and, in doing so, inhibits SG assembly (8). Fros and colleagues (9) also showed that the expression of the closely related Chikungunya virus (CHIKV) nsP3 blocks SG assembly by recruitment of G3BP-1 into cytoplasmic foci. These reports therefore describe a function for Old World alphavirus nsP3 in inhibition of the stress response. A surprising difference in the findings of the two groups was that the work of Fros and colleagues suggested that the CHIKV nsP3 sequence binding the SH3 domain of amphiphysins (398-PVAPRRRR-406 [10]) was essential for colocalization of nsP3 and G3BP (9). In contrast to that work, our study showed that the SFV nsP3 C-terminal L/ITFGDFD repeat motifs at positions 449 to 455 and 466 to 472, both well conserved in the Old World alphaviruses (11), were necessary and sufficient for formation of the nsP3/G3BP complex in infected cells (8). Both these regions, although situated within the hypervariable domain (HVD) of nsP3 (12–15), are highly conserved between both CHIKV and SFV, and it was therefore surprising that the viruses would differ in the region used to bind and recruit G3BP.

Recently, a variant of SFV (SFV-ΔP1+2) was described, lacking the proline-rich SH3-domain binding sequences of nsP3 (10). It was shown that the deletion impairs the recruitment of amphiphysin proteins to foci of nsP3 accumulation and delays replication complex formation (10). To determine if this domain of SFV nsP3 was important for the recruitment of G3BP-1, we infected mouse embryonic fibroblasts (MEFs) maintained as described previously (16) with SFV wild type (wt) or SFV-ΔP1+2 at a multiplicity of infection (MOI) of 1, fixed the cells at 8 h postinfection (hpi), and stained them for nsP3 and G3BP-1. Single-plane images were captured using a supercontinuum confocal TCS SP5 X microscope (Leica, Wetzlar, Germany) with a pulsed white light laser. No deficiency in G3BP-1 recruitment to nsP3-positive foci was detected, relative to SFV wt (Fig. 1A). We did not observe the

persistence of TIA-1-positive SGs in SFV-ΔP1+2-infected cells (data not shown) as we had done previously in cells infected with SFV-Δ789, which does not recruit G3BP to viral replication complexes or similar structures (8). These data show that the proline-rich region of SFV was not important for the interaction between nsP3 and G3BP-1 in MEFs. G3BP-1 also strongly colocalized with nsP3 in both SFV wt- and SFV-ΔP1+2-infected baby hamster kidney (BHK) cells (data not shown).

To test in direct comparison whether the SH3-domain binding domain of either SFV or CHIKV was important for the formation of a complex with G3BP-1 in the absence of other viral sequences, we transfected BHK cells with constructs encoding either wild-type nsP3 or nsP3 lacking the SH3-domain binding domains from both viruses (SFV-wt, SFV-ΔP1+2, CHIKV-wt, and CHIKV-ΔP1), N terminally tagged with the biotin acceptor peptide (BAP), originally described in reference 10. Transfections were performed as described previously (8). The boundaries of each of the deletions are presented in Fig. 1B. For SFV, we also included a C-terminally truncated construct lacking both the SH3-domain binding domain and downstream sequences, including the repeat motifs (SFV-ΔC). When lysates were precipitated with streptavidin-coated beads, we detected G3BP-1 in complex with wt nsP3 and with the constructs lacking the SH3-domain binding domains from both viruses (Fig. 1C). Interestingly, for both SFV and CHIKV nsP3, the ΔP1+2 and ΔP1 constructs, respectively, bound more G3BP-1 than did wt nsP3, suggesting that the deleted sequences were in fact antagonistic to G3BP-1 binding. Densitometric analysis of data from three separate experiments revealed that SFV-nsP3-ΔP1+2 bound 3.7 times (standard deviation, 2.1) and CHIKV-nsP3-ΔP1 bound 9.3 times (standard deviation, 5.0) more G3BP-1 than did their respective wt constructs. We did not detect G3BP-1 in complex with SFV-ΔC, consistent with our previous observation that the L/ITFGDFD repeat motifs represent the G3BP-binding site in this virus (8). These data suggest that the

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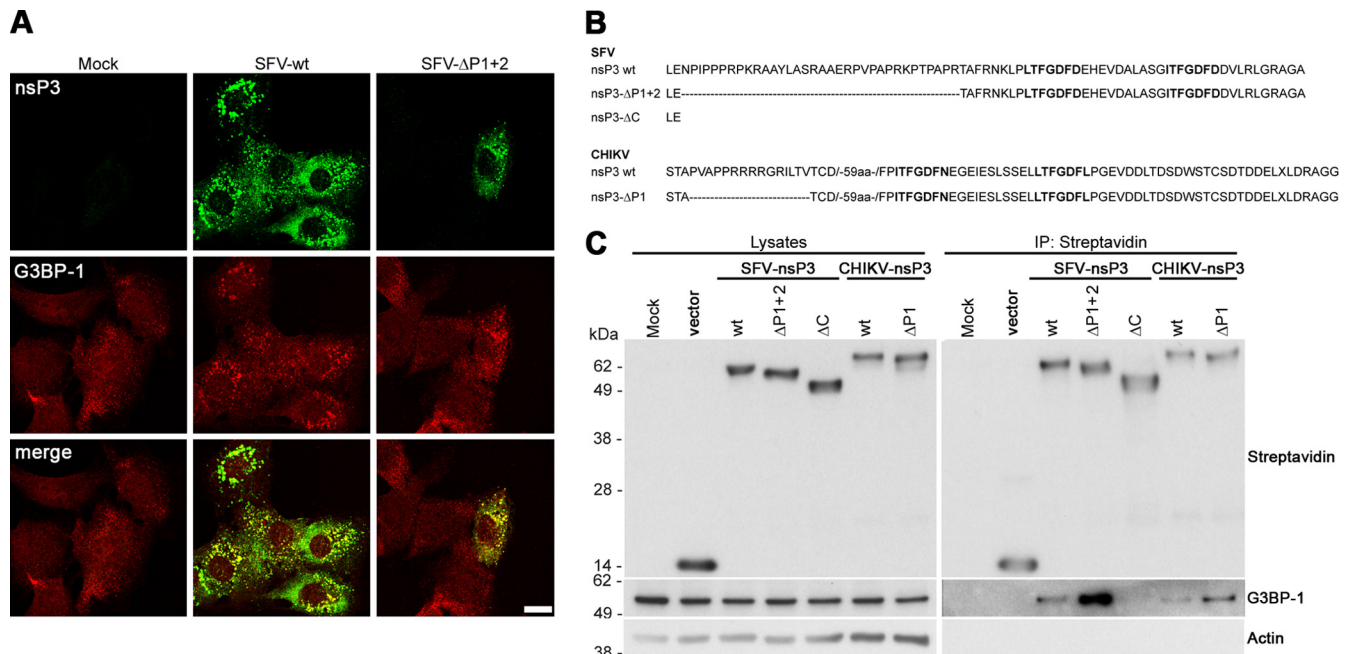
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**FIG 1** SFV and CHIKV nsP3 sequences binding the SH3 domain of amphiphysins are not required for G3BP-1 binding. (A) MEFs were infected with SFV wt or SFV-ΔP1+2 at an MOI of 1. At 8 hpi, cells were fixed and stained for nsP3 and G3BP-1. Results are representative of two independent experiments. Images of single focal planes were processed using Adobe Photoshop. Bar, 10  $\mu$ m. (B) Extreme C-terminal sequences of SFV wt nsP3, nsP3-ΔP1+2, nsP3-ΔC, and CHIKV wt nsP3 and nsP3-ΔP1. The C-terminal repeat sequences are in bold type. (C) BHK cells were mock transfected or transfected with the indicated constructs. Cell lysates were prepared 16 h after transfection, precipitated with streptavidin-coated beads, and separated by SDS-PAGE. Lysates and precipitates were probed with streptavidin and with G3BP-1 and actin antisera. Results are representative of three independent experiments. IP, immunoprecipitation.

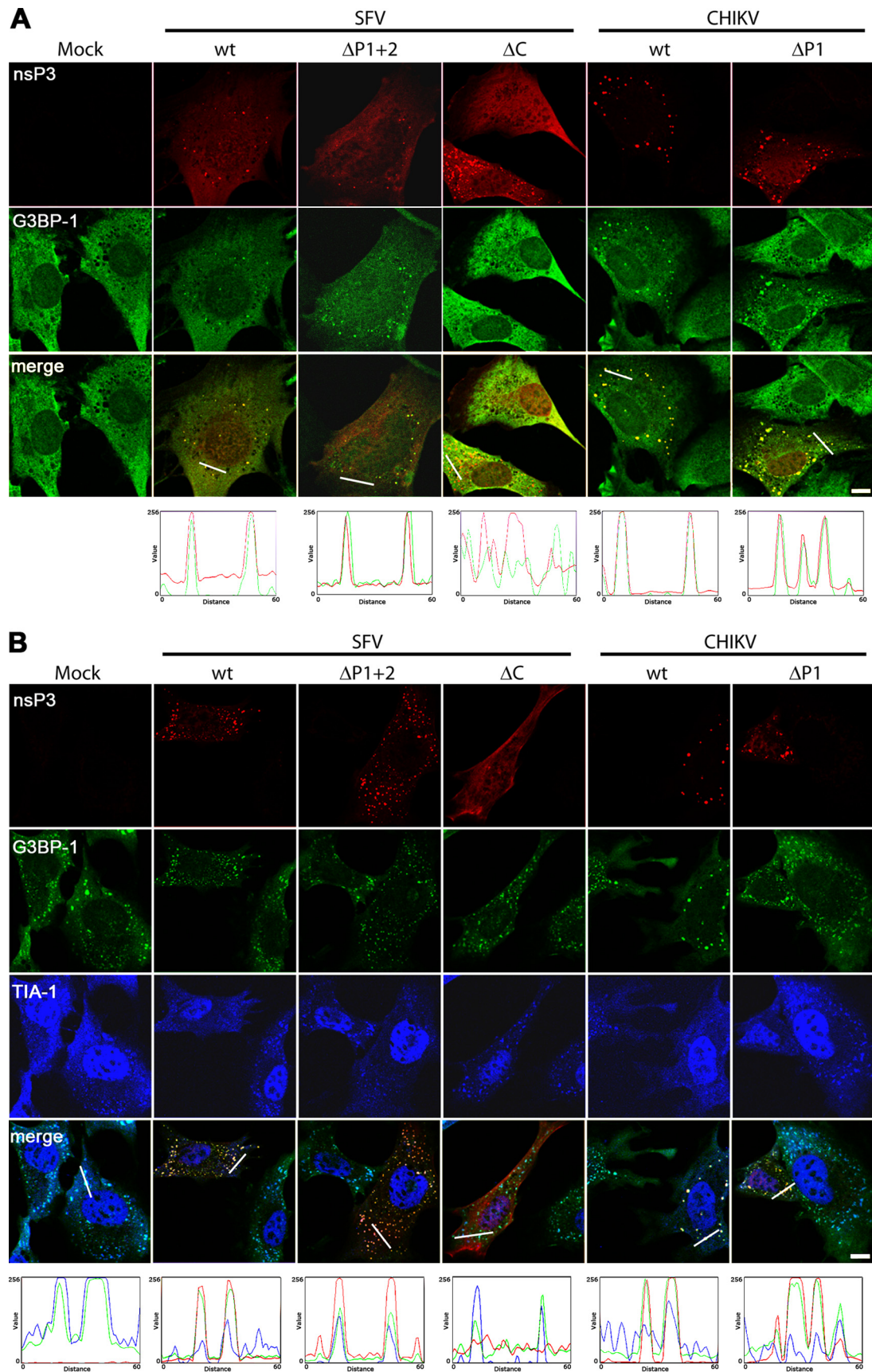
formation of a complex containing nsP3 and G3BP-1 does not require the proline-rich, SH3-domain binding domain of SFV or CHIKV nsP3 protein.

The subcellular localization of the proteins was also analyzed after transfection into MEFs (Fig. 2A). We have used these cells in the past for analysis of viral protein localization and SG formation (6, 8). Cells were fixed 22 h after transfection and stained for nsP3, G3BP-1, and TIA-1. All three SFV nsP3 constructs exhibited generally diffuse localization with occasional punctate staining. These puncta are foci of accumulation of the viral protein with its cellular interacting partners that have been observed in other studies on alphavirus nsP3 function (8–11, 17, 18). SFV wt nsP3 appeared in foci in about 50% of transfected MEFs, while foci in cells transfected with nsP3-ΔP1+2 and nsP3-ΔC were much more rare. Since, in this experiment, we were primarily interested in the interaction and localization of G3BP-1 with nsP3, we chose to restrict our analyses to cells displaying these foci. Colocalization studies are more meaningful when the proteins are not displaying completely diffuse cytoplasmic staining. The nsP3 puncta were, in the case of SFV wt nsP3 and nsP3-ΔP1+2, also G3BP-1 positive (Fig. 2A) but not TIA-1 positive (data not shown). This can be more easily appreciated in the RGD profile analyses shown under the merged images. Foci of nsP3-ΔC staining did not contain G3BP-1, as expected from the lack of interaction in the coimmunoprecipitation experiment (Fig. 1C). Punctate localization was more obvious for CHIKV nsP3 wt and ΔP1, where almost all transfected cells displayed such localization. Both CHIKV nsP3 variants colocalized very well with G3BP-1 but not with TIA-1.

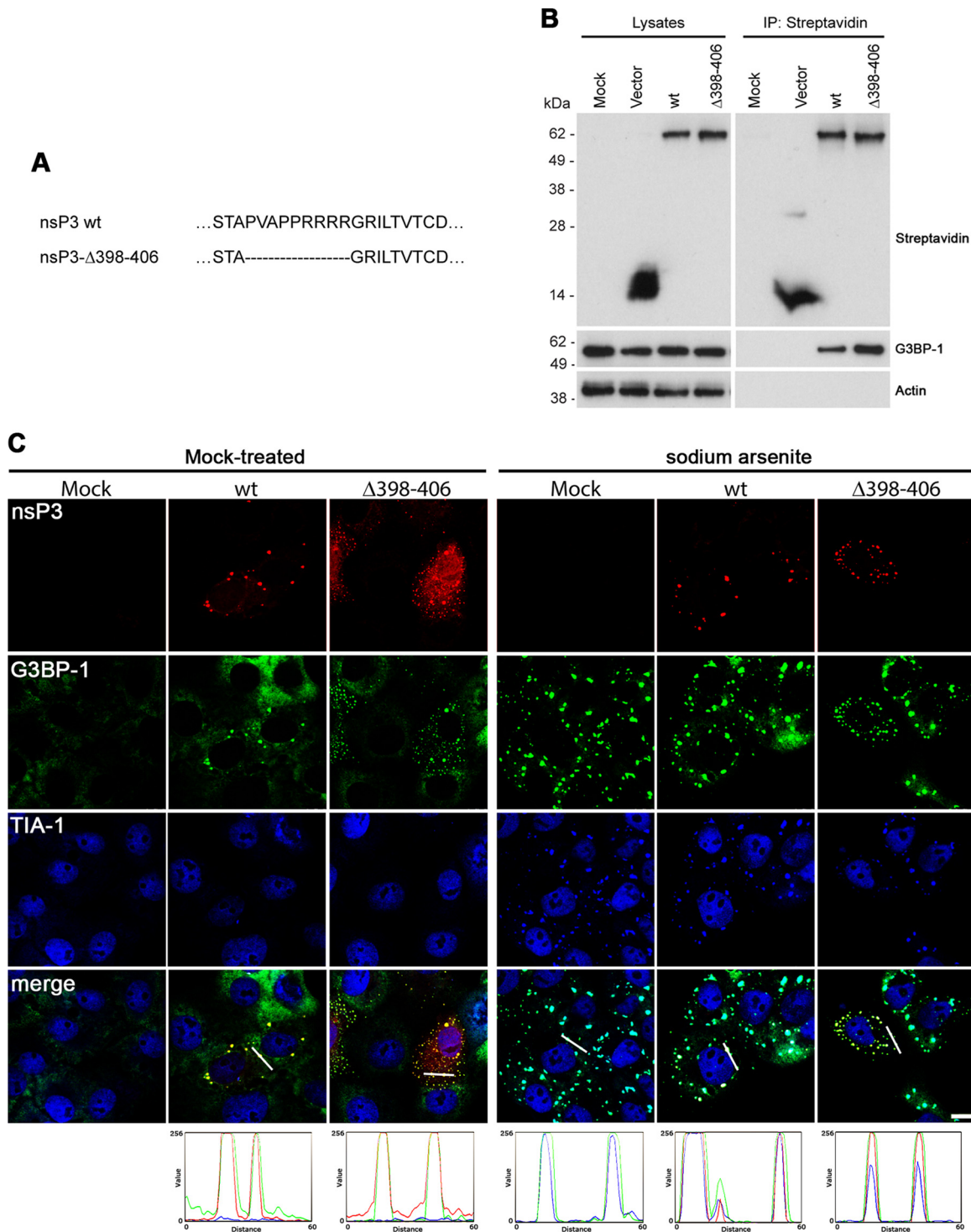
In our previous work (8), we showed that nsP3, when expressed in the absence of other viral sequences in an inducible cell

line, also colocalized with G3BP-1 but not with TIA-1. When those cells were treated for 1 h with sodium arsenite, however, wt nsP3 was found in foci that were G3BP-1 and also TIA-1 positive, representing SGs, suggesting that nsP3 binding alone does not completely block the function of G3BP-1 in SG formation (8). To test whether MEFs transfected with the BAP-nsP3 fusion constructs were capable of mounting a stress response, we treated them with 0.5 mM sodium arsenite to induce the formation of SGs. We found that while mock-transfected cells efficiently formed G3BP-1- and TIA-1-positive SGs, all nsP3 variants which were capable of G3BP-1 binding (SFV wt, SFV-ΔP1+2, CHIKV wt, and CHIKV-ΔP1) remained colocalized with G3BP-1 but also displayed some enrichment of TIA-1 in those foci (Fig. 2B). In cells transfected with these constructs, the localization of TIA-1 with G3BP-1 was weaker than that in nearby nontransfected cells or in mock-transfected cells but readily detectable. This was consistent with our previous work and suggested that nsP3–G3BP-1 binding *per se* is not enough to fully inhibit SG formation in response to sodium arsenite. SFV nsP3-ΔC, which does not bind G3BP-1, did not inhibit the formation of SGs and did not colocalize with G3BP-1 or TIA-1, as expected. Together, the results in Fig. 2 show that both SFV and CHIKV nsP3 colocalized with G3BP-1 under normal and stress conditions and that this colocalization was not dependent on the proline-rich domain but rather on sequences downstream.

In contrast to these results, Fros and colleagues suggested that the sequence between amino acids (aa) 398 and 406 of CHIKV nsP3, including the SH3-domain binding domain, was essential for the formation of nsP3/G3BP-1 foci in Vero cells (9). Our CHIKV-ΔP1 construct contained a larger deletion (residues 398



**FIG 2** SFV and CHIKV nsP3 sequences binding the SH3 domain of amphiphysins are not required for localization to G3BP-1-positive foci. MEFs were mock transfected or transfected with pEBB/PP-SFVnsP3, nsP3- $\Delta P1+2$ , or nsP3- $\Delta C$  or pEBB/PP-CHIKVnsP3 or nsP3- $\Delta P1$ . After 22 h, transfected cells were mock treated (A) or treated with sodium arsenite (B) for 1 h, fixed and stained with streptavidin (for nsP3) and with G3BP-1 and TIA-1 antisera, and analyzed by confocal microscopy. Images of single focal planes were processed using Adobe Photoshop. Bars, 10  $\mu$ m. Profiles were calculated using the RGB profiler tool in ImageJ.

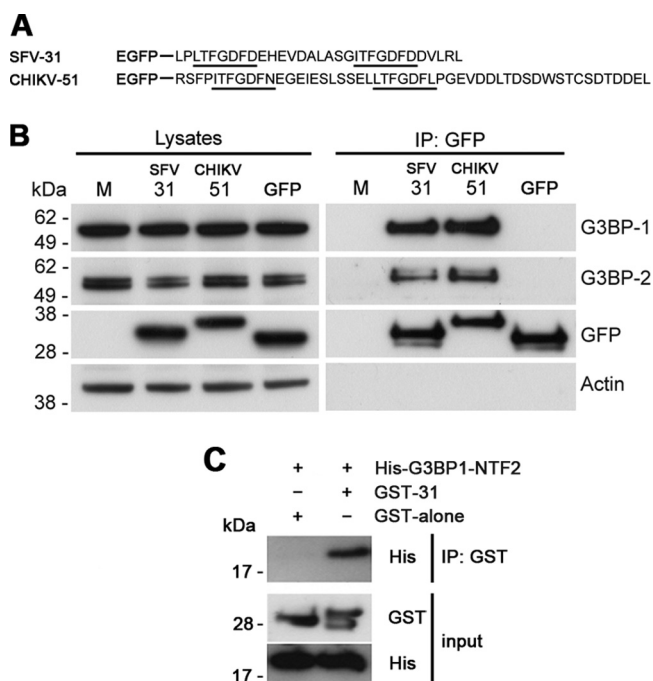


**FIG 3** CHIKV nsP3 sequences binding the SH3 domain of amphiphysins are not required for localization to G3BP-1-positive foci in Vero cells. (A) Amino acid sequence of  $\Delta$ 398–406 mutation. (B) Vero cells were mock transfected or transfected with pEBB/PP vector or with CHIKVnsP3 or nsP3- $\Delta$ 398–406. Cell lysates were prepared 22 h after transfection, precipitated with streptavidin-coated beads, and separated by SDS-PAGE. Lysates and precipitates were probed with streptavidin and with G3BP-1 and actin antisera. Results are representative of three independent experiments. (C) Vero cells were mock transfected or transfected with pEBB/PP-CHIKVnsP3 or nsP3- $\Delta$ 398–406. At 22 h, transfected cells were mock treated (left panels) or treated with sodium arsenite (right panels) for 1 h, fixed and stained with Cy3-streptavidin (for nsP3) and with G3BP-1 and TIA-1 antisera, and analyzed by confocal microscopy. Images of single focal planes were processed using Adobe Photoshop. Bar, 10  $\mu$ m. Profiles were calculated using the RGB profiler tool in ImageJ.

to 412), but we nevertheless demonstrated strong binding of that mutant protein to G3BP-1 in BHK cells. To address the discrepancy directly, we constructed a CHIKV-nsP3- $\Delta$ 398–406 construct (Fig. 3A) and analyzed its ability to bind G3BP-1 in Vero cells. Cells were transfected with constructs encoding BAP tag alone or BAP-tagged CHIKV-nsP3-wt or CHIKV-nsP3- $\Delta$ 398–406, and lysates were precipitated with streptavidin-coated beads and analyzed by immunoblotting as done before. Again, we detected somewhat stronger binding of the CHIKV nsP3 construct lacking the proline-rich domain to G3BP-1, relative to the wt protein (Fig. 3B), indicating that deletion of the proline-rich domain did not inhibit nsP3's ability to form a complex with G3BP-1 in Vero cells. We also analyzed the localization of the nsP3 variants in Vero cells (Fig. 3C). Similarly to their localization in transfected MEFs, both CHIKV nsP3 wt and  $\Delta$ 398–406 localized to predominantly punctate structures in Vero cells with some diffuse staining visible, more obviously so for the mutant (Fig. 3C, left panels). Puncta formed by both wt and mutant proteins contained G3BP-1, confirming the observation that both nsP3 variants formed a complex with the cellular protein. These puncta were likely not SGs, since TIA-1 was never found in wt nsP3- or nsP3 $\Delta$ 398–406- and G3BP-1-positive foci under these conditions. When similarly transfected cells were treated with sodium arsenite, however, both variants of nsP3 were found in foci that were G3BP-1 and TIA-1 positive, which we therefore identified as SGs (Fig. 3C, right panels). The diffuse staining for the viral proteins was no longer observed, indicating that the majority of the protein, whether or not diffuse under normal conditions, was recruited to SGs upon arsenite stress. In conclusion to the data shown in Fig. 3, despite repeating very closely the experiment of Fros and colleagues, we were not able to repeat their observations and conclude that the formation of a complex containing nsP3 and G3BP-1 in Vero cells does not require the proline-rich, SH3-domain binding domain of Old World alphavirus nsP3 protein.

We have previously shown that the C-terminal L/ITFGDFD repeat domain of SFV nsP3 is necessary and sufficient for formation of a complex with G3BP-1 (8). To determine whether the C-terminal repeat domain of CHIKV nsP3 was also sufficient for G3BP-1 complex formation in the absence of the proline-rich SH3-domain binding domain or other viral sequences, we generated an enhanced green fluorescent protein (EGFP) construct fused to 51 amino acids from the C terminus of CHIKV nsP3 (CHIKV-51), similar to the SFV-31 construct previously described (8) (Fig. 3A). After transfection of these constructs into BHK cells, immunoprecipitation with GFP antisera revealed that both constructs efficiently formed complexes with G3BP-1 and also with G3BP-2 (Fig. 3B).

Several studies have previously demonstrated an interaction between nsP3 from the Old World alphaviruses and G3BP-1 and -2 (8, 9, 19–21). These studies examined the interaction in infected cells or in cells transfected with mutant constructs, and in no case can it be excluded that the binding is indirect and that another protein or RNA may be involved. In order to determine if the nsP3-G3BP interaction involves direct binding, we constructed vectors for expression in *Escherichia coli* of the nuclear transport factor 2 (NTF2)-like domain of G3BP-1 (residues 1 to 139; shown to be necessary for the nsP3 interaction [19]) fused to the His tag (His-G3BP1-NTF2) and 31 residues of the SFV nsP3 C terminus fused to the C terminus of glutathione *S*-transferase (GST-31). The fusion proteins were separately expressed in *E. coli* BL21 T7



**FIG 4** The C-terminal repeat motifs of SFV and CHIKV nsP3 are sufficient for G3BP binding. (A) Amino acid sequences of the C termini of the SFV-31 and CHIKV-51 constructs used in this study. The repeat sequences are underlined. (B) BHK cells were mock transfected or transfected with pEGFP-SFV-31, pEGFP-CHIKV-51, or pEGFP-C1. Cell lysates were prepared 16 h after transfection, immunoprecipitated with GFP antisera, and separated by SDS-PAGE. Lysates and precipitates were probed for G3BP-1, G3BP-2, GFP, and actin antisera. Results are representative of three independent experiments. (C) Purified GST, GST-nsP3-31, or His-G3BP-1-NTF2 protein was mixed, immunoprecipitated with GST antisera, and separated by SDS-PAGE. Lysates and precipitates were probed with GST or His antisera. Data are representative of three independent experiments.

Express and purified using an ÄKTAprime Plus unit with HisTrap or GST GraviTrap columns (GE Healthcare). His-G3BP1-NTF2 (2 mg/ml) was mixed with GST (2 mg/ml) or GST-31 (2 mg/ml) in 20 mM Tris HCl, 300 mM NaCl, 30 mM imidazole, 5 mM MgCl<sub>2</sub>, 10% glycerol, pH 8, and incubated for 90 min with GST antisera (Abcam) at room temperature. When immunoprecipitates were analyzed by SDS-PAGE, we detected His-G3BP1-NTF2 in the sample containing GST-31 but not in that containing GST alone (Fig. 4C). These data show that the C-terminal 31 residues of SFV nsP3 were capable of binding to the NTF2-like domain of G3BP-1 in the absence of other cellular or viral factors and suggest, therefore, that in Old World alphavirus-infected cells, the binding of nsP3 to G3BP is direct.

In this work, we have shown that the C-terminal L/ITFGDFD repeat regions of both SFV and CHIKV nsP3 are necessary and sufficient for G3BP binding. Supporting our findings, recently published work with chimeric Sindbis virus and Venezuelan equine encephalitis virus (VEEV) revealed that the HVD of VEEV, which contains a proline-rich sequence potentially binding SH3 domains but lacks repeat motifs with homology to the SFV L/ITFGDFD repeats, does not form a complex with G3BP (17, 18). We conclude, therefore, that for the Old World but not the New World alphaviruses, the G3BP binding site resides in the C-terminal repeat motifs of nsP3. This contributes to an emerging picture

of distinct sets of host interactions for the New and Old World alphaviruses (17, 22).

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