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Participation of eIF4F complex in Junin virus infection: blockage of eIF4E does not impair virus replication

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Summary

Translation efficiency of viral mRNAs is a key factor defining both cytopathogenicity and virulence of viruses, which are entirely dependent on the cellular translation machinery to synthesize their proteins. This dependence has led them to develop different translational reprogramming strategies to ensure viral mRNAs can effectively compete with cellular mRNAs. Junin virus (JUNV) is a member of the family *Arenaviridae*, whose mRNAs are capped but not polyadenylated. In this work we evaluated the relevance to JUNV replication of the main components of the eIF4F complex: eIF4A, eIF4GI and eIF4E. We found the viral nucleoprotein (N) of JUNV colocalized with eIF4A and eIF4GI but not with eIF4E. Moreover, N could be immunoprecipitated in association with eIF4A and eIF4GI but not with eIF4E. Accordingly, functional impairment of eIF4A as well as eIF4GI reduced JUNV multiplication. By contrast, inhibition of eIF4E did not show a significant effect on JUNV protein synthesis. A similar situation was observed for another two members of arenaviruses: Tacaribe (TCRV) and Pichinde (PICV) viruses. Finally, the nucleoproteins of JUNV, TCRV and PICV were able to interact with 7 methyl-guanosine (cap), suggesting that the independence of JUNV multiplication on eIF4E, the cap-binding protein, may be due to the replacement of this factor by N protein.

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Introduction

Viruses, as obligate intracellular parasites, have developed a number of mechanisms to optimize their replication by taking advantage of cellular metabolism. Of these, mechanisms related to protein synthesis are of interest because modifications displayed by viruses should be subtle enough to allow continued use of the cellular machinery but robust enough to gain advantage over the synthesis of cellular proteins. Also, in many cases, these mechanisms allow viruses to avoid the blockage of translation that occurs in infected cells as a consequence of the innate immune response (Komarova *et al*., 2009; Walsh and Mohr, 2011). Although the translation process comprises three stages – initiation, elongation and termination – it is the first step that mainly regulates the whole process (Malys and McCarthy, 2011). During initiation of translation, Met-tRNAi, together with the small ribosomal subunit (40S) and eukaryotic initiation factors (eIFs), form a complex onto cellular mRNAs bearing a 5′ cap structure. Recruitment of the 40S subunit by the 5′ cap structure is mediated by eIF4F, a heterotrimer composed of eIF4G (multivalent scaffolding protein), eIF4E (cap-binding protein) and eIF4A (ATP-dependent helicase). eIF4E, the small subunit of eIF4F, specifically recognizes the 5′ cap of the mRNA and is, therefore, necessary for the start of cap-dependent translation (Van der Kelen *et al*., 2009). Although many viral capped mRNAs can be translated by a cap-dependent mechanism as explained above, others, particularly uncapped monocystronic mRNAs or capped polycystronic mRNAs, employ non-canonical initiation mechanisms such as the internal ribosome entry site (IRES) translation. Non-canonical translation may be also exploited by viral capped mRNAs that replace cellular eIF4E or other eIFs by a viral protein (Firth and Brierley, 2012; Valášek, 2012).

Junin virus (JUNV), a member of the *Arenaviridae* family, is the etiological agent of a severe human hemorrhagic fever endemo-epidemic in agricultural areas of the central pampas in Argentina. The virus is transmitted to rural workers by aerosols contaminated with the excreta of JUNV persistently infected field mice, mostly of the genus *Calomys* (Peters, 2002). The *Arenaviridae* family comprises a group of enveloped viruses with a genome composed of two negative-sense single stranded RNA segments that encode the genes of viral proteins, N (nucleoprotein), L (polymerase), GPC (glycoprotein

precursor) which is cleaved into G1 (main glycoprotein) and G2 (fusogenic glycoprotein), and Z (matrix protein), using an ambisense coding strategy (Buchmeier, 2002; Meyer *et al*., 2002). Although infection of culture cells by JUNV has been studied from diverse points of view (reviewed in Gómez *et al*., 2011), the exact mechanism of translation of viral mRNAs still remains obscure. Arenaviruses posses capped subgenomic mRNAs that lack a poly-A tail (Meyer *et al*., 2002). This characteristic would suggest a cap-dependent translation mechanism employing eIF4E for the initiation of translation of JUNV mRNAs. However, replication of the *Arenaviridae* prototype, lymphocytic choriomeningitis virus (LCMV), is not impaired by treatment of infected cells with rapamycin (Urata *et al*., 2012), a drug that inhibits mammalian target of rapamycin (mTOR) phosphorylation mediated by phosphorylated Akt. Taking into account that activated mTOR phosphorylates eIF4E for full activity (Van Der Kelen *et al*., 2009), the resistance of LCMV to rapamycin means that a likely alternative mechanism to eIF4E-dependent translation should be considered. This reasoning is also supported by the fact that the inhibitor Ly294002 that impairs phosphorylation of Akt is effective against JUNV multiplication when used during the early steps of infection, suggesting that viral protein synthesis is not affected by inhibition of Akt activation (Linero and Scolaro, 2009).

In the present work we investigated the participation of eIF4E in the replication of JUNV and found that this factor does not play a relevant role during translation of JUNV mRNAs. On the contrary, eIF4G and eIF4A are necessary for this process. A probable replacement of eIF4E by the viral nucleoprotein N is proposed.

Results

1. JUNV infection does not alter eIF4F components during infection but impairs their recruitment to stress granules

In order to investigate the modulation of the eIF4F complex during virus replication, we firstly assessed whether JUNV was able to alter the overall amount of the main factors that compose this complex. To this end, Vero cells were infected with JUNV and at different times post-infection (p.i.) eIF4F components were analysed by WB. As shown in Fig. 1, no changes in the abundance of eIF4E, eIF4A and eIF4GI were observed up to 48 h.p.i., time at which the synthesis of the viral proteins, N and G1, could be readily observed. These results suggest that replication of JUNV takes place without inducing a significant modification in the amount of the main components of eIF4F involved in cap-dependent translation. Next, we investigated whether JUNV was capable of relocalizing any of these components. To test this possibility, JUNV

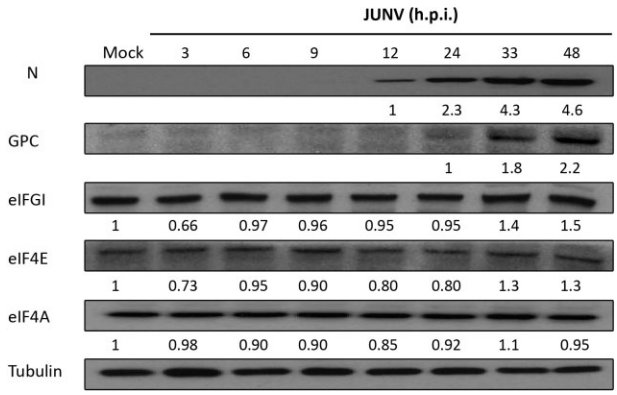


Fig. 1. Synthesis of the main components of eIF4F in JUNV infected cells. Vero cells were mock infected or infected with JUNV at an MOI of 1 pfu cell^{-1}. A different hours post-infection (h.p.i.), cells were lysed and proteins were separated by SDS-PAGE and analysed by WB. The translation initiation factors eIF4GI, eIF4A and eIF4E were evaluated, while N and GPC (G1) were assessed as viral proteins. Tubulin was used as a loading control. Numbers under images indicate the fold change of expression in arbitrary units calculated as the ratio between tubulin and each protein with the Image J program.

infected Vero cells were analysed by confocal microscopy and the pattern of expression of eIFs was characterized by IFA. A similar pattern of eIF4A, eIF4GI and eIF4E was observed in JUNV infected cells in comparison with mock infected cells (Fig. 2a–c), indicating that JUNV did not alter the cellular localization of these factors. However, in accordance with data recently reported for TACV (Baird *et al*., 2012), a strong colocalization of N with eIF4A and eIF4GI (Fig. 2a and b, Pearson coefficient 0.65 and 0.7 respectively) but not with eIF4E was observed (Fig. 2c, Pearson coefficient of 0.2). These results suggest that although JUNV infection of Vero cells did not significantly modify the amount of the main components of the eIF4F complex, it induced the colocalization of eIF4A and eIF4GI with N, suggesting that these factors are probably required during JUNV replication.

We have previously reported that JUNV impairs stress granules (SGs) formation when cells are stressed with sodium arsenite (Linero *et al*., 2011). This impairment might benefit the virus by allowing access to eIFs that otherwise would be sequestered into these silencing cytoplasmic structures. In order to ascertain the impact of oxidative stress on eIF4F components during infection, Vero cells were mock infected or infected with JUNV and, at 24 h.p.i., cells were stressed with sodium arsenite for 1 h and then processed for IFA. In first place we evaluated SGs induction by using the well characterized TIA-1 marker and as can be observed mock infected cells showed a 93 $\pm$ 7% of cells with SGs (Fig. 3a, upper panel) while JUNV infected cells showed only a 15 $\pm$ 7% of cells with SGs that are coincident with cells that showed a low expression of N (Fig. 3a, lower panels). Coincidently

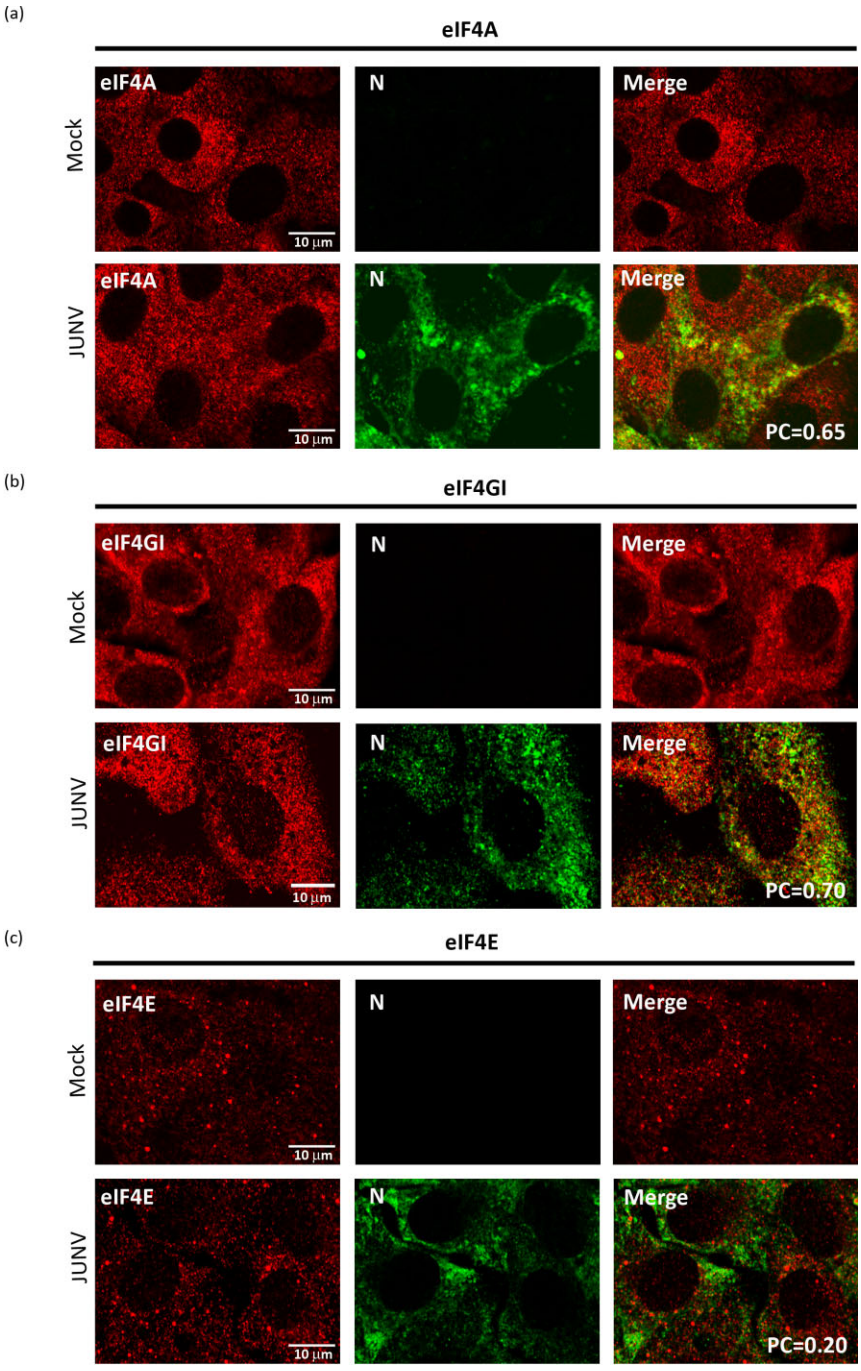


Fig. 2. Localization of eIF4F components during JUNV infection. Vero cells were mock infected or infected with JUNV at an MOI of 2 pfu cell^{-1} and at 24 h.p.i. cells were processed for IFA. Colocalization of the viral protein N with the translation initiation factors eIF4A (a), eIF4GI (b) and eIF4E (c) was evaluated. Numbers in the images indicate colocalization expressed by Pearson coefficient. Values between 0.5 and 1 indicate colocalization and values between –1 and 0.5 indicate absence of colocalization.

when infected cultures were analysed for SGs positives for eIFs, only 26 ± 5% of cells treated with sodium arsenite exhibited localization of eIF4A in SGs in comparison with 86 ± 11% of cells in mock-infected cultures (Fig. 3b). Similar results were observed for eIF4GI and eIF4E, showing 28 ± 7% and 18 ± 9%, respectively, of cells with SGs containing these factors in stressed-JUNV infected cells, in comparison with 90 ± 8% and 84 ± 10%, respectively, in stressed-mock infected cells (Fig. 3c and d). This finding supports data previously reported and strongly suggests that the modulation of stress response exerted by the virus upon arsenite treatment would reflect the necessity of JUNV for any of the eIFs sequestered in these structures.

2. JUNV nucleoprotein interacts with eIF4AI and eIF4GI but not with eIF4E

Colocalization of JUNV nucleoprotein, N, with eIF4A and eIF4GI might implicate an interaction between these

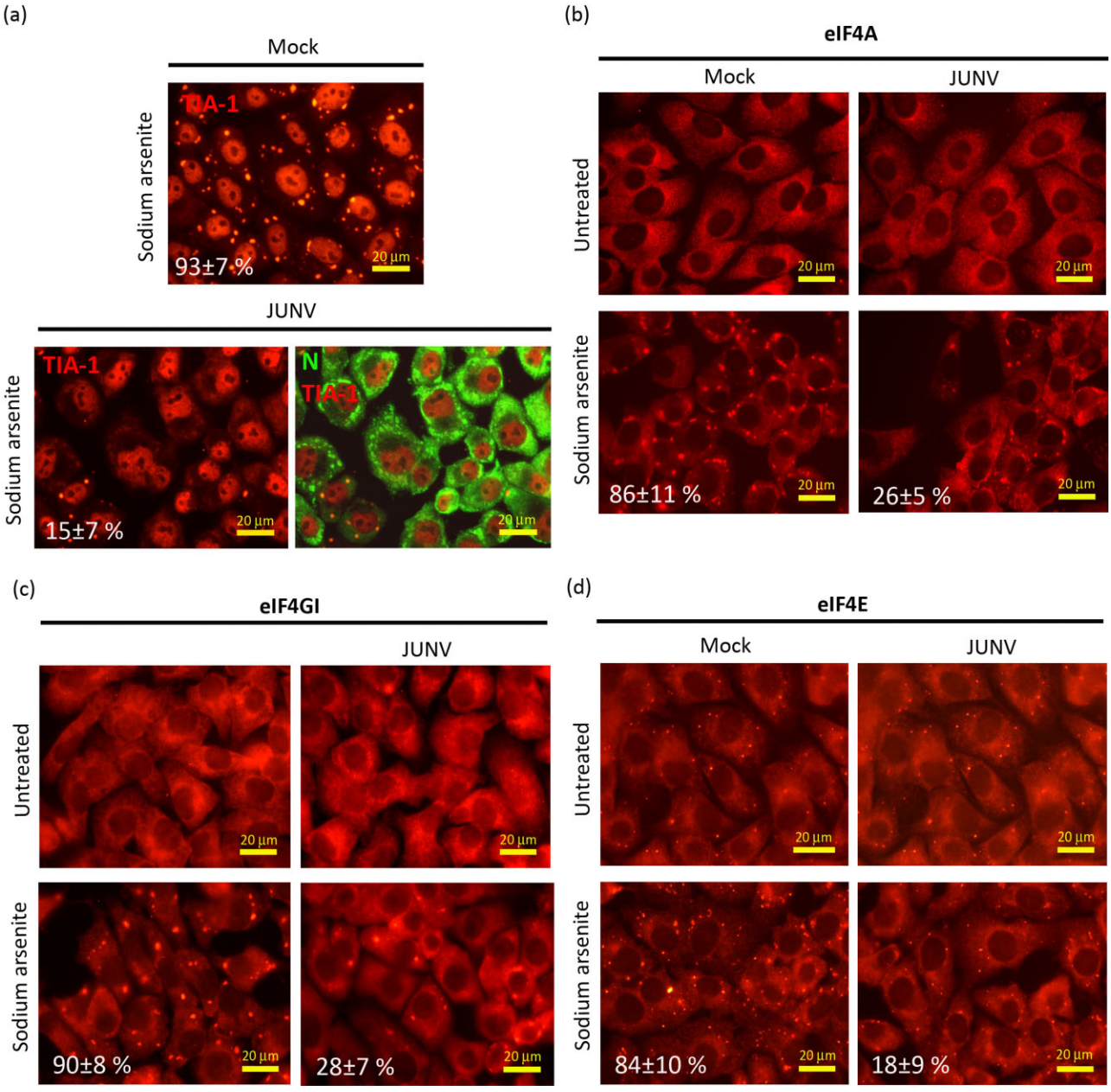


Fig. 3. Localization of eIF4F components in JUNV infected cells stressed with arsenite. Vero cells were mock infected or infected with JUNV at an MOI of 2 pfu cell^{-1} and at 24 h.p.i. cells were untreated or treated with 500 µM of sodium arsenite for 1 h. After treatment cells were fixed and evaluated by IFA to analyse the expression of N and the recruitment of TIA-1 (a) eIF4A (b), eIF4GI (c) or eIF4E (d) to stress granules. TIA-1 and eIF4F components in red and N in green. Numbers in the images indicate the percentage of cells with stress granules for each marker.

proteins during JUNV replication. In order to evaluate this hypothesis an immunoprecipitation assay was performed. Vero cells were mock infected or infected with JUNV and, at 24 h.p.i., cells were lysed and immunoprecipitated with antibodies against eIF4A, eI4GI and eIF4E. Immunoprecipitated (IP) proteins were separated by SDS-PAGE and analysed by WB. N was present in the IP fraction obtained with anti-eIF4GI (Fig. 4a, IP-JUNV) and anti-eIF4A (Fig. 4b, IP-JUNV). On the contrary, in the case of eIF4E-IP proteins, N was detected in the unbound fraction (Fig. 4c, SN-JUNV), and no band was observed in the IP fraction (Fig. 4c, IP-JUNV), even though most of eIF4E was present in this fraction. In order to ensure that the presence of N in the IP proteins was not due to a non-specific interaction, we studied reciprocally the presence of translation factors in IP proteins obtained with anti-N antibodies. As expected, both eIF4A and eIF4GI were readily observed in the IP fraction, whereas eIF4E could

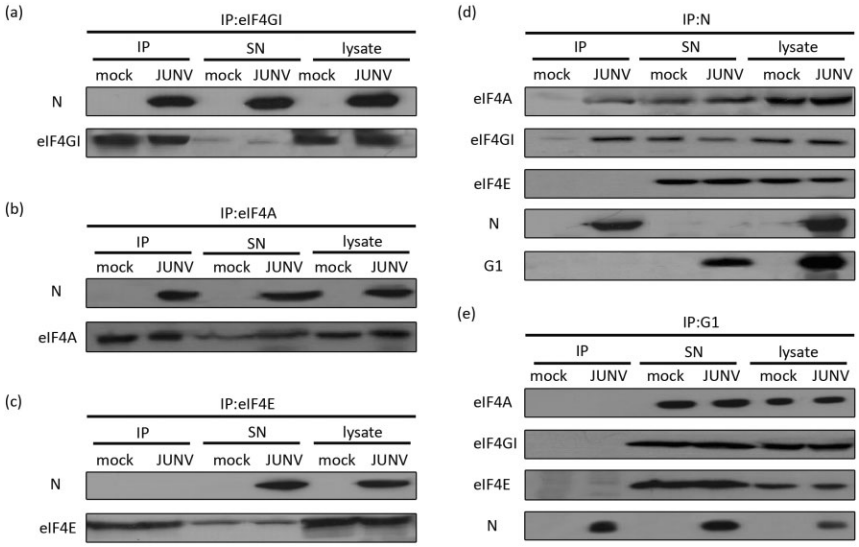


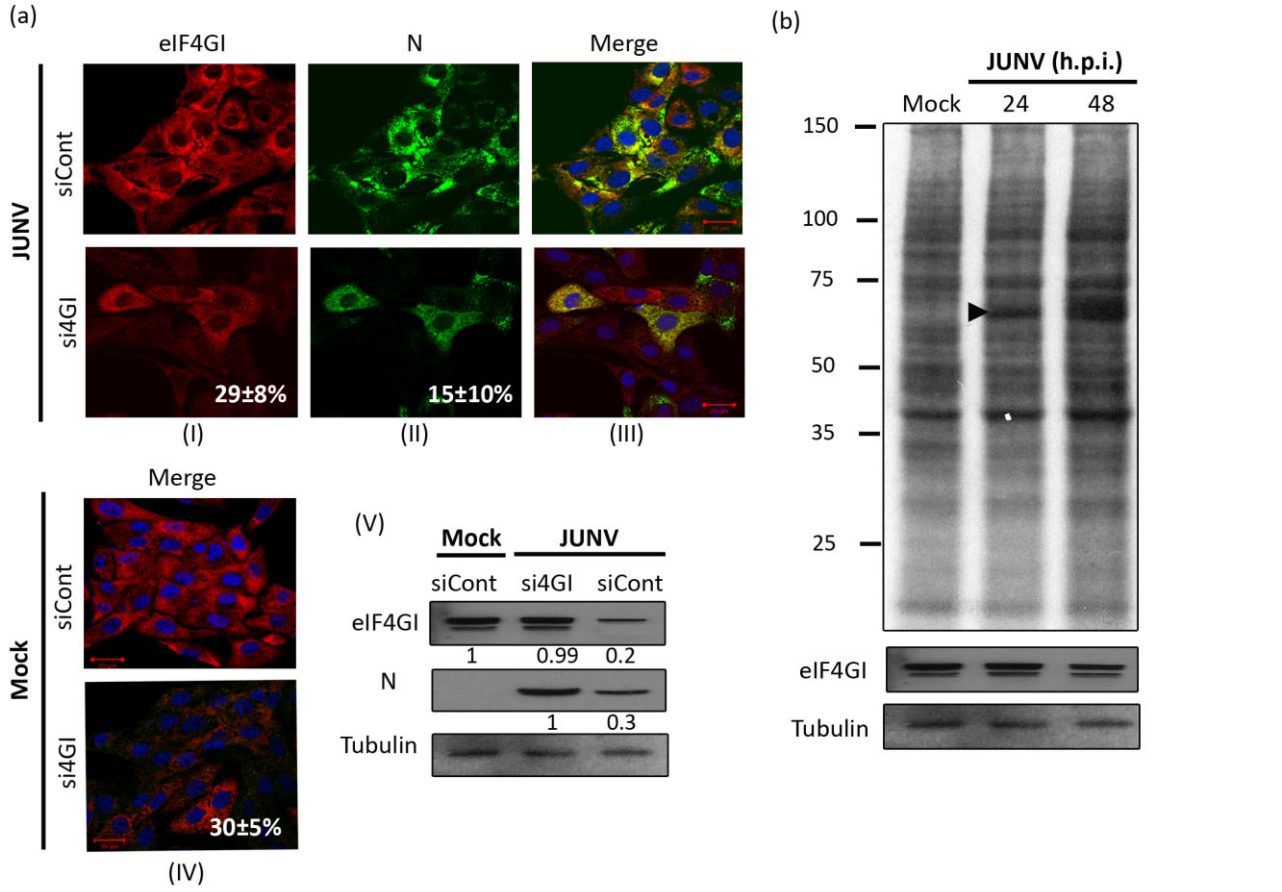
Fig. 4. JUNV nucleoprotein is present in eIF4GI and eIF4A but not in eIF4E immunocomplexes. Vero cells were mock infected or infected with JUNV at an MOI of 1 pfu cell^{-1} and at 24 h.p.i. cells were lysed with RIPA buffer and immunoprecipitated with protein-A sepharose beads previously bound to anti eIF4GI (a), eIF4A (b), eIF4E (c) antibodies or antibodies against the viral proteins N (d) or G1 (e). After immunoprecipitation, proteins were separated by SDS-PAGE and analysed by WB. IP, fraction bound to IgG-protein A-sepharose (immunoprecipitated); SN, fraction unbound to IgG-protein A-sepharose respective to each IP assay; lysate, whole cell lysate.

not be detected among IP proteins, while most of N was present (Fig. 4d, IP-JUNV). These results were complemented when the presence of the factors was examined in IP proteins obtained with anti-viral glycoprotein 1 (G1) antibodies. As shown in Fig. 4e (lane IP-JUNV), no IF4GI, eIF4A or eIF4E was detected in the IP fraction of G1. These results strongly support that the presence of N in the IP proteins obtained with anti-eIF4A and anti-eIF4GI antibodies would reflect a specific interaction of N with both factors.

3. JUNV protein synthesis requires the participation of the scaffolding protein eIF4GI

Results obtained from colocalization and immunoprecipitation assays suggest participation of eIF4GI during JUNV multiplication. In order to verify the involvement of this factor, we first evaluated the effect of eIF4GI depletion by transfecting with siRNAs. Vero cells were transfected with a specific siRNA directed against eIF4GI (si-eIF4GI) (Welnowska *et al.*, 2009) or a non-specific siRNA (siCont) and then infected with JUNV. At 24 h.p.i. cells were processed for IFA and infectivity in supernatants was quantified by a plaque assay. A reduction of about 75% in eIF4GI expression was detected in si-eIF4GI JUNV-infected cells (Fig. 5a-I), where in addition only a 15 ± 10% of N expression was detected in comparison with non-silenced cells (siCont) (Fig. 5a-II). A similar level of depletion, in the range of 70%, was observed in mock infected cultures (Fig. 5a-IV). As control depletion of eIF4GI was also evaluated by WB, showing a reduction on N synthesis in si-eIF4GI JUNV infected cells (Fig. 5a-V). These results were in accordance with data obtained from plaque assay, rendering a reduction of 85 ± 10% (data not shown) of virus yield in eIF4GI depleted cells. The effect of silencing eIF4GI was also evaluated by metabolic labelling with [^{35}S] Met-Cys. In order to find out the condition for the assay, we first evaluated the incorporation of [^{35}S] Met-Cys in JUNV infected cells at 24 and 48 h.p.i. As shown in Fig. 5b, viral protein synthesis, inferred through the presence of a band corresponding to N, could be readily observed both at 24 h as well as 48 h.p.i. In view of these results, Vero cells were transfected with si-eIF4GI or a non-specific siRNA (siCont) and then mock infected or infected with JUNV. At 24 h.p.i. cells were metabolically labelled with [^{35}S] Met-Cys and the synthesized proteins were analysed by SDS-PAGE. Only a very faint band of 60–64 kDa, corresponding to N, could be observed in eIF4GI depleted cells in comparison with cells transfected with the control siRNA (Fig. 5c).

The next approach to evaluate the role of eIF4GI in JUNV protein synthesis was to electroporate an *in vitro* transcribed mRNA containing the encephalomyocarditis virus IRES followed by the sequence of poliovirus 2A protease (2A^{pro}) (IRES-2A mRNA). With this method, quick hydrolysis of eIF4GI occurs (Castello *et al.*, 2006a,b). Vero cells were mock infected or infected with JUNV and at 24 h.p.i. cells were electroporated with two different amounts of IRES-2A mRNA. At 2 h post-electroporation (p.e.), proteins were labelled with [^{35}S] Met-Cys and then analysed by SDS-PAGE. One microgram of IRES-2A mRNA induced inhibition of cellular protein synthesis in both mock (70%) as well as JUNV infected cells (74%), which correlated with 50 and 69% of eIF4GI cleavage respectively (Fig. 5d, lines 2 and 5). When 10 μg of IRES-2A mRNA were electroporated, strong inhibition of cellular protein synthesis was observed (87–88%), which correlated with approximately 90% of cleavage of eIF4GI (Fig. 5d, lanes 3 and 6). Remarkably, JUNV proteins were strongly dependent on



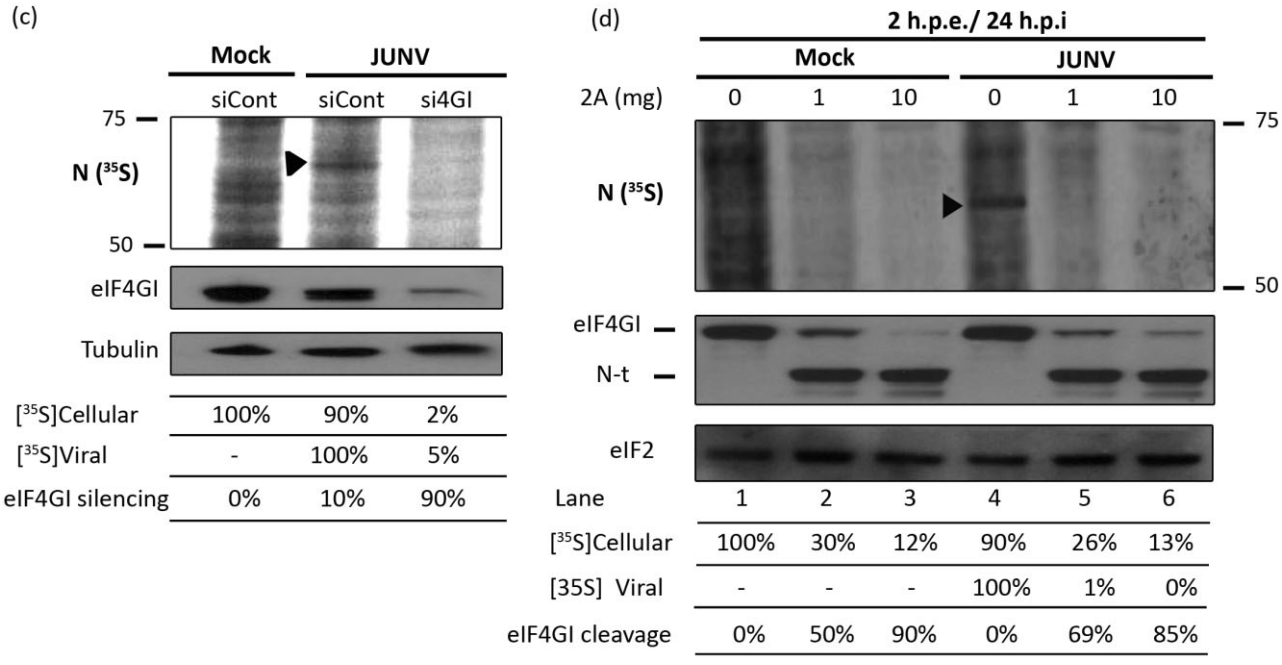


Fig. 5. The scaffolding protein eIF4GI is necessary for JUNV replication.
a. Vero cells transfected twice with a specific eIF4GI-siRNA (si4GI) or a control siRNA (siCont) were mock infected or infected with JUNV at an MOI of 2 pfu cell^{-1} and at 24 h.p.i. cells were fixed and processed for IFA and WB in order to evaluate eIF4GI and N expression. Numbers in column I and IV indicate the percentage of inhibition of eIF4GI expression calculated as the number of cells with lower or absent expression of this factor from 150 counted cells in three independent experiments. The number in column II indicates the percentage of N expression in si4GI in comparison with siCont transfected cells ($n = 3$). Column V showed eIF4GI and N synthesis from si4GI and siCont transfected cells by WB.
b. Vero cells were infected with JUNV at an MOI of 5 pfu cell^{-1} and at 24 and 48 h.p.i. cells were metabolically labelled with [^{35}S] Met-Cys during 1 h. Proteins were then resolved by SDS-PAGE. Arrow indicates the viral protein identified as the putative nucleoprotein.
c. Vero cells transfected twice with si4GI or siCont were mock infected or infected with JUNV at an MOI of 5 pfu cell^{-1} and at 24 h.p.i. cells were metabolically labelled with [^{35}S] Met-Cys during 1 h and proteins were resolved by SDS-PAGE. eIF4GI and tubulin were analysed by WB, as a control. Percentages were calculated by measuring pixels with Image J program ($n = 3$).
d. Vero cells were mock infected or infected with JUNV at an MOI of 5 pfu cell^{-1} and at 24 h.p.i. cells were electroporated with different amounts of the poliovirus 2A protease mRNA prior to in vitro transcription. After 2 h post-electroporation (h.p.e.) cells were metabolically labelled with [^{35}S] Met-Cys and proteins were resolved by SDS-PAGE. Cleavage of eIF4GI was evaluated by WB, and eIF2 was used as control. Percentages were calculated by measuring pixels with Image J program in independent experiments ($n = 2$).

eIF4GI since, both 1 μg and 10 μg of IRES-2A mRNA induced a profound inhibition of viral protein synthesis (Fig. 5d, lanes 5 and 6, upper panel). These results indicate that replication of JUNV requires the functional activity of eIF4GI, since both silencing and cleavage of this factor showed a marked detrimental effect on viral protein synthesis and infectivity.

4. JUNV protein synthesis requires eIF4A

The most abundant eIF is the RNA helicase eIF4A which facilitates translation initiation by unwinding the otherwise inhibitory mRNA secondary structure. In order to study the participation of this factor in JUNV multiplication we evaluated, in a first approach, the effect of the eIF4A inhibitor, hippuristanol. This compound interacts with eIF4A by blocking the RNA-dependent ATPase, RNA binding and helicase activities of this factor (Bordeleau *et al*., 2006). Vero cells were infected with JUNV and treated after infection with different concentrations of hippuristanol. At 24 h.p.i. cells were processed for WB and infectivity in supernatants was quantified by plaque assay. Treatment with hippuristanol induced a dose-dependent reduction of N synthesis (Fig. 6a, middle and lower panel) which was correlated with a strong decrease in virus production, with a 5% virus yield at 5 μM hippuristanol (Fig. 6a, upper panel). These results, indicating the participation of eIF4A in JUNV multiplication, were supported by data obtained from a metabolic labelling assay with [^{35}S] Met-Cys. Vero cells mock infected or infected with JUNV were labelled with [^{35}S] Met-Cys at 24 h.p.i. in the presence of hippuristanol (5 μM). Hippuristanol markedly inhibited cellular protein synthesis either in mock (66%, Fig. 6b, lane 2) and JUNV-infected cells (80%, Fig. 6b, lane 4). On the other hand, viral protein synthesis was completely impaired by hippuristanol (99%) (Fig. 6b, lane 3 versus lane 4), indicating that eIF4A activity is necessary for JUNV protein synthesis. However, it has been reported that hippuristanol is able to induce SGs formation in an eIF2-α phosphorylation independent mode. In fact, SGs induction was detected at 5 μM concentration under these experimental conditions (data not shown). For this reason, the impairment observed for viral and cellular protein synthesis induced by hippuristanol could be also exerted, at least in part, by sequestering the eIF4F components in SGs, eventually blocking translation.

With the aim of more specifically evaluating the requirements of eIF4A, we used a dominant-negative mutant of eIF4A defective in cycling through eIF4F complex (Pause *et al*., 1994). In this assay, poliovirus was used as control since a strong eIF4A-dependence has been previously reported for this virus (Bordeleau *et al*., 2006). Vero cells were transfected with plasmids of expression of eIF4A wild type (eIF4A wt) or mutant (eIF4A mut) and at 24 h post-transfection (p.t.), cells were infected with JUNV or poliovirus as a control. At 24 h.p.i. virus yield was evaluated by plaque assay. As can be seen in Fig. 6c, expression of eIF4A-mut decreased the yield of both JUNV and poliovirus by approximately 50%, indicating that this functional factor is necessary for an efficient virus multiplication.

Finally the requirement for eIF4A was also analysed by silencing this factor using a specific siRNA (Mazroui *et al*., 2006). Vero cells were transfected with the siRNA directed against eIF4A (si4A) or a control siRNA (siCont) and then infected with JUNV. At 24 h.p.i. cells were processed for WB and supernatant infectivity was quantified by plaque assay. A reduction of approximately 50% in eIF4A expression by si4A was observed in mock infected cultures (Fig. 6d, lane 2). A similar level of depletion, in the range of 60%, was detected in si4A JUNV-infected cells, where in addition, a reduction of approximately 50% in N expression was detected (Fig. 6d, lane 4). These results were in accordance with data obtained from plaque assay, rendering a reduction of 62 ± 10% in virus yield in eIF4A depleted cells (data not shown). Taken together, these results indicate that the helicase function of eIF4A would be required for JUNV multiplication.

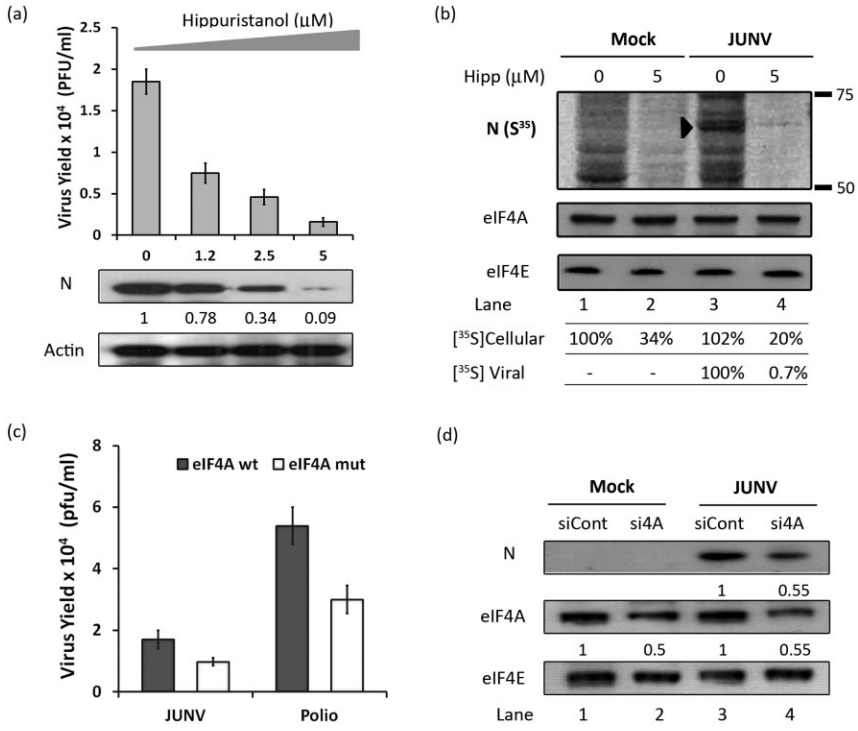


Fig. 6. The RNA helicase eIF4A is required for JUNV multiplication.
a. Vero cells were infected with JUNV at an MOI of 1 pfu cell^{-1} and after adsorption virus was removed and medium was replaced by fresh medium in absence or presence of hippuristanol (1.2–5 μM). At 24 h.p.i. cells were processed for WB (lower panel) and supernatants were quantified by plaque assay (upper panel).
b. Vero cells mock infected or infected with JUNV at an MOI of 2 pfu cell^{-1} were metabolically labelled at 24 h.p.i. with ^{35}S Met-Cys in the presence of 5 μM hippuristanol for 1.5 h and proteins were then resolved by SDS-PAGE. eIF4A and eIF4E synthesis were evaluated by WB. Percentages were calculated as the ratio of cellular or viral proteins pixels with eIF4A pixels, measured with Image J program ($n = 2$).
c. Vero cells were transfected with a plasmid coding for eIF4A-wild type (eIF4A-wt) or eIF4A-mutated (eIF4A-mut) and, at 24 h.p.t., cells were infected with JUNV or poliovirus at an MOI of 1 and 0.01 pfu cell^{-1} respectively. At 24 h.p.i. supernatants were quantified by plaque assay ($n = 3$).
d. Vero cells transfected twice with a specific eIF4A-siRNA (si4A) or a control siRNA (siCont) were mock infected or infected with JUNV at an MOI of 1 pfu cell^{-1} and at 24 h.p.i. cells were processed for WB and supernatants were quantified by plaque assay. Numbers indicate the fold change as the ratio of N or eIF4A with eIF4E used as control, measured with the Image J program.

5. Translation of JUNV mRNAs does not involve a cap-dependent canonical mechanism

The PI3K/Akt pathway is a key regulator of cap-dependent translation through the activation of mTOR/raptor complex, which in turn controls the availability of eIF4E through phosphorylation of 4E-BP (Fig. 7a). In previous studies, it has been shown that the PI3K/Akt signalling pathway is activated by JUNV during entry into the host cell (Linero and Scolaro, 2009). However, PI3K/Akt activation induced by JUNV could also have further implications in virus replication other than entry, for this reason, we extended the study to downstream effectors of Akt. We first evaluated the effect of rapamycin, an mTOR/raptor inhibitor, during JUNV multiplication. Serum starved Vero cells were pre-treated with rapamycin (range 50–250 nM) and then infected with JUNV in the presence of the drug. As shown in Fig. 7b no changes were observed in virus yield (upper panel) or N protein synthesis (lower panel) after 24 h of treatment at all tested concentrations. Similar results were obtained by IFA (Fig. 7c), where a similar level of N expression was observed in 250 nM rapamycin JUNV-infected cells in comparison with untreated cells (85 ± 10% of control). As expected, treatment with Ly294002, a PI3K inhibitor, showed a significant reduction in N expression (15 ± 10% of control). This finding, is in agreement with previously reported data (Linero and Scolaro, 2009) that demonstrated that PI3K/Akt pathway plays a key role during the early steps of virus multiplication. Even though Ly294002 was added after adsorption, progeny virus could have been blocked impairing this way a second round of infection and thus justifying a reduction in N expression detected by IFA (Fig. 7c). To verify the effect of rapamycin in protein synthesis, a radioactive labelling assay was carried out. Serum starved Vero cells were mock infected or infected with JUNV and at 24 h.p.i., pre-treated for 1 h with rapamycin (250 nM) or Ly294002 (40 μM) and then metabolically labelled with [^{35}S] Met-Cys

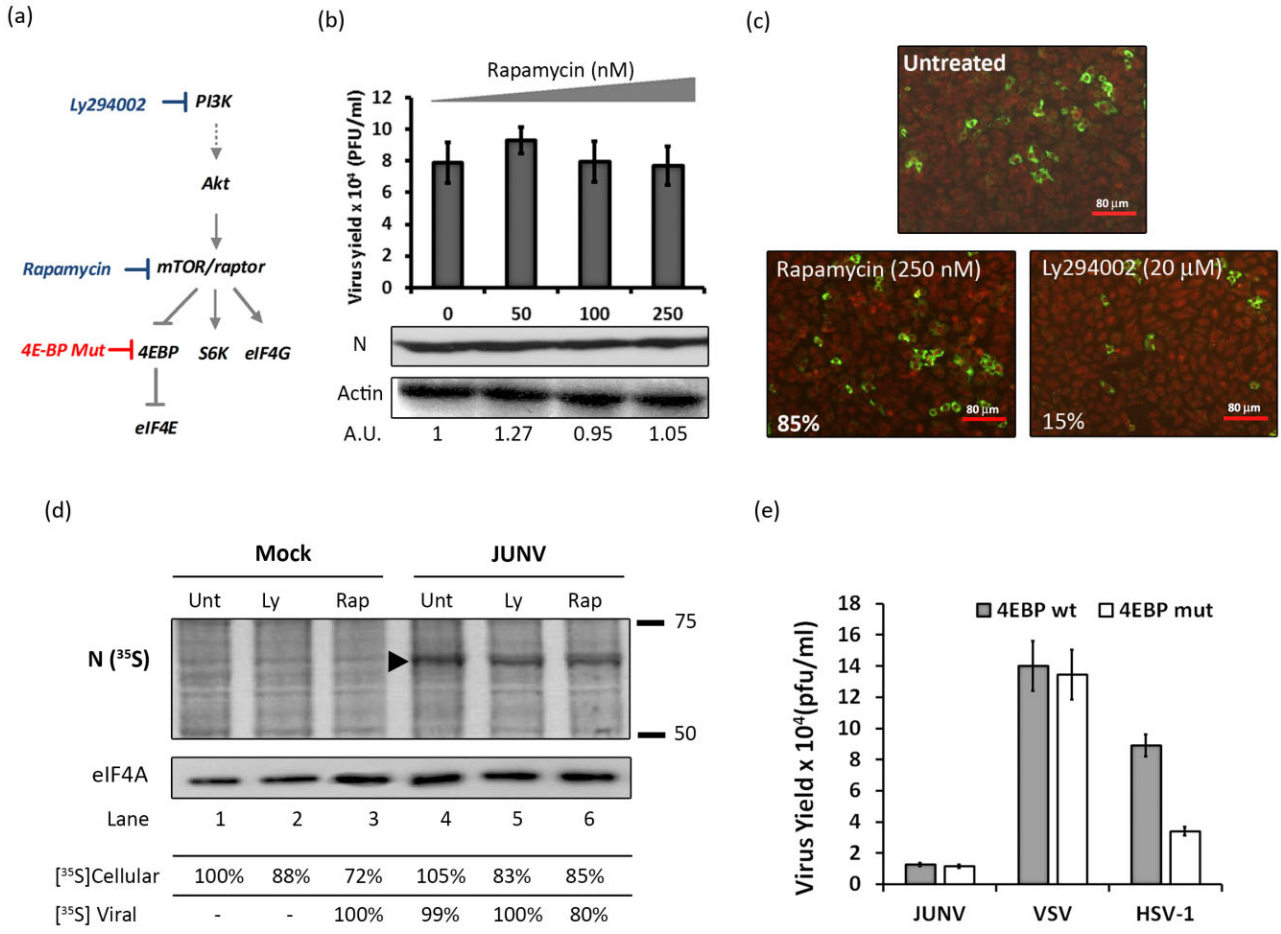


Fig. 7. Inhibition of PI3K/Akt/mTOR pathway does not affect JUNV multiplication.
a. Schematic of the PI3K/Akt/mTOR pathway.
b. Serum starved Vero cells were mock infected or infected with JUNV at an MOI of 1 pfu cell^{-1} and, after virus adsorption, cells were treated with different concentrations of rapamycin (50–250 nM). At 24 h.p.i. cells were processed for WB (lower panel) and supernatants were quantified by plaque assay (upper panel).
c. Serum starved Vero cells were mock infected or infected with JUNV at an MOI of 1 pfu cell^{-1} and, after virus adsorption, cells were treated with 250 nM of rapamycin or 20 µM of Ly294002. At 24 h.p.i. cells were fixed and N expression was evaluated by IFA. Numbers in the images indicate the percentage of cells positives for N expression.
d. Vero cells were mock infected or infected with JUNV at an MOI of 1 pfu cell^{-1} and at 24 h.p.i. cells were pre-treated during 1 h with 250 nM rapamycin or 40 µM Ly294002. Cells were then metabolically labelled with ^{35}S Met-Cys in the presence of the drugs and proteins were resolved by SDS-PAGE. Percentages were calculated as the ratio between viral or cellular proteins and eIF4A measured as pixels with Image J.
e. Vero cells were transfected with a pcDNA3 vector coding for wild-type 4E-BP (4E-BP wt) or a mutated 4E-BP (4E-BP mut). At 24 h.p.t. cells were infected with JUNV (MOI 1 pfu cell^{-1}), VSV (MOI 0.001 pfu cell^{-1}) or HSV (MOI 0.01 pfu cell^{-1}) and at 24 h.p.i. supernatants were quantified by plaque assay ($n = 3$).

in the presence of each compound. The synthesized proteins were analysed by SDS-PAGE. According with the results shown above, rapamycin treatment did not significantly alter viral protein synthesis and only had a slight effect on cellular protein synthesis, rendering an inhibition of about 20% (Fig. 7d, lanes 3 and 6). On the other hand, the inhibition of PI3K by Ly294002 induced a reduction in cellular protein synthesis in the range of 30% (Fig. 7d, lanes 2 and 5), but practically had no effect on JUNV N protein synthesis (Fig. 7d, lane 5). These results suggest that the PI3K/Akt/mTOR pathway would not contribute significantly to JUNV translation apart from the early activation of Akt induced by the virus previously reported (Linero and Scolaro, 2009).

As mentioned above, phosphorylation of 4E-BPs, mediated by mTOR/raptor complex, promotes the release of eIF4E enabling the assembly of eIF4F complex and the eventual delivery of mRNAs to this complex (Zimmer *et al.*, 2000; Li *et al.*, 2002; de Benedetti and Graff, 2004). According to our results, the

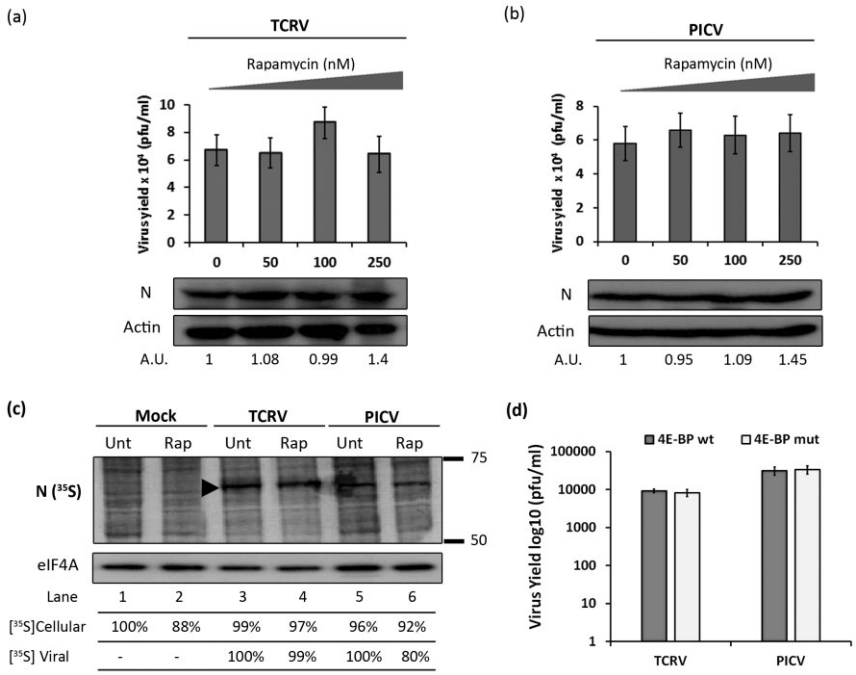


Fig. 8. The New World arenaviruses TCRV and PICV show a similar independence for mTOR pathway as JUNV. Serum starved Vero cells were mock infected with TCRV (a) or PICV (b) at an MOI of 1 pfu cell^{-1} and, after virus adsorption, cells were treated with different concentrations of rapamycin (50–250 nM). At 24 h.p.i. cells were processed for WB (lower panels) and supernatants were quantified by plaque assay (upper panels) ($n = 3$).
c. Vero cells were mock infected or infected with TCRV or PICV at an MOI of 1 pfu cell^{-1} and at 24 h.p.i. cells were pre-treated during 1 h with 250 nM rapamycin. Cells were then metabolically labelled with ^{35}S Met-Cys in the presence of the drug and proteins were resolved by SDS-PAGE. Percentages were calculated as the ratio between viral or cellular proteins and eIF4A measured as pixels with Image J.
d. Vero cells were transfected with a pcDNA3 vector coding for wild-type 4E-BP (4E-BP wt) or a mutated 4E-BP (4E-BP mut) and at 24 h.p.t. cells were infected with TCRV or PICV at an MOI of 1 pfu cell^{-1}. At 24 h.p.i. virus was quantified by plaque assay ($n = 3$).

mTOR/Raptor complex would not significantly participate in JUNV protein synthesis. For a more specific analysis of the requirement for eIF4E in JUNV replication, we studied the effect of overexpression of a constitutively unphosphorylatable 4E-BP1 (Burgui *et al.*, 2007) which associates with eIF4E very strongly preventing the formation of the eIF4E/eIF4G complex and impairing protein translation. Vero cells were transfected with plasmids expressing wild-type 4E-BP1 (4EBP wt) or a non-phosphorylatable 4E-BP1 mutant (4EBP mut) and then infected with JUNV. As shown in Fig. 7e, overexpression of 4EBP-mut had no effect on JUNV multiplication in comparison with 4EBP-wt since virus yield was similar in both cases. As a control, we tested the effect of the 4EBP-mut overexpression on VSV and HSV-1 replication, which initiate translation in an eIF4E-independent and in an eIF4E-dependent manner respectively. As expected, VSV multiplication was similar in cells expressing either 4EBP-mut or 4EBP-wt, whereas HSV-1 replication was strongly affected by the overexpression of 4EBP-mut (Fig. 7e). These results reinforce the idea that JUNV is not dependent on the availability of a functional eIF4E to reach an effective multiplication.

To extend the characterization of mTOR/raptor in arenavirus replication, we included the New World arenaviruses Tacaribe (TCRV) and Pichinde (PICV), belonging to the clades B and C respectively. To this end, Vero cells were pre-treated with rapamycin (range 50–250 nM) and then infected with TCRV or PICV in the presence of the drug. At 24 h.p.i, cells were processed for WB and supernatants were analysed by plaque assay. Neither TCRV nor PICV exhibited any reduction in virus yield in the case of infected cultures treated with rapamycin (Fig. 8a and b, upper panels). Similar results were observed by WB in which synthesis of the corresponding nucleoprotein was not affected by rapamycin at all concentrations tested (Fig. 8a and b, lower panels). Accordingly, treatment with rapamycin did not significantly alter the incorporation of [^{35}S] Met-Cys in viral protein when TCRV or PICV infected Vero cells were subjected to a radioactive labelling assay in the presence of the drug (Fig. 8c). Finally, overexpression of 4EBP-Mut did not have any effect on either TCRV or PICV multiplication (Fig. 8d). Taken together, these results reveal that replication of New World arenaviruses JUNV, TCRV and PICV would take place independently from the mTOR/Raptor regulation.

To further assess that the translation strategy employed by JUNV, TCRV and PICV is eIF4E independent, we examined the effect of silencing eIF4E with siRNA. To this end, Vero cells were transfected with a specific siRNA to deplete eIF4E (si-4E) (Mazroui *et al.*, 2006) and then infected with JUNV, TCRV or PICV. At 24 h.p.i., cells were processed for WB and supernatant infectivity was quantified by plaque assay. As shown in Fig. 9a silencing of eIF4E did not reduce viral nucleoprotein synthesis. In fact, increasing levels of nucleoprotein expression was observed for JUNV and for TCRV and PICV, rendering a 1.6-, 1.6- and 2-fold increase in comparison with control siRNA (siCont). These results were in accordance with data obtained for virus yield showing a moderate increase

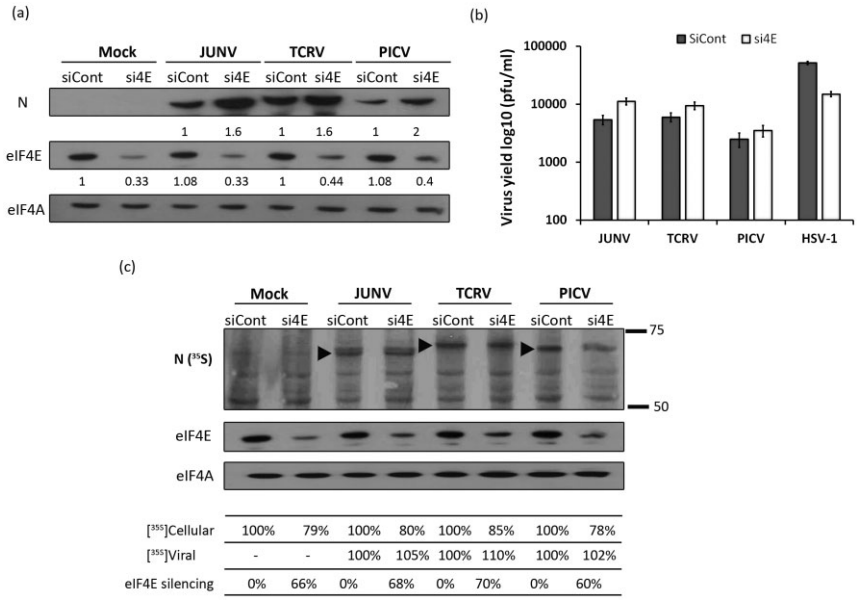


Fig. 9. JUNV, TCRV and PICV are able to replicate in eIF4E depleted cells.
a. Vero cells transfected twice with a specific eIF4E-siRNA (si4E) or a control siRNA (siCont) were mock infected or infected with JUNV, TCRV or PICV at an MOI of 2 pfu cell^{-1} and at 24 h.p.i. cells were processed for WB in order to analyse the synthesis of N, eIF4E and eIF4A. Numbers indicate the fold change as the ratio of N or eIF4E with eIF4A used as control, measured with the Image J program.
b. Alternatively, supernatants were quantified by plaque assay.
c. Vero cells transfected and infected in the conditions mentioned above, were radioactively labelled with ^{35}S Met-Cys at 24 h.p.i. Proteins were resolved by SDS-PAGE and quantified by Image J program. Percentages indicate the fold change of viral and cellular protein synthesis using eIF4A as control. eIF4E silencing was also calculated as the ratio between eIF4E and eIF4A.

in virus titre in JUNV, TCRV and PICV infected eIF4E-silenced cells (Fig. 9b). By contrast, silencing of eIF4E provoked a marked negative effect on HSV-1 showing a 70% of inhibition in virus yield (Fig. 9b). Finally, analysis of viral protein synthesis by metabolic labelling with [^{35}S] Met-Cys was carried out. Vero cells previously transfected with si-4E or siCont were infected with JUNV, TCRV or PICV and, at 24 h.p.i., cells were labelled with [^{35}S] Met-Cys for 1 h and the synthesized proteins were analysed by SDS-PAGE. As shown in Fig. 9c, although si-4E transfected cells showed a considerable reduction in the expression of eIF4E, viral protein synthesis was not affected for JUNV, TCRV or PICV.

In summary, these results indicate that although JUNV is able to activate one of the main signalling pathways responsible for regulation of cap-dependent translation and m7GpppN caps are present at the 5′ ends of the arenavirus mRNAs, virus replication can take place without functional eIF4E. A similar conclusion would also apply for TCRV and PICV.

6. N proteins of JUNV, TCRV and PICV are able to bind to 7 methyl-cap

Previous studies reported the presence of a cavity to bind 7 methyl-cap in the amino terminal extremity of Lassa virus (LASV) nucleoprotein (Qi *et al.*, 2010). According with our results, JUNV is able to multiply under conditions that impair eIF4E functioning. Given that N is present among IP proteins using antibodies against eIF4A and eIF4GI but not eIF4E it could be possible that this viral protein interacts with 7 methyl-cap structures, constituting a translation complex of viral cap-mRNAs. To evaluate this hypothesis, we performed a cap-binding assay using 7 methyl-cap sepharose beads. Lysates of Vero cells mock infected or infected with JUNV were incubated with 7 methyl-cap sepharose beads and, after incubation, fractions bound and unbound to the 7 methyl-cap beads were analysed by SDS-PAGE. Notably, the 7 methyl-cap sepharose beads retained the nucleoprotein of JUNV but not the viral

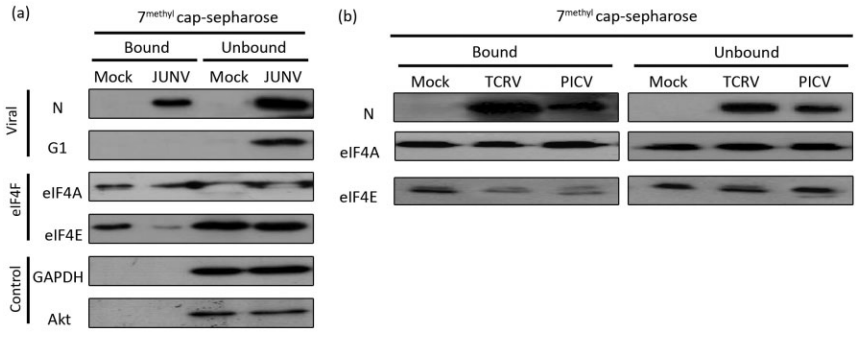


Fig. 10. The viral nucleoprotein is able to interact with 7 methyl-cap. Vero cells were infected with JUNV (a) or TCRV and PICV (b) at an MOI of 1 pfu cell^{-1} and at 24 h.p.i. cells were lysed and incubated with 7 methyl guanosine-protein A-sepharose beads. Proteins retained on the beads (bound) and proteins non-retained on the beads (unbound) were resolved by SDS PAGE and N, G1, eIF4E, eIF4A, GAPDH and Akt proteins were analysed by WB.

glycoprotein G1 (Fig. 10a). In this fraction the presence of eIF4A and eIF4E was also detected. As control the presence of the non-related proteins Akt and GAPDH were also evaluated and as can be seen both proteins were absent in the fraction bound to the 7 methyl-cap sepharose beads. Similar results were obtained for TCRV and PICV, in which the presence of both nucleoproteins was detected in the 7 methyl-cap bound fraction (Fig. 10b). In all cases, non-specific association of N and beads was discarded (data not shown). Taken together, these results support the notion that arenaviral nucleoproteins are able to associate, either direct or indirectly, to the 7 methyl-cap sepharose beads.

Discussion

Unveiling the exact mechanisms used by viral mRNAs to be efficiently translated in the infected cells is of great interest to further understand the strategies followed by animal virus replication. In this sense, viral translational reprogramming strategies include a broad range of mechanisms, from the regulation of signalling pathways that control the functional activity of translation factors to the regulation of the availability of these factors either by controlling their synthesis, degradation and/or translocation (Gale *et al*., 2000).

In the present work, we have studied the participation of the main components of the eIF4F complex in the replication of JUNV in order to characterize the translation mechanism employed by this virus to achieve an efficient viral protein synthesis. Subgenomic mRNAs of arenaviruses have a short additional sequence (1 to 5 nt) with a cap structure at the 5′ end but lack a poly(A) sequence at the 3′ end (Banerjee *et al*., 1975–1976; Kolakofsky and Garcin, 1993; Meyer and Southern, 1993). Previous findings reported a slight cellular shut-off during JUNV replication in cell cultures (Candurra *et al*., 1990) suggesting that the eIF4F complex should not be mainly affected during infection. In fact, our results revealed that JUNV infection did not alter the overall amount of eIF4A, eIF4E and eIF4GI. Taking into account that arenaviral mRNAs possess a cap structure, arenaviruses would likely employ an eIF4E-dependent translation mechanism similar to that used for cellular mRNAs. However, at present, there is growing evidence of viruses which, in spite of having capped mRNAs, exhibit a low dependence of eIF4E, such as Influenza virus (Burgui *et al*., 2007) and hantavirus (Mir and Panganiban, 2008). For hantavirus, whose mRNAs are capped but not polyadenylated, a translational strategy has been proposed in which the viral nucleoprotein is able to replace eIF4E. The hantavirus nucleoprotein binds to the cap of viral mRNAs with higher affinity than to cellular mRNAs, because of the presence of a short conserved sequence following the cap structure. The functions of eIF4A and eIF4G are also replaced by the hantavirus nucleoprotein (Mir and Panganiban, 2008).

Our present findings support the idea that JUNV infection did not significantly modify the subcellular localization of eIF4A, eIF4GI and eIF4E within the cell, however a marked colocalization of N was observed with eIF4A and eIF4GI, but not with eIF4E. These data are strongly supported by the fact that N was able to immunoprecipitate in association with eIF4A and eIF4GI but not with eIF4E. These findings are in agreement with data published recently by Baird *et al*. (2012) describing the presence of cytosolic structures constituted by aggregates of TCRV nucleoprotein. The nucleoprotein in such structures colocalizes with eIF4A and eIF4GI but not with eIF4E and would play a role in the ongoing viral genomic and antigenomic RNA synthesis. However, despite the presence of these translation factors, viral mRNAs would be excluded of these cytosolic structures. The interaction of viral proteins with cellular translation factors has been widely described. For rotavirus, for example, the interaction of the viral protein NSP3A with eIF4E, eIF4A and eIF4GI allows the circularization of the viral capped mRNAs through interaction with the non-polyadenylated 3′ UTR (Piron *et al*., 1998). For Influenza virus, the interaction of the viral proteins NS1 and the viral polymerase (PB2) with translation initiation complexes has been reported, favouring viral translation by the recruitment of cellular factors (Aragón *et al*., 2000; Burgui *et al*., 2003).

The interaction of N protein with eIF4A and eIF4GI suggests a probable functional requirement for both factors during JUNV replication. Accordingly, the alteration of eIF4A, either by silencing or overexpression of an eIF4A dominant negative variant, diminished JUNV multiplication. Treatment with the eIF4A inhibitor, hippuristanol, impaired JUNV protein synthesis, suggesting this factor is necessary for viral protein synthesis. However, taking into account that hippuristanol is able to induce the formation of SGs (Mazroui *et al*., 2006), it is difficult to conclude whether the effect of this compound on JUNV protein synthesis is due to inhibition of eIF4A or a consequence of a global translation blockade because of the hijacking of eIF4F components in SGs. We have previously demonstrated that JUNV infection impairs markedly the formation of SGs when cells are stressed with sodium arsenite but not with hippuristanol (Linero *et al*., 2011). As expected, we demonstrated that eIF4F components were not sequestered into SGs when JUNV infected cells were stressed with arsenite, suggesting the functional requirement of any of the factors that otherwise would be stalled in these silencing structures. In agreement with this observation, silencing of eIF4GI showed detrimental effects on JUNV multiplication. Along these lines, the

expression of the poliovirus 2A protease strongly diminished JUNV protein synthesis. Expression of this protease induced the proteolytic cleavage of eIF4GI in two fragments, an amino-terminal region which contains the binding domains for eIF4E and PABP and a carboxy-terminal region which conserves the binding sites for eIF4A and eIF3 (Castelló *et al*., 2011; Valášek, 2012). The carboxy-terminal product conserves its capacity to bind to some viral mRNAs, representing an important advantage for a cap-independent virus, such as picornaviruses, for which this cleavage product is enough to maintain the IRES dependent translation of viral mRNAs along with a strong inhibition of cellular cap-translation (Ziegler *et al*., 1995; Pestova *et al*., 2000; Castelló *et al*., 2011). In this manner, results obtained for JUNV indicate that the integrity of this factor is a requisite for efficient viral protein synthesis, allowing the speculation that the sites of binding of eIF4E and/or PABP could play a role in JUNV mRNA translation. The absence of a poly-A sequence in arenaviral 3′ mRNAs allows to speculate that the participation of PABP would not be necessary for arenavirus translation. However, it has been reported the binding of PABP to the non-poly-A sequence 3′ mRNAs of dengue virus playing an important role in viral translation (Polacek *et al*., 2008). On the other hand despite depletion of eIF4A and eIF4GI diminished JUNV replication, the effect on global protein translation must be also considered as a possible reason for the detrimental effect on viral protein synthesis observed.

JUNV induces the activation of the PI3K/Akt pathway during viral entry into cells (Linero and Scolaro, 2009). Activation of this pathway phosphorylates the mTOR/Raptor complex, which in turn hyperphosphorylates 4E-BP, allowing the release of eIF4E and hence the activation of cap-dependent translation. Despite the positive effect of this activation on eIF4E-dependent translation, here we demonstrate that JUNV replication could efficiently take place under conditions in which eIF4E-dependent translation is impaired. Treatment of cells with rapamycin as well as the overexpression of a constitutively underphosphorylated 4E-BP1 did not show a severe effect on JUNV multiplication. According to these findings, it has been reported that rapamycin treatment has no effect on LCMV replication, whereas the dual inhibitor of PI3K type I and mTOR, BEZ-2, impairs mainly LCMV budding (Urata *et al*., 2012). We also provide evidence that the independence of viral translation from functional eIF4E was not restricted to JUNV, but was also observed for the New World arenaviruses TCRV and PICV, which were able to multiply under eIF4E unfavourable conditions. In fact, replication of JUNV, TCRV and PICV seemed to be enhanced when cells were depleted of eIF4E after silencing of this factor. This finding would be supported by the fact that Z protein of arenaviruses exerts a repressive function on eIF4E. The interaction of Z with the ribosomal protein P together with the hijacking of eIF4E would be responsible for the translational repression of some cellular proteins, such as cyclin D1 and cyclin 2 (Borden *et al*., 1998; Campbell Dwyer *et al*., 2000).

The independence from eIF4E for JUNV multiplication should involve the intervention of a viral protein that replaces this factor for the binding to the viral cap-mRNAs. The presence of a cap-domain motif in the amino-terminal domain of the LASV nucleoprotein (Qi *et al*., 2010) together with our observation of JUNV nucleoprotein in association with complexes of eIF4A and eIF4GI, makes this protein a key candidate for replacing eIF4E. As speculated, N of JUNV as well as TCRV and PICV strongly bound to a 7 methyl-guanosine sepharose beads. These results would appear to be contradictory with data previously reported for Lassa (LASV) nucleoprotein (Brunotte *et al*., 2011; Hastie *et al*., 2011) in which the cap-binding capacity of N could not be proved. Hastie *et al*. (2011), showed that LASV-N expressed from bacteria was unable to bind to 7 methyl-guanosine sepharose beads while eIF4E, also expressed from bacteria, was readily bound. In this regard, the differential affinity for 7-m GTP between viral cap-binding proteins and eIF4E, as reviewed by Fechter and Brownlee (2005) may be a suitable explanation for this finding. Differences in the experimental approach to visualized N-cap interactions may be also considered as a possible explanation for this apparent discrepancy. Brunotte and co-workers (2011) reported that mutations in the putative cap-binding pocket of LASV-N diminished not only viral mRNA but also genomic RNA synthesis as well, using a minireplicon system. Although this last author did not evaluate the cap-binding capacity of N, the possible loss of N functionality due to conformational changes induced by mutations is highlighted. In this line, the pleiotropic functions conferred to the N protein by means of its interaction with RNAs or other cellular or viral proteins might also explain the apparent lack of consistency between our results and data reported. Although our present results do not fully prove the participation of N as a translation factor, based in our findings, we suggest the involvement of this protein during arenaviral mRNA translation.

Collectively, we propose an eIF4E-independent mode of translation for arenavirus protein synthesis. A putative model for this process might consider that N would replace eIF4E, recruiting eIF4A and eIF4GI to the viral mRNAs. Z protein would help the competition between N and eIF4E by sequestering this cellular factor to inactive sites of cellular translation. Taking into account the lack of a 3′ poly(A) sequence in viral mRNAs, PABP would be unlikely to play a role in JUNV translation. However, requirement for an entire eIF4GI would suggest that N

binds to the amino-terminal domain of this factor in order to achieve efficient replication.

Experimental procedures

Cells, drugs and viruses

Vero cells were grown in minimum essential medium (MEM) containing 5% fetal bovine serum (FBS) (Invitrogen) and 50 μg ml^{-1} gentamicin. Cells were subcultured weekly and maintained in MEM 1.5% FBS after infection.

Infection of Junin virus (JUNV) strain XJCl3, Tacaribe virus (TCRV) strain TLRV 11573 and Pichinde virus (PICV), were performed at the indicated multiplicity of infection (MOI). As controls, Herpes simplex- 1 virus (HSV-1) strain F and poliovirus type III (PV) Sabin strain were used at MOI of 0.01 plaque-forming unit (pfu) cell^{-1} and vesicular stomatitis virus (VSV) Indiana strain was used at MOI of 0.001 pfu cell^{-1}.

Virus titrations were performed by a plaque-forming unit (PFU) assay in Vero cells. In all experiments virus titration was obtained from three independent experiments and was expressed as titre $\pm 2\sigma$ being $\sigma =$ (average number of plaques/number of duplicates)$^{1/2}$ this represents a $P < 0.05$. Ly294002 20–40 μM (GE Healthcare Biosciences) was used as a PI3K inhibitor, rapamycin 50–250 nM (Sigma) was used as mTOR/Raptor inhibitor and hippuristanol 1–5 μM (kindly supplied by Dr J. Pelletier, McGill University, Montreal, Quebec, Canada) was employed as a eIF4A inhibitor. For experiments with Ly294002 and rapamycin, cells were serum starved during 24 h and experiments were performed in MEM 0% FBS in order to reduce the level of activation of PI3K/Akt/mTOR/raptor pathways triggered by serum.

Metabolic labelling and Western blot assay (WB)

For metabolic labelling, Vero cells (1×10^5 cells dish^{-1}) were incubated for 1 h with 300 μl of methionine–cysteine free MEM with 25 μCi of a $^{[35]}$ S-methionine–cysteine mixture (Perkin-Elmer). Cells were then harvested with 30 μl of SDS-PAGE loading buffer (Bio-Rad), boiled for 5 min, and fractionated by SDS-PAGE, followed by autoradiography and fluorography. For inhibitor treatment experiments, drugs were added at the indicated concentrations together with the $^{[35]}$ S-methionine–cysteine mixture. When pre-treatment was needed, drugs were added in methionine–cysteine free MEM at the indicated time and then $^{[35]}$ S-methionine–cysteine mixture was added.

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 20 μl SDS-PAGE loading buffer (Bio-Rad). Proteins in cell lysates were separated by 10% SDS-PAGE and transferred to a PVDF (polyvinylidene difluoride) membrane (Hybond P, Amersham Pharmacia) in a dry system (Multiphor II; LKB Instruments) or in a tank system for proteins of high molecular weight. Membranes were blocked for 1 h with TBS containing 0.1% Tween and 5% bovine serum albumin (BSA, GE). Then, membranes were washed with 0.1% Tween in TBS and incubated with the indicated primary antibody in blocking buffer overnight at 4°C. After rinsing in 0.1% Tween in TBS, secondary antibodies were diluted in blocking buffer and incubated with membranes 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, mix of monoclonal antibodies anti-JUNV N (SA02-BG12, QB06-AE05, NA05-AG12 and IC06-BE10) (Sanchez *et al*., 1989) was used for N-TCRV and N-PICV at 1:600, mouse monoclonal anti-JUNV G1 (GB03BE08; Sanchez *et al*., 1989) at 1:600, rabbit polyclonal anti-eIF4E (Cell Signaling Technology) at 1:1000, rabbit polyclonal anti-eIF4A (Cell Signaling Technology) at 1:1000, rabbit polyclonal antisera raised against peptides derived from the N- and C-terminal regions of human eIF4GI (Aldabe *et al*., 1995) at 1:1000, rabbit polyclonal anti B-actin (Cell Signaling Technology) at 1:1000, mouse monoclonal antibody anti-β-tubulin (Sigma) at 1:50 000, rabbit polyclonal Akt (Cell Signaling) at 1:1000, mouse monoclonal GAPDH (Abcam) at 1:10 000 and rabbit polyclonal eIF2α (Santa Cruz Biotechnology) at 1:1000 dilution. HRP-conjugated goat anti-mouse (Sigma) at 1:1000 and HRP-conjugated goat anti-rabbit (Amersham) at 1:1000 were used as secondary antibodies. The synthesis of cellular protein was measured by densitometry of the lanes in the autoradiography selecting proteic bands comprised between 50 and 55 kDa, a range where no viral proteins are expected. The amount of viral protein was measured by densitometry of the band observed at 60 kDa. Load control was measured by densitometry from WB for each experiment. Cellular protein percentage ([^{35}S cellular]) was calculated as the ratio between the amount of cellular protein and load control and normalized to mock infected cells. The amount of viral protein percentage ([^{35}S viral]) was calculated as the ratio between the amount viral protein and the load control and normalized to JUNV infected cells either untreated or siCont transfected cells. Measures were performed by using ImageJ program.

siRNA and plasmid transfection

siRNA transfection was performed as described previously (Welnowska *et al*., 2009). Briefly, Vero cells were grown at a 60% of confluence and transfected twice with a mixture of 250 nM of siRNA and 0.5 μg of lipofectamine 2000 (Invitrogen) in 100 μl of Optimem I (Invitrogen). Cells were incubated with the mixture at 37°C for 4 h and then transferred in a 1:2 ratio to another 24-well plate and incubated overnight with MEM 5% BSF. At 24 h.p.t., cells were retransfected in the same conditions. At 24 h.p.t. cells, were mock infected or infected and processed according with the experiment. The following siRNAs were employed: si-eIF4E 5′-GGACGAUGGCUAAUUACAUdTdT-3′ (Svitkin *et al*., 2005), si-eIF4GI-3 5′-GAGCGAAGCUGCUGC AGAAdTdT-3′ (Welnowska *et al*., 2009), si-eIF4AI-1 5′-GCC CAAUCUGGGACUGGGAdTdT-3′ (Mazroui *et al*., 2006) and si-Control 5′-CGUACCGUGGAAUAGUUCCdTdT-3′ (Svitkin *et al*., 2005).

For plasmid transfection, Vero cells grown at 60% of confluence were transfected with 0.5 μg plasmid using lipofectamine 2000. At 24 h.p.t. cells were infected and at 24 h.p.i. cells and supernatants were processed accordingly for each specific experiment. The following plasmids were used: pcDNA3-3HA wild-type 4E-BP1 (4EBP wt), pcDNA3-3HA nonphosphorylatable 4E-BP1 mutant with alanine substitutions at T37, T46, S65, and T70 (4EBP mut) kindly supplied by Dr Amelia Nieto (Burgui *et al*., 2007), Wild-type eIF4A (eIF4A-wt) and R362Q eIF4A

(eIF4A-mut) kindly supplied by Dr Antonito Panganiban (Pause *et al*., 1994).

In vitro *transcription assay*

The plasmid pTM1-2A was used as template to synthesize in vitro IRES-2A mRNAs. The in vitro transcription reaction and transfection of resulting mRNAs were carried out as described previously (Castello *et al*., 2006a). Briefly, Vero cells mock infected or infected with JUNV were electroporated at 24 h.p.i. with transcription buffer (0) or 1 μg and 10 μg of IRES-2A mRNAs, respectively, and at 2 h post-electroporation, cells were metabolically label with $^{[35]}$S-methionine–cysteine mixture.

Indirect immunofluorescence assay (IFA)

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 15 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, mouse monoclonal anti-JUNV N (NA05AG12; Sanchez *et al*., 1989) at 1:1000, mouse monoclonal anti-JUNV G1 (GB03BE08; Sanchez *et al*., 1989) at 1:1000, goat anti-TIA-1 (Santa Cruz Biotechnology) at 1:200, rabbit polyclonal anti-eIF4E (Cell Signaling Technology) at 1:50, rabbit polyclonal anti-eIF4A (Cell Signaling Technology) at 1:50 and rabbit polyclonal antisera raised against peptides derived from the N- and C-terminal regions of human eIF4GI (Aldabe *et al*., 1995) at 1:100 were used. As secondary antibodies, donkey anti-rabbit–Alexa 488, donkey anti-goat–Alexa 488 and donkey anti-mouse–Alexa 568 (Molecular Probes) at 1:1000 were used.

Immunoprecipitation assay and binding to 7 methyl-cap sepharose beads

Vero cells grown in 6-well microtitre plates were mock infected or infected with JUNV at an MOI of 1 pfu cell^{-1} and at 24 h.p.i. cells were lysed with 300 μl of RIPA buffer (Ellenberg *et al*., 2004) containing PMSF and aprotinin. Lysates were subjected to three cycles of frozen and thawed, and then clarified by centrifugation at 10 000 r.p.m. during 10 min. Supernatants were incubated with 1:100 antibody 30 min at 37°C followed by 90 min at 4°C. Subsequently, 30 μl of protein-A sepharose beads (100 mg ml^{-1}) were added followed by a new incubation of 30 min at 37°C and 90 min at 4°C. After incubation, beads were centrifuged at 10 000 r.p.m. for 10 min. At this point, supernatants were collected and resuspended in SDS-PAGE sample buffers (unbound fraction), while the pellet was washed three times con lysis buffer and finally resuspended in 30 μl of SDS-PAGE sample loading buffer (IP fraction). Proteins were subsequently analysed by WB.

For precipitation with 7 methyl-GTP sepharose B beads (GE, Healthcare Biosciences), Vero cells grown in 6-well microtitre plates were mock infected or infected with JUNV, TCRV or PICV at an MOI of 1 pfu cell^{-1}. At 24 h.p.i. cells were lysed with 300 μl of RIPA buffer containing PMSF and aprotinin. Lysates were subjected to three freeze-thaw cycles and then clarified by centrifugation at 10 000 r.p.m. during 10 min. Supernatants were incubated with 50 μl of 7 methyl-GTP sepharose beads (0.2 μmol ml^{-1}) overnight at 4°C. After incubation, the beads were centrifuged at 10 000 r.p.m. during 10 min, the pellet was then washed three times with lysis buffer and finally resuspended in 30 μl of SDS-PAGE sample loading buffer. Proteins were subsequently analysed by WB.

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