Role of the phosphatidylinositol-3-kinase/Akt/target of rapamycin pathway during ambidensovirus infection of insect cells

F. Salasc,1,2 D. Mutuel,2 S. Debaissieux,2 A. Perrin,2,3 T. Dupressoir,1,2 A.-S. Gosselin Grenet2,4 and M. Ogliastro2

1EPHE, Pathologie Comparée des Invertébrés, UMR 1333, 34000 Montpellier, France
2INRA, UMR 1333, 34000 Montpellier, France
3Invivo Agrosolutions, 06560 Valbonne, France
4Université de Montpellier, UMR 1333, 34000 Montpellier, France

The phosphatidylinositol-3-kinase (PI3K)/Akt/target of rapamycin (TOR) signalling pathway controls cell growth and survival, and is targeted by a number of viruses at different phases of their infection cycle to control translation. Whether and how insect viruses interact with this pathway remain poorly addressed. Here, we investigated the role of PI3K/Akt/TOR signalling during lethal infection of insect cells with an insect parovirus. Using Junonia coenia densovirus (JcDV; lepidopteran ambidensovirus 1) and susceptible insect cells as experimental models, we first described JcDV cytopathology, and showed that viral infection affects cell size, cell proliferation and survival. We deciphered the role of PI3K/Akt/TOR signalling in the course of infection and found that non-structural (NS) protein expression correlates with the inhibition of TOR and the shutdown of cellular synthesis, concomitant with the burst of viral protein expression. Together, these results suggest that NS proteins control the cellular translational machinery to favour the translation of viral mRNAs at the expense of cellular mRNAs. As a consequence of TOR inhibition, cell autophagy is activated. These results highlight new functions for NS proteins in the course of multiplication of an insect parovirus.

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INTRODUCTION

Viruses are obligate intracellular parasites that rely entirely on host cell resources for their multiplication; one major challenge is thus to control translation. Very different viruses may use convergent strategies to hijack translational components, either targeting key signal transduction pathways and/or antagonizing cell antiviral response (Buchkovich et al., 2008).

The majority of cellular mRNAs require a cap initiation complex to initiate translation and many viruses target this process, either to maintain cap-dependent translation and/or to divert translational components (Schneider & Mohr, 2003). The phosphatidylinositol-3-kinase (PI3K)/serine/threonine kinase target of rapamycin (TOR) pathway is a major transduction signalling cascade controlling cap-dependent translation (Laplante & Sabatini, 2009); its activation propagates to downstream effectors through a coordinated cascade of phosphorylation. PI3K activation is initiated at the membrane by the phosphorylation of a three phosphate-lipid product, which activates the 3-phosphoinositide-dependent protein kinase 1 (PDK1) and protein kinase B (PKB)/Akt kinases. The signalling is next relayed, via the TOR pathway, to major effectors: eukaryotic translation initiation factor 4E (eIF4E)-binding protein (4E-BP; a repressor of eIF4E that is released upon phosphorylation), S6 kinases (S6Ks) and ribosomal protein S6 (rpS6) composing the 40S ribosomal subunit, which ultimately activate translation of capped mRNAs (Dibble & Cantley, 2015). PI3K/Akt/TOR signalling also controls cell death and survival, making this pathway a target of choice for many viruses that have developed exquisite strategies to maintain cell metabolic activity or counteract detrimental early death signalling (Cooray, 2004; Diehl & Schaal, 2013).

These signalling pathways are conserved amongst phyla, yet most of our knowledge about how they are manipulated by viruses concerns infections of vertebrates or plants; very few studies concern other phyla.

Viruses infecting insects, including insect-borne viruses, have long been studied because of economic or health threats. The fact that viruses can infect several hosts, where they can be lethal, asymptomatic or mutualistic, questions the molecular mechanisms underlying adaptation, specificity and virulence (Ryabov et al., 2009; Xu et al., 2014). At the molecular level, lethal infections of
insects analysed so far show a global shutoff of cellular synthesis (Katsuma et al., 2007; Garrey et al., 2010). The most advanced knowledge we have about the mechanisms involved comes from studies with discistroviruses, particularly the cricket paralysis virus (CrPV) (Jan, 2006; Nakashima & Uchiumi, 2009). CrPV infection inactivates the eIF4E complex which selectively inhibits host translation and favours the translation of viral mRNA containing a specialized internal ribosome entry site (RES) (Garrey et al., 2010). By contrast, baculovirus infections activate the PI3K/Akt signalling pathway, but the mechanism involved to shut off cellular synthesis is unknown (Katsuma et al., 2007; Xiao et al., 2009).

Densoviruses are small naked viruses with a 4–6 kb linear ssDNA genome ended by inverted terminal structures of different sizes and complexity (Bergoin & Tijssen, 2008). They belong to the family Paroviridae, where the subfamily Densovirinae infects arthropods (Cotmore et al., 2014). Like all paroviruses, the genome of densoviruses is relatively simple, with two sets of open ORFs coding for non-structural (NS) and structural proteins (VPs), arranged in a tandem array, except for ambidensovirus that have an original ambivalent NS (NS) and structural proteins (VPs), arranged in a tandem array, except for ambidensovirus that have an original ambivalent genome. Like their vertebrate counterparts, densovirus NS proteins is a pivotal role in viral genome replication, parvoviruses. They belong to the family Paroviridae, where the subfamily Densovirinae infects arthropods (Cotmore et al., 2014). Like all paroviruses, the genome of densoviruses is relatively simple, with two sets of open ORFs coding for non-structural (NS) and structural proteins (VPs), arranged in a tandem array, except for ambidensovirus that have an original ambivalent genome. Like their vertebrate counterparts, densovirus NS2 protein function has been attributed so far to densovirus NS2 protein (Wang et al., 2013a). The main function attributed to NS proteins is a pivotal role in viral genome replication, particularly for NS1 (Ding et al., 2002; Abd-Alla et al., 2004). No function has been attributed so far to densovirus NS2 protein and no homology exists amongst paroviruses. In summary, JcDV multiplication relies on seven proteins only.

We analysed the temporal progression of JcDV lethal infection and the main cytopathic effects (CPEs) on cell size, growth and survival. Our results showed that JcDV requires a functional PI3K/Akt/TOR pathway to initiate infection. NS proteins induce the inhibition of the TOR pathway, thereby favouring viral translation at the expense of cellular proteins. These results highlight new functions for NS proteins in the course of multiplication of an insect parvovirus.

RESULTS

JcDV replication affects cell size, cell proliferation and survival

JcDV replication was analysed daily until 6 days post-infection (p.i.). Viral genome amplification was detected within 2 days p.i. and accumulated >300 000 copies per cell within 6 days (Fig. 1a). Comparing daily measurements showed that viral genome amplification occurred between days 1 and 3 p.i., before pausing from day 4 p.i. (Fig. 1a). A modest increase was noted again at day 6 p.i., which might reveal secondary infections within the cell population and thus asynchronous infection. If so, accurate timing of the infection cycle is impossible. We tentatively estimated that the duration of the virus cycle is 5 days, with 3 phases: (i) an early phase (day 1 p.i.), including entry and initiation of transcription/replication of the viral genome, (ii) a burst phase of amplification (days 1–3 p.i.), and (iii) a productive phase (days 4–5 p.i.); new viral particles can initiate secondary infections.

We next characterized the CPEs associated with JcDV infection. Cells typically display a roughly spherical shape with short pseudopodia (Fig. 1b). Upon infection, there was an overall enlargement and modifications of shape accompanied by cell morphological changes. Infected cells rounded up and stacked to form clusters with cells developing long extensions connecting clusters (Fig. 1b). At 4 days p.i. we observed that infected cells immunolabelled with anti-VP antibodies displayed different fluorescence intensities in the population, reflecting different stages of infection (Fig. 1c). It is noteworthy that cellular extensions were strongly labelled with anti-VP antibody, suggesting some role in cell-to-cell transmission of the virus (Fig. 1d). Although all cells were infected at 30 min p.i. (data not shown), we estimated that ~70 % displayed typical virus labelling at 4 days p.i. Conversely, ~30 % of the cells had no clear labelling and were either in the early phase of infection or non-infected, which supported asynchronous infections.

To calculate cellular volume changes during infection, cell nuclei were labelled with Hoechst, and cellular and nuclear diameters were measured in non-infected and infected cells at 4 days p.i. Cellular and nuclear volumes increased three- and fourfold respectively (not shown), giving a threefold increase of the nucleo/cytoplasmic size ratio following viral replication (0.71 ± 0.22 in infected cells compared with 0.38 ± 0.16 in non-infected cells, P<0.001). We then examined cell proliferation and survival with a Trypan blue exclusion assay. As shown in Fig. 1(f), cell proliferation stopped from day 3 p.i., which corresponded to the burst of JcDV amplification (Fig. 1a). Asynchronous infections probably blurred this timing as cells non-infected at primary infection keep proliferating. Regarding cell death, a slightly increased mortality was noticed at 1 day p.i. (Fig. 1g) that might be induced by virion entry and/or their intracellular traffic. No significantly increased mortality followed the burst phase of viral replication (2–3 days p.i.); mortality increased significantly from 5 days p.i. as expected (Fig. 1g), correlating with the end of primary infections and early secondary infections. These data support the JcDV cycle estimated from quantitative viral replication.
Fig. 1. JcDV replication affects cell size, cell proliferation and survival. (a) JcDV replication in Ld652Y cells. Viral replication was measured by quantitative (q) PCR using VP4 primers. The amount of viral genomes was normalized with those of β-actin gene copies to obtain viral genomes per cell. Values represent the mean value of three independent experiments and three technical replicates. Daily amplification was calculated by dividing the amount of viral genomes per cell at day \( n + 1 \) by the amount at day \( n \). (b) Microscopic observation of cell morphological changes in non-infected (NI) or JcDV-infected cells at 4 days. DIC, differential interference contrast. Bar, 50 μm. (c) Immunolabelling of non- and JcDV-infected cells at 4 days. Cells were labelled with anti-VP and Alexa Fluor 594-conjugated goat anti-rabbit antibodies (red). Nuclei were labelled with Hoechst. (d) Nucleo/cytoplasmic ratio. (e) Cell proliferation (cells ml−1). (f) Cell mortality (%).
These results showed that JcDV infection affects cell size, cell proliferation and survival. These key cellular processes are mainly controlled by the PI3K/Akt/TOR signalling cascade, which prompted us to analyse this pathway in the course of JcDV infection.

**JcDV controls cellular translation machinery to favour viral protein syntheses**

To test whether JcDV infection modulates PI3K/Akt/TOR signalling, we first used a biochemical approach. Several specific antibodies have been previously validated in insect cells (Arsic & Guerin, 2008; Nagata et al., 2008; Xiao et al., 2009; Liu et al., 2010). We thus analysed the phosphorylation status of key regulators of the pathway, i.e. Akt, TOR, 4E-BP, p70S6K and rpS6, in infected and non-infected Ld652Y cells by Western blotting. Cells were harvested at different times p.i. and whole lysates analysed with the different antibodies, including antiviral antibodies, to follow infection. NS1 and VP proteins were clearly detected from day 2 p.i. and accumulated in cells up to day 6 p.i. (Fig. 2), paralleling the replication of the viral genome (Fig. 1a). Levels of Akt phosphorylated on Ser473 were likely constant over infection (Fig. 2), indicating the maintenance of a metabolic activity rather than an induction by infection. Downstream of Akt, the phosphorylation of TOR on Ser2448 was maintained until 2 days p.i., but was completely inhibited from 3 days p.i. (Fig. 2b). This inhibition correlated with the hypo-phosphorylation of TOR downstream targets 4E-BP and p70S6K on Thr37/46 and Thr389, respectively. We cannot exclude that 4E-BP phosphorylation may occur on other residues that could promote either its release from eIF4E or the activation of p70S6K. Intriguingly, in addition to the 70 kDa protein, the p-p70S6K antibody mainly revealed a 40 kDa product (Fig. 2a, b). This product was also revealed by an antibody raised against the total (unphosphorylated) form of p70S6K, suggesting proteolytic cleavage of this protein, concomitant with the burst of viral replication. We observed that the p-p70S6K antibody recognized a single band at 70 kDa, whilst the p70S6K antibody recognized a doublet at 70 kDa and three bands were observed from 2 days p.i. These bands may correspond to different post-translational modifications of p70S6K, i.e. other than phosphorylation of Thr389. In insects, p70S6K contains between 446 and 550 aa depending on the species. As the 40 kDa protein includes Thr389, which is located in the C-terminal linker region, we concluded that truncation removed ~200 aa in the N terminus, including the activation loop and 100 aa of the catalytic domain (from residue 89 to 394 in *Bombyx mori*), which might cause the inactivation of the kinase function (Alessi et al., 1997). As a main target of S6K, we analysed the phosphorylation of the ribosomal protein rpS6. This protein can be phosphorylated on five clustered residues that are conserved amongst metazoans, including Ser236 and Ser244. These residues can be phosphorylated by p70S6K or p90 ribosomal S6K (Reggiori et al., 2010); interestingly, the ribosomal S6K family members are downstream effectors of the Ras-extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) signalling cascade and therefore independent of TOR (Anjum & Blenis, 2008). We thus analysed the phosphorylation of rpS6 on Ser236 and Ser244 in the course of JcDV infection. Results showed that phosphorylation of rpS6 on Ser236 was very low in non-infected and early infected cells compared with phosphorylation at Ser244 (Fig. 2b). More interestingly, from 2 days p.i., rpS6 was hyper-phosphorylated at the two positions tested, indicating that its activation can be mediated by an alternative mechanism to selectively support protein synthesis during infection.

To test whether TOR inhibition affected cellular protein syntheses, we analysed *de novo* protein synthesis by labelling nascent proteins with [35S]methionine and cysteine in the course of infection. At early times p.i., proteins synthesized *de novo* were rather similar in infected and non-infected cells (Fig. 3a, 1–3 days p.i.), but changed from 3 days p.i. where synthesis was likely dominated by the four VPs, identified based on their molecular mass (calculated sizes were 89, 58, 53 and 47 kDa for VP1–4, respectively; Fig. 3). VP4 was the most highly expressed, as expected from the VP molecular ratios in the capsid, but we noticed the overexpression of a 35 kDa protein from 3 days p.i. The pattern of proteins accumulating in cells per day was similar to *de novo* synthesis (Fig. 3b), although viral proteins were detected earlier (from 2 days p.i.), i.e. at the onset of viral replication. As 35 kDa might correspond to NS2, we analysed the gel by Western blotting with an anti-NS2 antibody. Fig. 3(c) shows that the NS2 antibody specifically recognized the 35 kDa protein. It is noteworthy that NS1 and NS3 (68 and 15 kDa, respectively) could not be identified with certainty in any of these experiments, suggesting their relatively low expression.

Although there was a relative overexpression of viral proteins from 3 days p.i., results showed JcDV did not trigger a clear global shutoff of cellular synthesis as observed for CrPV. To better assess host response to infection, we next analysed eIF2α phosphorylation. Results showed that unlike CrPV, eIF2α phosphorylation did not change over the course of infection, further suggesting that JcDV infection may be not as detrimental to cells as CrPV (Fig. 3d).
NS proteins are the key factors controlling cellular translation

To determine which viral product(s), amongst proteins and the genome, could control cellular translation, we tested the independent effect of NS/VP proteins or the genome on the phosphorylation patterns of 4E-BP, S6K and rpS6. Cells were transfected with non-replicative constructs coding for either NS (pJΔVP) or VP (pJΔNS) proteins, or with a replicative construct containing the entire viral genome (pBRJH). We analysed viral protein expression and phosphorylation of TOR, 4E-BP, p70S6K and rpS6 by Western blotting and Coomassie blue staining (Fig. 4a, b). Fig. 4(a) shows first that the stoichiometry of viral proteins, although expressed separately from

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**Fig. 2.** JcDV infection induces inhibition of the TOR pathway and activates translation by an alternative pathway. Analysis of PI3K and TOR effector activation. (a, b) Ld652Y cells were non-infected (NI) (a) or infected with JcDV (b), and total proteins were extracted at early time of infection, from 30 min to 24 h p.i., and from the burst of viral replication (2–6 days p.i). Protein expression and/or phosphorylation were analysed with the indicated antibodies. Arrowheads indicate the position of p70S6K (70 kDa) and its truncated form (40 kDa). Non- and JcDV-infected samples were loaded on the same gels. p, phosphorylated form.
could be processed independently of infection. Concerning cellular components, results showed that the hypophosphorylation of TOR and 4E-BP, the hyperphosphorylation of rpS6, and the cleavage of p70S6K were positively correlated with the expression of NS proteins, i.e. only observed in cells transfected with the construct deleted of the vp gene (pJAVP) or with the replicative construct pBRJH, but not in the absence of the ns gene (pJANS). This result suggested that NS proteins were sufficient to induce the inhibition of TOR and to activate an alternative mechanism to support translation. Which NS protein(s) is/are involved and what is the mechanism have yet to be determined.

**TOR is essential to initiate JcDV infection but not for its replication**

To clarify the role of TOR in infection, we developed a functional approach. As the genome of *Lymantria dispar*, the host from which Ld652Y cells originate, is not sequenced and the design of a small interfering RNA is still a hurdle in these cells, we thus used pharmacological inhibitors. Rapamycin and torin-1 target TOR by different mechanisms, although they both block cell division at the G1 phase and both affect protein synthesis (Aghdasi *et al.*, 2001). As these drugs have never been assayed in Ld652Y cells, we tested increasing concentrations of rapamycin or torin-1 (0.1, 1 and 10 μM), and measured cell proliferation and mortality. Both drugs significantly affected the cell proliferation rate at 1 and 10 μM (Fig. 5a, b), with a dose-dependent effect. No significant toxicity was observed at any of the doses tested compared with cells treated with the vehicle (DMSO); indeed 1 μM rapamycin likely decreased cell mortality (Fig. 5b). As Ld652Y cells respond to drugs over a wide range of concentrations, we considered 0.1–10 μM in the experiments reported below.

To test the specificity of the response to rapamycin, we analysed the levels of phosphorylation of TOR effectors as above, i.e. 4E-BP as a direct target and rpS6 that can also respond to mitogenic stimulation. As expected, the phosphorylation of 4E-BP was completely inhibited following 1 and 10 μM rapamycin treatments (Fig. 5c), whereas levels of phosphorylation of rpS6 in rapamycin-treated cells were similar to the untreated conditions. This result showed that rpS6 was not sensitive to rapamycin and might thus be activated independently of TOR in Ld652Y cells.

We then verified that rapamycin did not affect virus uptake. Cells were pre-treated with the drug and then infected for 30 min before immunolabelling with an anti-VP antibody to localize viral particles. Labelling was cytoplasmic in both treated and untreated cells, showing that rapamycin did not alter virus uptake (Fig. 5d).

We concluded that Ld652Y cells respond...
Fig. 4. NS proteins control cellular translation mechanisms. Ld652Y cells were transfected with pBRJH, pJΔNS or pJΔVP, or non-transfected (NT). At the indicated times post-transfection (p.t.), total proteins were separated by SDS-PAGE. (a) Expression of VP, NS1, NS2 and S6K proteins; phosphorylation of TOR, 4E-BP, S6K and rpS6 analysed by Western blot. (b) Total protein pattern of Ld652Y cells transfected with pBRJH, pJΔNS or pJΔVP analysed at 3 days post-transfection by Coomassie blue staining. Arrowheads indicate the positions of VP proteins.
(a) (b)

(c) 8 h

DMSO 0.1 μM
DMSO 1 μM
DMSO 10 μM
Rapa 0.1 μM
Rapa 1 μM
Rapa 10 μM
Torin 0.1 μM
Torin 1 μM
Torin 10 μM

Cell proliferation (cells ml⁻¹)

Cell mortality (%)

1 10 1 10 1 10 1 10 μM
Rapa DMSO Rapa

24 h (d)

JcDV Hoechst

Hoechst Merge

Merge

JcDV

Viral genomes (treated/untreated ratio)

1.0

0.8

0.6

0.4

0.2

0.0

Unt Unt

2 da

SI

1 h before infection

Rapa

1 μM

Rapa

10 μM

Rapa

0.1 μM

Torin

1 μM

Torin

10 μM

(f)

Viral genomes (treated/untreated ratio)

1.0

0.8

0.6

0.4

0.2

0.0

Unt Rapa Rapa Torin Torin

1 h before infection

2 days p.i.
specifically to rapamycin, making the pharmacological approach suitable.

To analyse the function of TOR at early (initiation) and late (post-replication) phases, cells were treated with TOR inhibitors before and after viral replication, respectively. Fig. 5(e) shows that treating cells before infection significantly decreased JcDV replication in a dose-dependent manner (similar effects were observed with rapamycin and torin-1; we thus used rapamycin in the following experiments). In contrast, treating cells with rapamycin at 2 days p.i. (i.e. after initiation of viral replication) had no impact on virus replication (Fig. 5f). Taken together, these results suggest that the early phase of infection only depends on TOR, but not the late phase, which supports the results above. As rapamycin induced cell cycle arrest at the G1 phase, the more likely explanation for the inhibition of virus replication is that the G1 phase is not compatible with early infection. It is noteworthy that the drug release experiment showed that infection recovered with time after removing the drug, further suggesting that the viral growth defect was not due to cytotoxicity (Fig. 5g).

We next assessed viral gene transcription (at 3 days p.i.) by reverse transcription (RT)-quantitative qPCR and viral particles released in the medium at cell death (at 6 days p.i.) by qPCR. Rapamycin treatment significantly affected the transcription of viral genes and the production of viral particles compared with untreated infected cells, as a consequence of virus replication default (Fig. 5h, i).

The PI3K/Akt/TOR pathway is required for early infection, probably to drive the cells to the appropriate phase of the cell cycle to initiate virus replication and NS expression. NS proteins then likely trigger the inhibition of TOR to favour the translation of viral mRNAs and boost viral replication.

**JcDV infection induces cell autophagy**

Autophagy is a highly conserved cell survival mechanism amongst eukaryotes and activated by TOR inhibition. It
is regulated by a family of proteins encoded by autophagy-related (atg) genes and has been characterized in the lepidopteran B. mori (Zhang et al., 2009). Atg8 is a ubiquitin-like protein controlling autophagosome formation and size, and is widely used to monitor autophagy, including in lepidopteran cells (Gai et al., 2013). To test whether autophagy was activated in response to NS-induced inhibition of TOR, we analysed Atg8 localization by immunofluorescence in non-infected and rapamycin-treated cells at 4 days p.i. Typical cytoplasmic punctae containing Atg8 were enhanced in rapamycin-treated cells as a response to the chemical inhibition of TOR (Fig. 6). This labelling was further enhanced in infected cells, suggesting virus-induced inhibition of TOR activated autophagy. However, Atg8 labelling was also localized in the nucleus of infected cells, which might indicate its relocalization as a consequence of infection. These results showed that after the burst of virus replication, there is a positive correlation between the inhibition of TOR and the activation of autophagy in Ld652Y cells.

**DISCUSSION**

Here, we investigated the interaction of an insect parovirus with the host PI3K/Akt/TOR signalling pathway. Our results show that PI3K/Akt signalling is maintained throughout infection, whereas NS proteins likely inactivate the TOR pathway, which consequently triggers cell autophagy. This inhibition of cap-dependent translation by NS proteins might favour the high level expression of viral proteins, particularly NS2 and VPs, although these mechanisms remain to be clarified.

The selective inhibition of cellular synthesis by insect viruses to favour the translation of their own mRNAs has been exemplified by dicistroviruses (Garrey et al., 2010). The lack of a typical IRES structure in densovirus mRNAs and similarities with vertebrate parvoviruses, has so far favoured the hypothesis that translation occurs by leaky scanning by the ribosomes of the mRNAs and translation initiation depends on the context of the initiation codons (Bergoin & Tijssen, 2000). VP4 or NS2 are in a more favourable context compared to VP1 and NS1, which supports their respective abundant expression (Wang et al., 2013a). However, the translation of viral mRNAs may be more complex. Indeed, transfection experiments highlight that translation of NS and VP proteins is independently processed, and can also process independently of virus replication. On the one hand, the translation of VP mRNA is independent of TOR, i.e. the relative abundance of the four VPs is similar whether...
TOR is active or not, although VP expression is boosted by viral replication, i.e. when TOR is inhibited. On the other hand, NS proteins triggered TOR inhibition, suggesting that a specific, NS-dependent mechanism interferes with the translation machinery. We speculate that the synthesis of NS and VP proteins may involve different mechanisms that cooperate to boost virus production. The use of independent strategies to produce viral proteins is original amongst paroviruses and may result from the acquisition across evolution of an ambisense genome organization, which may limit competition for cellular factors and thus improve expression.

A key point in infection likewise depends on the capacity of NS proteins to subvert translational components, particularly their amount. It has been proposed that the NS1 concentration determines, by a feedback regulation, a switch between transcription and replication of the viral DNA (Bergoin & Tijssen, 2000). We propose that NS protein concentration may also determine a switch between early and late translation mechanisms in order to face the high translational demand of viral mRNAs. Which NS protein(s) is/are involved and which cellular interactions determine their function in translation are questions currently being studied in our laboratory.

We inadvertently found that NS protein expression correlates with the cleavage of p70S6K, which probably inactivates its function. However, our data suggest that p70S6K is not the main physiological S6K operating in Ld652Y cells. Indeed, the phosphorylation of rpS6 is not sensitive to rapamycin and may thus depend on an alternative pathway, independent of TOR and p70S6K, which further supports that p90 ribosomal S6K might be the S6K involved. We now need to investigate the role of PDK1 and ERK/MAPK pathways during JcDV infection as their role in parovirus infection has already been shown (Riolobos et al., 2010; Chen et al., 2011; Bär et al., 2015). Interestingly, a previous study showed that p70S6K was cleaved by caspase-3 at an unconventional motif during DNA damage-induced apoptosis (DNA damage response; DDR; Dhar et al., 2014). It has been shown that DDR is activated during parovirus replication and is required for infection (Adyemii et al., 2010; Cotmore & Tattersall, 2013); p70S6K cleavage might be an outcome of JcDV-induced DDR activation.

Lastly, our results showed that JcDV infection affected cell survival and induced autophagy. These results are in agreement with TOR inhibition; however, the role of autophagy in the JcDV infection process remains to be assessed. The nuclear localization of Atg8 has been known for a long time, although its significance in starvation-induced autophagy has been assessed only recently (Huang et al., 2015). We cannot exclude that JcDV modifies Atg8 relocalization and/or that nuclear Atg8 may have other functions in the virus cycle independently of autophagy, as shown for other viruses (Reggiori et al., 2010; Robinson et al., 2014).

Data provided in this work highlight how the densivirus lytic cycle requires a strict coordination of direct or indirect interactions of NS proteins with the PI3K/Akt/TOR pathway, determining a quantitative switch in virus amplification with associated cytolysis. Whether and how these molecular interactions are orchestrated in the context of mutativistic or persistent densivirus infections would be interesting questions to consider.

**METHODS**

**Cells, virus and infections.** L. dispar ovarian cells, IPLB-Ld652 cells (Ld652Y) (Goodwin et al., 1978) and JcDV were used in this study as described previously (Vendeville et al., 2009). JcDV titres were determined on Ld652Y cells by TCID<sub>50</sub> assay in 96-well plates as described previously (Li et al., 1996). Infections were performed for 1 h at 4 °C in serum-free medium in order to allow virus adsorption (m.o.i. 5). The inoculum was then removed and cells grown in 10 % serum TC100 medium at 28 °C for the indicated times.

**Plasmids and transfections.** The pBRJH construct carries the full-length WT genome of JcDV (GenBank accession number NC004284) (Dumas et al., 1992; Rollings, 1992). Constructs carrying ns or vp genes driven by their respective promoters were derived from the pJA construct where inverted terminal repeats had been initially deleted (Wang et al., 2013a), and further digested with BamHI/HpaI or with KpnI/BsmI to generate plNS and plVP, respectively. Ld652Y cells (4×10<sup>5</sup>) were transfected with pBRJH, pJNS or plVP using FuGENE HD Transfection reagent (1 μg; 6 μl; Promega) and incubated at 28 °C for 24–72 h. Proteins were extracted in 100 μl lysis buffer (150 mM NaCl, 10 mM Tris/HCl, pH 8.3, 0.1 % SDS, 1 % Triton X-100 and 1 % sodium deoxycholate) supplemented with protease and phosphatase inhibitors (Roche).

**Antibodies.** Anti-VP and anti-NS antibodies were rabbit polyclonal, obtained from Eurogentec or kindly provided (Abd-Alla et al., 2004), and used at 1 : 1000. Primary antibodies were used at the indicated concentrations: anti-Akt (1 : 200), anti-phospho-Ser473-Akt (p-Akt; 1 : 200), anti-TOR (1 : 200), anti-phospho-Ser2448-TOR (p-TOR; 1 : 200), anti-phospho-Thr389-p70S6K (p-p70S6K; 1 : 400), anti-rpS6 (1 : 200) and anti-phospho-Ser236 or Ser244-rpS6 (p-rpS6; 1 : 200), anti-eIF2α (1 : 200), anti-phospho-Thr37/46-4E-BP1 (p-4EBP; 1 : 300), anti-rpS6 (1 : 200), anti-phospho-Thr389-p70S6K (p-p70S6K; 1 : 400), anti-rpS6 (1 : 200) and anti-phospho-Ser236 or Ser244-rpS6 (p-rpS6; 1 : 200), anti-eIF2α (1 : 200), anti-phospho-Ser2448-TOR (p-TOR; 1 : 200), anti-phospho-Thr389-p70S6K (p-p70S6K; 1 : 400), anti-rpS6 (1 : 200) and anti-phospho-Ser236 or Ser244-rpS6 (p-rpS6; 1 : 200), anti-eIF2α (1 : 200), anti-phospho-Thr37/46-4E-BP1 (p-4EBP; 1 : 300) and anti-phospho-Ser51-eIF2α (p-eIF2α; 1 : 500) (Santa Cruz Biotechnology); anti-4E-BP1 (4EBP; 1 : 200), anti-phospho-Thr37/46-4E-BP1 (p-4EBP; 1 : 300) and anti-phospho-Ser51-eIF2α (p-eIF2α; 1 : 500) (Cell Signalling); anti-tubulin (1 : 2000) (Sigma) and anti-Atg8 (LC3B) (1 : 1000) (Molecular Probes). Secondary antibodies were purchased from Bio-Rad (goat anti-rabbit and anti-mouse HRP-conjugated IgG; 1 : 3000) and Molecular Probes (Alexa Fluor 594-conjugated goat anti-mouse or Alexa Fluor 488-conjugated anti-rabbit antibodies; 1 : 1000).

**Cell size, cell proliferation and cell survival analyses.** Cell and nucleus size was analysed using Hoechst 33342 at 4 days p.i.; images were taken using an Andor CSU-W1 spinning disk confocal microscope. For each condition, cell diameters of 20 cells were measured on three independent images. Volumes were calculated assuming the cell and nucleus to be spheres (volume=4/3πr<sup>3</sup>).

**Viral DNA and RNA quantification.** Total DNA or RNA was extracted from cells with the aDNeasy Blood & Tissue kit (Qiagen) or RNeasy Mini kit (Qiagen), respectively. RNA samples were treated with a DNase (Turbo DNA-free kit; Ambion). cDNAs were synthesized using SuperScript III reverse transcriptase (Invitrogen). Viral replication and VP transcription were measured using VP4 primers as described previously (Wang et al., 2013b). Expression of the ns1 gene was measured with forward (5′-CTGCCCAGCATCTGATCT and reverse (3′-CAGGGGCTGAGCGAATTG) primers.
A CG-3') and reverse (5'-TTGTACGAAACGGGGAGAG-3') primers designed to amplify from position 563 to 752 of the ns1 gene. Cellular β-actin forward (5'-CTCACCCTCCCTGGAAGTC-3') and reverse (5'-GTGATGACCTGCAGCTCGGC-3') primers were designed from the L. dispar β-actin cDNA sequence (GenBank accession number AF182715.1). qPCRs and RT-qPCRs were performed using SYBR Green I Master (Roche) and analysed on a LightCycler 480 Instrument (Roche). Each sample was assayed in triplicate, and data from three biological replicates and three technical replicates were analysed with LightCycler 480 software (version 1.5). The number of viral genomes or viral transcripts obtained was normalized against the number of copies of the β-actin gene in order to obtain the number of viral copies per cell.

**Immunofluorescence.** Ld652Y cells were seeded onto coverslips, pre-treated or not with 10 μM rapamycin (Pfizer) for 1 h at 28 °C and then infected or not with JcDV. At the dedicated times, cells were labelled with primary antibodies overnight at 4 °C and then for 1 h at room temperature with secondary Alexa Fluor antibodies. Fluorescence was analysed using a confocal microscope Leica SPE DM 2500.

**SDS-PAGE and Western blot analyses.** Ld652Y cells (2.5 x 10^6) were infected and harvested at different indicated times p.i. Protein concentration was assessed by Bradford analysis and equal amounts of lysates were separated onto 4–15 % gel to run SDS-PAGE. Gels were stained with Coomassie blue or transferred to PVDF membranes (Immobilon-P; Millipore) for Western blot analysis and were stained with primary antibodies overnight at 4 °C and then infected or not with 10 μM rapamycin (Pfizer) for 1 h at 28 °C and then infected or not with JcDV. At the dedicated times, cells were assayed in triplicate, and data from three biological replicates and three technical replicates were analysed with LightCycler 480 software (version 1.5). The number of viral genomes or viral transcripts obtained was normalized against the number of copies of the β-actin gene in order to obtain the number of viral copies per cell.

**Analysis of protein synthesis by radioactive labelling.** Ld652Y cells (2.5 x 10^6) were infected. At the indicated times, cells were washed and incubating for 1 h with 1.5 ml PBS supplemented with 10 μl EXPRESS 35S Protein Labelling mix, [35S]Met/Cys (11 mCi ml⁻¹; Perkin Elmer) per flask. Total proteins were analysed by SDS-PAGE; gels were subsequently dried and revealed by autoradiography.

**Inhibition assays.** Rapamycin (Pfizer) and torin-1 (Tocris) were dissolved in DMSO. Controls cells were treated with an equivalent volume of DMSO. Three independent experiments with three technical replicates in each experiment were performed for each condition.

**Statistical analyses.** Analyses were performed with R software (version 3.1.1) using the Mann–Whitney Wilcoxon non-parametric test, with an z risk equal to 0.05 and a degree of freedom equal to 1.95.

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**REFERENCES**


