Article

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Abstract

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The cellular protein TIP47 restricts *Respirovirus* multiplication leading to decreased virus particle production

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ABSTRACT

The cellular tail-interacting 47-kDa protein (TIP47) acts positively on HIV-1 and vaccinia virus production. We show here that TIP47, in contrast, acts as a restriction factor for Sendai virus production. This conclusion is supported by the occurrence of increased or decreased virus production upon its suppression or overexpression, respectively. Pulse-chase metabolic labeling of viral proteins under conditions of TIP47 suppression reveals an increased rate of viral protein synthesis followed by increased incorporation of viral proteins into virus particles. TIP47 is here described for the first time as a viral restriction factor that acts by limiting viral protein synthesis.

1. Introduction

Sendai virus (SeV) is an enveloped virus, the prototype of the *Paramyxoviridae* family, *Respirovirus* genus. The SeV particle contains two glycoproteins protruding from the virus envelope; these are the hemagglutinin-neuraminidase (HN) protein, which is involved in cellular receptor binding, and the fusion (F) protein. Both of these proteins are required for delivery of the viral genome to the cytoplasm of target cells. The matrix (M) protein forms a layer beneath the lipid envelope at the interface between the glycoproteins and the viral nucleocapsid. The nucleocapsid represents the active viral genome, to which the RNA-dependent RNA polymerase complex, which is composed of a large subunit (L) and the cofactor (P), is attached. A minor component of the SeV particle is the C protein. Among other functions, the C protein is thought to participate in the formation of viral particles (for a detailed description of the Paramyxovirus life cycle, see Lamb and Parks (2007).

It is generally thought that virus particle formation occurs at the plasma membrane, from which the newly formed particle buds. The involvement of ESCRT (endosomal sorting complexes required for transport) proteins in this process is still controversial (Sakaguchi et al., 2005; Irie et al., 2008; Gosselin-Grenet et al., 2007), as is the use of detergent-resistant membranes to represent assembly platforms (Gosselin-Grenet et al., 2006). The M protein is viewed as the central organizer of virus particle formation; its suppression leads to a significant drop in particle production (Mottet-Osman et al., 2007). Suppression of F or alteration of its cytoplasmic tail also reduces virus particle production. Surprisingly, although HN suppression *per se* has no effect on virus particle formation, the suppression of both F and HN has a greater effect on viral production than suppression of F alone (Fouillot-Coriou and Roux, 2000; Gosselin-Grenet et al., 2010).

In its cytoplasmic domain, SeV HN contains a SYWST motif that targets the protein to the virus particle and allows its partial internalization to late endosomes, from which a minor fraction of the protein is redirected to virions (Fouillot-Coriou and Roux, 2000; Gosselin-Grenet et al., 2010). In an attempt to investigate the route that internalized HN follows to reach virions, we suppressed the tail-interacting protein of 47 kDa (TIP47). TIP47, also called perilipin 3,which is associated with retrograde transport of the mannose-6 phosphate receptor (MPR) from late endosomes (LE) to the trans-Golgi network (TGN) in association with Rab9 GTPase (Diaz and Pfeffer, 1998; Ganley et al., 2004). TIP47 is also involved in lipid droplet formation (Bulankina et al., 2009). In particular, activation of TLR9 in macrophages enhances TIP47 expression and

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promotes lipid accumulation (Gu et al., 2010). Finally, TIP47 has been shown to protect mitochondrial integrity and limit stress-induced cell death (Hocsak et al., 2010a,b). In HIV-1 infected cells, "TIP47 acts as a trafficking adaptor for Env retrograde transport from late endosomes to the TGN and brings Gag and Env together during HIV-1 morphogenesis" (Bauby et al., 2010). Interaction of Env with TIP47 involves a YW motif, and suppression of TIP47 leads to a deficit of Env incorporation in particles, which decreases HIV infectivity (Lopez-Verges et al., 2006; Blot et al., 2003). For vaccinia virus morphogenesis, the viral protein p37 also interacts with TIP47 via a YW motif. Mutation of this motif blocks its association with TIP47 and inhibits viral plaque formation (Chen et al., 2009).

In contrast to the observations that TIP47 is beneficial for virus multiplication and propagation, analysis of TIP47 in the context of SeV infection has led to opposite conclusions. We report here that TIP47 suppression results in a 5-to-20-fold increase in the production of infectious virions, making TIP47 a restriction factor for SeV production. This effect was also observed for human parainfluenza 3 virus (HPIV3, another respirovirus), but not for parainfluenza 5 virus (PIV5, a rubulavirus), measles virus (MeV, a morbillivirus) or vesicular stomatitis virus (VSV, a rhabdovirus). Pulse-chase experiments showed that SeV protein synthesis was increased upon TIP47 suppression, leading to a higher rate of protein incorporation into virus particles and an increase in SeV particle production.

2. Materials and methods

2.1. Cells and viruses

Cells were grown at 37 °C in a 5% CO_2 atmosphere in Dulbecco's Modified Eagle Medium (DMEM; Gibco-Invitrogen) supplemented with 10% fetal calf serum (FCS) and 1% penicillin–streptomycin (PS-Sigma). Sendai virus (H and rSeV, Z strains) were prepared in embryonated eggs and characterized as described previously (Roux and Holland, 1979). Recombinant rSeV-HA-TIP47 was recovered from plasmid pFL5-HA-TIP47 by a slight modification of a previously described method (Fouillot-Coriou and Roux, 2000). The pFL5-HA-TIP47 plasmid was transfected (Fugene, Roche) in BSR-T7 cells (Conzelmann and Schnell, 1994) along with pGem plasmids expressing the N, L and P/Cstop helper virus proteins. Twenty-four hours after transfection, cells were injected into 9-day old embryonated chicken eggs. After 3 days of incubation at 33 °C, the allantoic fluid was collected; the clarified fluid constituted the viral stock. Parainfluenza virus 5 (PIV5; WR strain), vesicular stomatitis virus (VSV, Mudd-Summer strain), human parainfluenza virus 3 (HPIV3) and measles virus (MeV, Hu6) were kindly provided by Machiko Nishio (Mie University Graduate School of Medicine, Japan), Jacques Perrault (San Diego State University, USA), Peter Collins (NIAID, Bethesda, USA), and Bertus Rima (Queens University, Belfast), respectively.

2.2. Virus titration

SeV-infected cell supernatants were collected and treated for 30 min at 37 °C with 3 μg/ml of acetylated trypsin. Infectious virus titers were estimated by two methods. In the first, a plaque-forming unit detection assay was performed on LLC-MK2 cells under a 0.3% agarose overlay in the presence of 3 μg/ml of acetylated trypsin according to Sugita and co-workers (Sugita et al., 1974). Infectious units were also determined by detection of virus-infected cells by flow cytometry after a single round of infection; viral titer was calculated according to Poisson's law as described by Grigorov et al. (2011).

2.3. Antibodies and reagents

The antibodies used in this study included rabbit sera raised against SeV SDS-denatured HN, F or N proteins (α-HN_{SDS}, α-F_{SDS}, α-N_{SDS}), anti-SeV N_{Vac}, anti-SeV F_{vac}, anti-SeV HN_{vac} and anti-SeV M_{vac}, [rabbit sera raised against N, F, HN and M proteins expressed from vaccinia recombinant virus (Kast et al., 1991)], a mouse monoclonal antibody, anti-SeV M (MAb 383, obtained from Claes Örvell, Laboratory of Clinical Virology, Huddinge Hospital, Huddinge, Sweden) and anti-influenza virus HA epitope MAb (16b12, Covance Research Products Inc.). Anti-TIP47 was purchased from Sigma–Aldrich and monoclonal anti-actin antibody, clone C4, from Millipore. Antibodies against PIV5-HN, -NP and -M proteins were a gift from R.E. Randall (University of St. Andrews, United Kingdom). Peroxidase-conjugated secondary antibodies were purchased from Bio-Rad. MG132 proteasome inhibitor was purchased from Sigma. Mouse monoclonal anti-ubiquitin (P4D1 sc-8017) was from Santa Cruz Biotechnology. Anti-LC3 antibodies were purchased from Sigma–Aldrich.

2.4. Plasmid DNA constructs

The plasmids pAS1B and pAS1B-HATPI47 were kindly provided by Dr. Clarisse Berlioz-Torrent (Institut Cochin-INSERM U567, Paris, France). The siRNA target sequence in TIP47 was modified to create the pAS1B-HATIP47$_{simut}$, which escapes from siRNA silencing, by changing the sequence AAGGACACGGTGGCCACCCAA encoding the Lys–Asp–Thr–Val–Ala–Thr–Gln peptide to AA**A**GA**T**AC**A**GT**C**GC**T**AC**G**CA**G**. In pAS1B plasmids the HA-TIP47 proteins are expressed form a CMV promoter. The plasmid pFl5-HA-TIP47 was generated by inserting the HA-TIP47$_{simut}$ coding sequence amplified by PCR at the MluI restriction site of pFl5-F-gfpt plasmid (Gosselin-Grenet et al., 2010).

2.5. Cellular extracts and viral particle purification

Transfected or infected cells were collected and disrupted in 300 μl of RIPA buffer (150 mM NaCl, 1% deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 10 mM Tris–HCl, pH 7.4) supplemented with protease inhibitors (Sigma). After a 10-s sonication (Branson Sonic Sonifer B-12, lowest speed), the cell extracts were centrifuged for 10 min at 12,000 rpm in a microfuge. The supernatants were then processed for Western blotting analysis or immunoprecipitation. Virus particles were isolated from the clarified cell supernatants by centrifugation through a 25% glycerol cushion (Beckman SW55 rotor, 1 h, 43,000 rpm, 4C) and directly resuspended in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer.

2.6. Virus infection and radiolabeling

Infections were performed at 33 °C. Virus stocks were adequately diluted (multiplicity of infection is indicated in the figure legends) in DMEM without FCS and laid over the cells for 1 h. The viral inoculum was removed and replaced with fresh DMEM supplemented with 2% FCS or with no FCS when the titration assay was to be performed subsequently. For metabolic radiolabeling, cells were incubated with 40 μCi/ml of L-^{35}S-methionine and L-^{35}S-cysteine (Pro-mix-[^{35}S]-Amersham Biosciences) in DMEM containing 1/10th the normal methionine and cysteine content plus 0.2% FCS, from 16 to 40 h post-infection. Culture medium and cells were harvested at the times indicated in the figure legends and analyzed as described below.

2.7. Pulse-chase assay

At 18 h post-infection, cells were washed twice with DMEM lacking cysteine and methionine (DMEM-C/-M) (Sigma) and then starved for 30 min at 37 °C in DMEM-C/-M. A 10-min pulse was then performed in DMEM-C/-M with 300 μCi/ml of L-^{35}S-methionine and L-^{35}S-cysteine (Pro-mix-[^{35}S]-Amersham Biosciences). Cells were washed twice with DMEM enriched with 10 mM methionine, 10 mM cysteine and supplemented with 2% FCS and 1% PS. Cells were then incubated for 0, 1, 3, or 6 h in the same medium and harvested. Cell extracts were lysed in RIPA buffer containing 1% aprotinin and 20 mM AEBSF as described (Mottet et al., 1986). Viral proteins were recovered by immunoprecipitation. Total cellular extracts were first incubated with the specific antisera (α-HN_{Vac} + α-N_{SDS}, α-F_{Vac} + M_{Vac} antibodies) overnight at 4 °C, then with a 50% suspension of protein A-Sepharose (Roche) for 2 h at 4 °C. Virus particles were harvested as described above. The samples were analyzed by SDS-PAGE and autoradiography.

2.8. Transfections and suppression complementation assay

Forty-eight hours before SeV infection, HeLa cells in 35-mm dishes were transfected with 150 nM of anti-TIP47 siRNA (target sequence position 495–512: 5′-AAGGACACGGTGGCCACCCAA-3′, Qiagen), with scrambled siRNA (Qiagen) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. For suppression complementation assays, which were conducted 24 h post-siRNA transfection, cells were transfected with pAS1B or pAS1B-HATIP47$_{simut}$ using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions for 24 h and then infected with rSeV viruses for 24 h.

2.9. GFP expression assay

HeLa cells in 24-well plates were transfected with anti-TIP47 siRNA or scrambled siRNA (Qiagen) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Forty-eight hours post-transfection, cells were either infected with rSeV-GFP for 24 h or rVSV-GFP for 18 h or transfected with pEBS-GFP for 24 h. Cells were then harvested and analyzed for GFP expression using a BD Accuri C6 flow cytometer.

2.10. Total and cell surface immune precipitations

Samples of infected cells were labeled with 75 μCi/ml of L-^{35}S-methionine and L-^{35}S-cysteine (Pro-mix-[^{35}S]-Amersham Biosciences) from 15 to 24 h post-infection in DMEM containing 1/10 the normal content of cysteine and methionine and containing 0.2% FCS. At the end of the labeling period, the cell supernatants were removed and the labeled virus particles were purified. The cells were rinsed twice with DMEM without FCS (-FCS) and incubated for 1 h at 33 °C with 1 ml of the same medium containing 50 μl of anti-SeV N_{Vac} or anti-SeV F_{vac} or 10 μl of anti-SeV M MAb 383. At the end of the incubation period, the cell samples were disrupted in 650 μl of RIPA buffer, of which 50 μl were set aside for direct PAGE analysis, 300 μl were directly incubated with a 50% suspension of protein A-Sepharose for 2 h at 4 °C [surface immune precipitation (surface IP)]; the remaining 300 μl were incubated again with 50 μl of anti-SeV N_{Vac} or anti-SeV F_{vac} or 10 μl of anti-M 383 for 6 h at 4 °C (total IP) before incubation with the protein A-Sepharose mix (total IP). The immune precipitates were treated and analyzed as described above (pulse-chase assay).

2.11. Western blotting and autoradiography

Total cellular protein extracts and virus particles were resolved by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore) using a semi-dry system (Bio-Rad). The blots were incubated with the specific antibodies described above and then with HRP-conjugated secondary antibodies (Bio-Rad). Bound antibodies were detected using an enhanced chemiluminescence system (Amersham Biosciences), and the signal was analyzed using a FugiFilm Imager. The radiolabeled virus particle samples and the surface and total immune precipitates (IP) were analyzed by SDS-PAGE. The gels were treated for enhanced fluorography (DMSO plus 5% 2,5-diphenyloxazol – PPO) and exposed to Hyperfilm MP (Amersham Biosciences). The films were scanned, and the band signals were quantified with MultiGauge (FugiFilm).

2.12. Real-time RT-PCR

HeLa cells transfected with anti-TIP47 siRNA or control siRNA (Qiagen) were rSeV infected for 24 h. Total RNA was extracted from cells with Trizol (Invitrogen) according to the manufacturer's instructions. The integrity of the resulting RNA was checked on an agarose gel. The RNA was used as template for the synthesis of cDNA with oligo dT (Promega) at 42 °C using the M-MLV RNase (H-) point mutant reverse transcriptase (Promega). The cDNAs were quantified using SYBR Green-based real-time PCR on the CFX96 Real Time System/C1000 Thermal Cycler (Bio-Rad) using the following program: 95 °C for 2 min, 40 cycles of 95 °C for 10 s, 55 °C for 1 min and the following primers:

- F-mRNA: sense CAGAACTTGACAGTCGGTC, antisense GTGCTTTCTCAAGCTCAGC;
- HN-mRNA: sense ACCCGTTCTCTGCACTATA, antisense GGTTAGTCCACCATACCCAAG;
- M-mRNA: sense CACCGAAACAAACAACCAATC, antisense CGGAGCCGCATATTGAGTAG;
- N-mRNA: sense AGAAGCCTCATAGACACCTAC, antisense TGGAGCAAATTCACCATGAAC;
- AOZ1-mRNA: sense GAGGGAATAGTCAGAGGGATCAC, antisense GAATCCTCGTCTTGTCGTTGG. GAPDH-mRNA: sense TGCACCACCAACTGCTTAGC, antisense GGCATGGACTGTGGTCATGAG used as an endogenous controls.

3. Results

3.1. TIP47 suppression or overexpression and SeV particle production

To examine a possible role of the late-endosome-to-trans-Golgi network transport pathway in the incorporation of internalized HN into SeV particles (Gosselin-Grenet et al., 2010), we suppressed TIP47, a cellular transport effector of this pathway, prior to infection. In cells in which TIP47 was suppressed, we observed no difference in the incorporation of HN into SeV particles relative to F and M (Fig. 1A, compare lanes 3 and 4–5 and 6–7, and 1B, lanes 3 and 4, Virus particles). However, the total number of particles produced was significantly increased upon TIP47 suppression (Fig. 1A and B, Virus particles). These results were obtained by monitoring viral protein expression by Western blotting (Fig. 1A) and by autoradiography of labeled proteins, a method that more directly scores protein amount (Fig. 1B). For the two experiments presented in Fig. 1A, TIP47 suppression reached 15–10% (Fig. 1C, white bars) of control treated samples [(−)α-siRNA = 100%], while virus particle quantification showed a 20-to-30-fold increase in virus particle production (Fig. 1C, black bars) relative to control treated samples

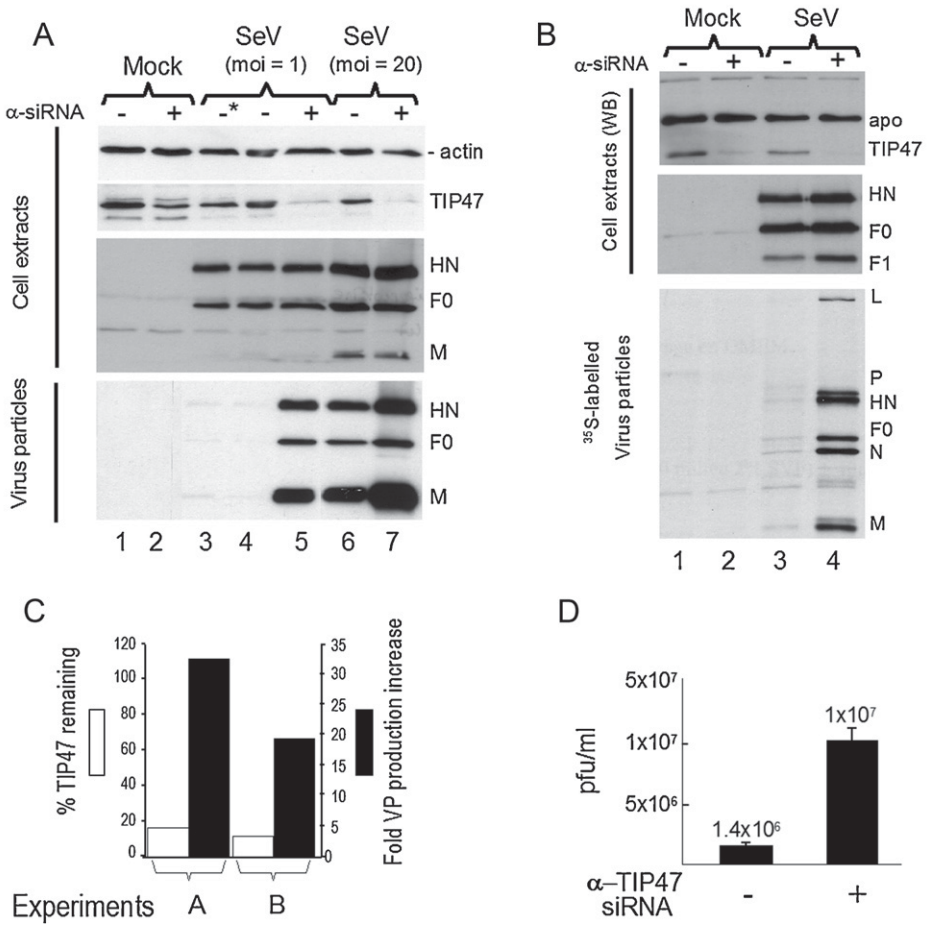

Fig. 1. Effects of TIP47 suppression on SeV production. HeLa cells grown in 35-mm dishes were transfected with α-TIP47 (+) or scrambled siRNA (−*) or left untransfected (−); they were then infected with SeV at different multiplicities of infection (moi). Thirty-six hours post-infection, cells and virus particles in the supernatants were collected and analyzed as described in Section 2. (A) Western blot analysis of cellular extracts (1/60th of total sample) and concentrated virus particles (1/8th of total sample) using primary antibodies against the indicated proteins. Actin is used as a loading control. HN, F and M: viral proteins. (B) Cells were transfected as in A and infected at moi = 3. From 15 h post-infection, cells were radiolabelled with ^{35}S-methionine and cysteine. At 36 h post-infection, cells and supernatants were collected and treated as in A. Cellular extracts (1/60th) were analyzed by Western blotting using primary antibodies against the indicated proteins. The concentrated labeled virus particles in the supernatants were directly analyzed (half the total sample) by SDS-PAGE and detected by autoradiography. apo: apolipoprotein used as a loading control. (C) From panels A (moi = 1) and B, the signals obtained for TIP47 and viral protein expression in culture supernatants were quantified. The percent remaining of TIP47 [white bars, α-TIP47 siRNA (−) value = 100%] or the fold increase in virus particle production [black bars, α-TIP47 siRNA (−) value = 1] is displayed graphically. (D) Independent experiments, identical to panel B (SeV, moi = 3), were used to titrate the number of infectious virus particles in the cell supernatants (see Section 2). Error bars indicate the standard deviation of the mean of three experiments.

[(−)α-siRNA = 1]. The increase in viral physical particle production paralleled an increase in the number of infectious particles (Fig. 1D). It is noteworthy that the levels of cell-associated HN, F and M were not affected by TIP47 suppression (Fig. 1A and B). In these experiments, control samples [(−)α-siRNA] were either transfected with scrambled si-RNAs (Fig. 1A, lane 3*) or were mock transfected (i.e. treated with transfection conditions, but with no siRNA added, Fig. 1A, lanes 1, 4, 6); in the control samples, no significant differences in TIP47 detection were observed. In the remainder of the study, therefore, transfections with scrambled RNA or cells treated without RNA were used indiscriminately as controls.

To confirm that the increase in virus particle production was due to TIP47 suppression, TIP47 expression was reintroduced. The rationale for this was that the reintroduction of TIP47 expression should restrain the positive effect observed after suppression. An HA-tagged version of TIP47 that escapes si-RNA suppression was first expressed from transfected plasmids along with TIP47 suppression. Fig. 2A shows that increased expression of HA-TIP47 (3.8 μg of plasmid transfected, lanes 5 and 6) could partially limit the positive effect of TIP47 suppression on virus particle production (Fig. 2A, compare lanes 2 and 4 to lane 6, Virus particles, Fig. 2B for quantification). As shown in Fig. 2C, the limitation of VP production upon a 3.8 μg transfection corresponds to about a doubling of the normal amount of TIP47 (HA-TIP47 + TIP47), with the caveat that this estimation is biased by the fact that not all the infected cells are transfected with the plasmid expressing exogenous HA-TIP47. A second approach was then developed that utilized the construction of a recombinant SeV harboring an extra transcriptional unit expressing HA-TIP47. HeLa cells were infected with this recombinant SeV without suppression of endogenous TIP47 (Fig. 2D, HA-TIP47). Under these conditions, virus particle production was diminished by approximately 10-fold compared to infection with regular virus (Fig. 2D, virus particles). As in the case in which increased virus production occurred, the decrease in virus production did not lead to a detectable change in the level of cell-associated viral protein (Fig. 2D, cellular extracts). This is likely due to the small fraction of total viral proteins incorporated into virus particles (∼5%). Taken together, these results confirm the involvement of TIP47 in the restriction of SeV particle production.

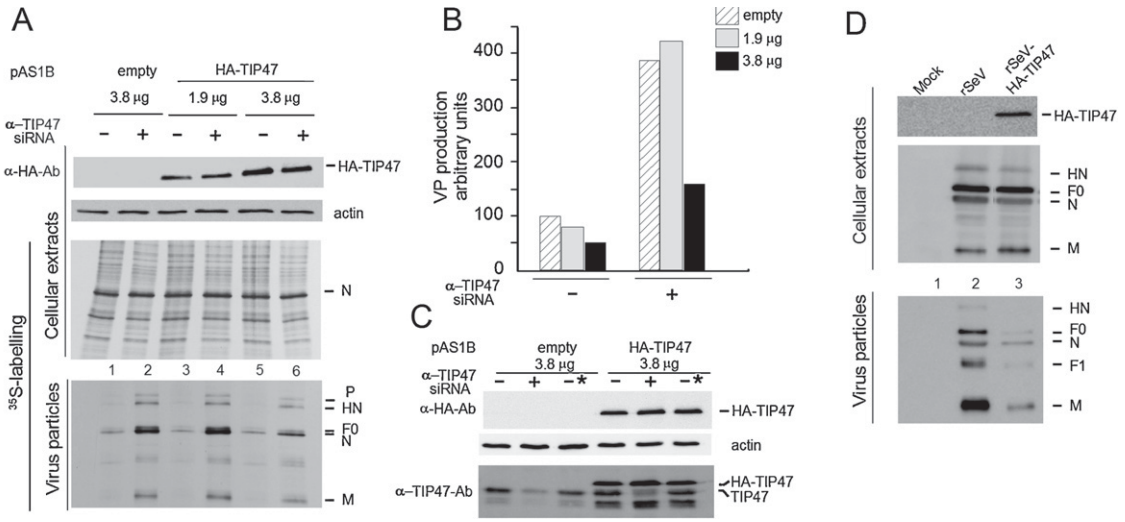

Fig. 2. Effects of TIP47 overexpression. (A) HeLa cells were transfected with α-TIP47 siRNA, infected and ^{35}S-labeled as in Fig. 1B. Samples were also transfected as indicated (1.9 or 3.8 μg) with a pAS1B plasmid, either empty or carrying a gene encoding HA-tagged TIP47 protein (HA-TIP47) modified to escape siRNA suppression. Cellular extracts (1/60th) and concentrated virus particles (half the sample) were directly analyzed by SDS-PAGE and detected by autoradiography as in Fig. 1B. One-sixtieth of the cell extract was also analyzed by Western blot to monitor HA-TIP47 expression. Actin expression was used as an internal loading control. (B) Quantification of the signals corresponding to N/F bands from the autoradiogram shown in Fig. 2A (virus particles). Values are presented in arbitrary units (signal in lane 1 was set to 100). (C.) HeLa cells were transfected as in A with α-TIP47 siRNA (+), mock transfected (−) or transfected with scrambled siRNA (−*). Cell samples were also transfected with pAS1B (3.8 μg) plasmids as indicated. Cellular extracts analyzed by Western blot, probing with α-HA, α-TIP47 and α − actin antibodies. (D) HeLa cells were mock-infected or infected with wt SeV (rSeV) or an rSeV strain carrying an extra gene encoding HA-TIP47 (rSeV-HA-TIP47). Cellular extracts (1/60th) were analyzed by Western blotting to monitor HA-TIP47 or viral proteins expression as indicated. Concentrated virus particles from culture supernatants (1/8th) were analyzed in a similar manner.

3.2. The TIP47 suppression effect appears restricted to respiroviruses

As mentioned above, TIP47 is a positive effector of infectious HIV and vaccinia virus particle production. It was therefore of interest to determine the range of the effect observed in the present study. Cells were infected with four other nonsegmented negative-stranded RNA viruses: measles virus (MeV), parainfluenza virus 5 (PIV5) and human parainfluenza type 3 (HPIV3) virus (these three viruses belong to the *Paramyxoviridae* family but to different genera; see above), and vesicular stomatitis virus (VSV), which belongs to the *Rhabdoviridae* family. Fig. 3 shows representative cells infected with each of these four viruses after TIP47 suppression. Due to the limited availability of specific antibodies, the experiments were performed either by ^{35}S-metabolic labeling of the infected cells (VSV and HPIV3) or by Western blotting (PIV5 and MeV). Using either method, and similarly to SeV infection, TIP47 suppression [(+)TIP47 siRNA samples] was not found to alter the levels of intracellular virus proteins in comparison with matched control samples [(−)TIP47 siRNA samples]. Only HPIV3 particle production was found to be significantly increased upon TIP47 suppression. These results indicate that, although the restrictive effect of TIP47 occurs in another *Respirovirus* (HPIV3), it does not extend to other genera of the *Paramyxoviridae* family (MeV and PIV5) or to VSV. Although the range of viruses tested is limited, this result supports an element of specificity in the mechanism involved.

3.3. Specific effects of TIP47 suppression on SeV infection

SeV particle production is known to be adjusted to intracellular levels of the envelope viral proteins because suppression of M and F individually or of F and HN in concert leads to a significant reduction in virus production (Gosselin-Grenet et al., 2010). To find out whether TIP47 suppression can exert its effect when the level of only one of these three proteins is suppressed, TIP47 suppression was performed in cells infected with recombinant SeV viruses for which the specific suppression of M, F, HN or F and HN proteins is possible (for a detailed description of this approach, see Gosselin-Grenet et al., 2010). The upper panel of Fig. 4 shows the intracellular viral protein levels achieved when TIP47 is or is not suppressed [siRNA TIP47 or scrambled (Scble), respectively] and demonstrates the efficiency of viral protein(s) suppression (in Mgfpt infection, suppression of M; in Fgfpt infection, suppression of F, etc.). Again, TIP47 suppression had no effect on intracellular viral protein levels (compare protein levels in the upper panel in lanes 3 and 4, lanes 5 and 6, etc.). The lower panel shows the production of virus particles in these cells. TIP47 suppression consistently resulted in increased virus particle production regardless of which viral protein was suppressed (compare odd-numbered with even-numbered lanes).

Even if the global availability of envelope virus proteins is not affected by TIP47 suppression, their proper positioning in the assembly process could be influenced by TIP47. In other words, because TIP47 is involved in protein trafficking, its suppression could affect the trafficking of viral glycoproteins to the cell surface. In this regard, glycoprotein F is of particular interest because a decrease in its availability clearly results in decreased virus production (Gosselin-Grenet et al., 2010). The availability of F at the cell surface was therefore measured under conditions of regular infection, during TIP47 suppression and upon infection with rSeV-TIP47. Fig. 5A (upper panel) shows once again that the levels of viral proteins in the cellular extracts did not differ under the different conditions (lanes 2–4), although TIP47 suppression or overexpression resulted in increased or decreased virus particle production, respectively (lower panel, lanes 3 and 4). Fig. 5B and C, however, show that variations in virus particle production are not accompanied by variations in F cell surface expression. The absence of M and N in the immune precipitates shown in Fig. 5B validates the results of the cell surface immune precipitation experiments (lanes 2–4). Taken together, these data support the notion that TIP47 restriction is not targeted to a particular surface viral protein that becomes more available upon TIP47 suppression but likely involves a more general process.

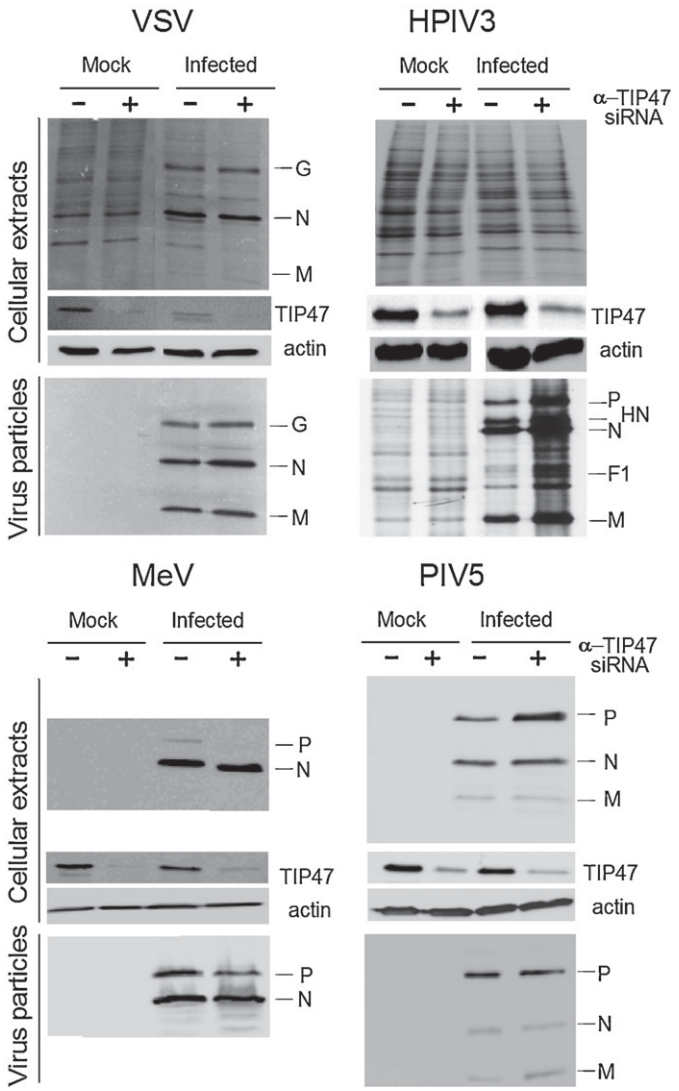

Fig. 3. Effects of TIP47 suppression on production of viruses. HeLa cells were transfected with α-TIP47 siRNA or were not transfected; they were then infected with the indicated viruses. VSV- and HPIV3-infected cells were ^{35}S-labeled from 6–15 h and 15–36 h post-infection, respectively. Cellular extracts (1/60th) and virus particles (1/8th) were directly analyzed by SDS-PAGE and detected by autoradiography as shown in Fig. 2A. PIV5 and MeV infected cells were kept unlabeled; at the end of the infection (respectively 36 and 48 h post-infection), cellular extracts (1/60th) and concentrated virus particles (1/8th) were analyzed by Western blot using appropriate antibodies. Samples (1/60th of the total of each cellular extract) were also used to monitor TIP47 suppression and actin as a loading control.

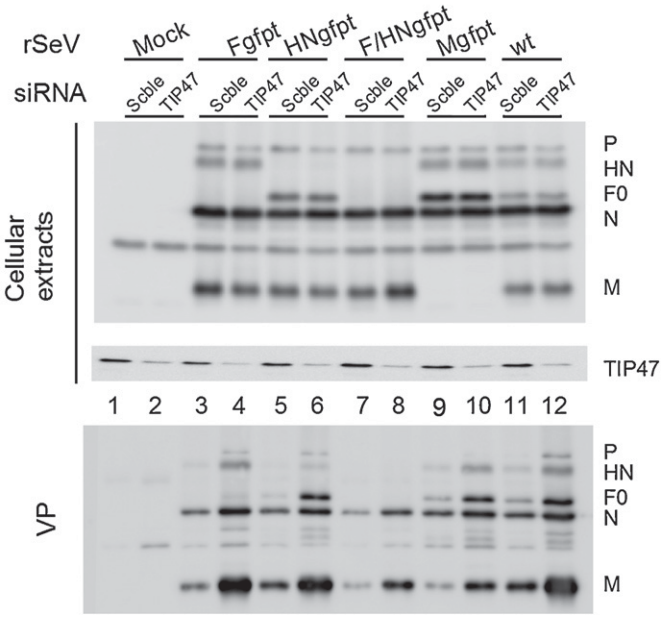

Fig. 4. TIP47 suppression is not related to the expression of a particular viral envelope protein. HeLa cells constitutively expressing siRNA against a GFP target sequence (gfpt, see Section 2) were transfected with α-TIP47 siRNA or with scrambled siRNA. The cells were then infected with recombinant Sendai viruses harboring the gfpt sequence in the indicated gene, M for Mgfpt, F for Fgfpt, HN for HN gfpt and F and HN for F/HNfgfpt or with wild type virus. Twenty-four hours post-infection, the cells and the viruses present in the cell supernatants were collected and analyzed by Western blots using primary antibodies against the indicated proteins. (A) Analysis of the cellular extracts (1/60th of the total sample). (B) Virus particles (VP) analysis (1/8th of the total sample).

3.4. *Mechanism accounting for increased virion progeny upon TIP47 suppression*

The known functions of TIP47 do not provide an immediate clue to explain the observed effects of its suppression. We first postulated that TIP47 could directly or indirectly promote suboptimal production of virus progeny by inducing the degradation of viral proteins. To examine this possibility, infected cells were first treated with the proteasome inhibitor MG132 to determine whether this treatment could restore virion production to the level observed after TIP47 suppression. Fig. 6 shows that, although MG132 treatment was efficient as attested by ubiquitinylated protein accumulation (Fig. 6A), virion production was not increased (Fig. 6B). Second, virus infections of mouse embryo fibroblast cells (MEF) that are incompetent for autophagy [by knockout of autophagy-related (ATG) genes (Yorimitsu and Klionsky, 2007; Huang and Klionsky, 2007)] were performed to test whether autophagy, possibly controlled by TIP47, normally lowers the level of viral constituents. This possibility was not verified. In absence of autophagy (as shown by lack of LC3-I to LC3-II cleavage, Fig. 6C), virion production not only did not increase (Fig. 6C, VP), but rather significantly dropped. These results therefore do not support a putative participation of TIP47 in lowering viral protein levels by inducing their partial degradation. As for the apparent positive role of autophagy in promoting SeV particle production, this has been observed before for influenza A virus for which pharmacological inhibition of autophagy decreased M1 and M2 levels and virus particle production (Zhou et al., 2009, reviewed in Miazza and Roux, 2009). Although this has not been the focus of the present study, it is interesting to point to lower levels of SeV M (relative to NP) in MEF Agt5 −/− at 24 and 48 h post infection (Fig. 6C, cellular extracts).

Next, the possibility that TIP47 can restrict viral protein synthesis was explored. Infected cells were pulse-labeled with radioactive methionine and cysteine under normal conditions and after TIP47 suppression and viral proteins were recovered by immune precipitation immediately after the pulse or after increasing chase periods. Interestingly, higher levels of radiolabeled viral proteins were recovered after the labeling pulse (Fig. 7A, time 0′) under conditions of TIP47 suppression. The level of radiolabeled viral proteins then decreased until, at later time points, it equaled the level recovered under normal conditions. In cells in which TIP47 was suppressed, incorporation of the radiolabeled viral proteins into virus particles was increased (right panel). The particular behavior of HN is caused by a conformational change that progressively increases its reactivity with the antibodies used to detect it (Mottet et al., 1986). This change is apparent in both conditions and does not invalidate the results.

Viral mRNA levels were measured under normal conditions and after TIP47 suppression using real-time quantitative PCR analysis

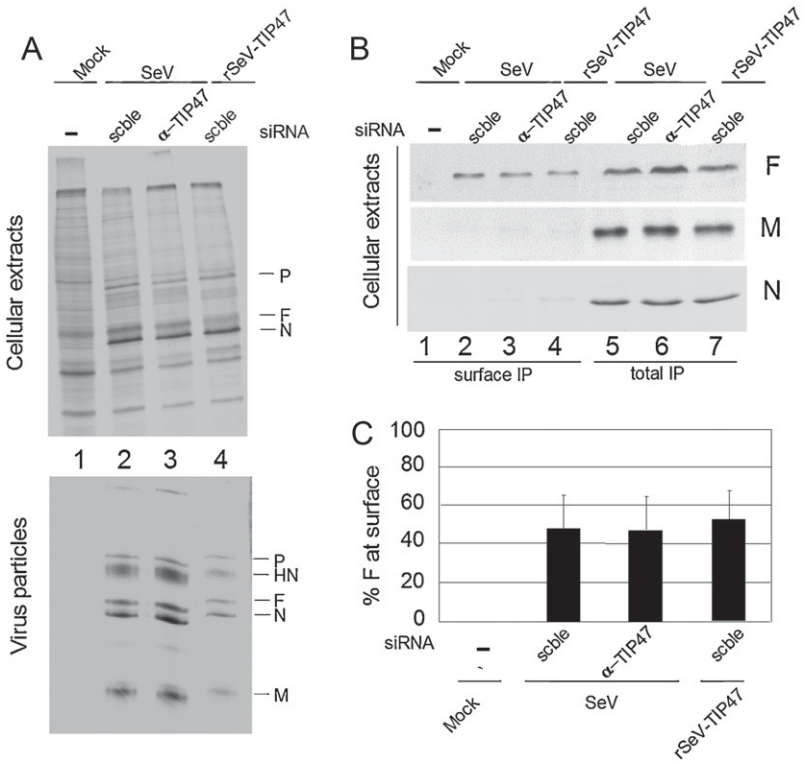

Fig. 5. TIP47 suppression or overexpression does not affect steady-state viral protein levels. HeLa cells were infected with wild type Sendai virus (SeV) after transfection with α-TIP47 or scrambled (scble) siRNAs, infected with rSeV-HA-TIP47 (SeV-TIP47) after transfection with scrambled (scble) siRNA, or mock infected and not transfected. At 15 h post-infection, the cells were metabolically labeled with ^{35}S-methionine and cysteine (see Section 2) for 20 h. (A) Fractions of the collected cells (1/80th) and virus particles (1/5th) present in the cell supernatants were directly analyzed by SDS-PAGE, and labeled proteins were visualized by phosphorimager detection (GE-Typhoon FLA 7600). (B) Remaining cells were split into three equal aliquots and subjected to cell surface (lanes 1–4, surface IP) and total cell immunoprecipitation (lanes 5–7, total IP) using α-F, α-M and α-N antibodies (see Section 2). The immune precipitates were analyzed by SDS-PAGE and phosphorimager detection. (C) The F cell surface and total cell immune precipitates presented in B (lanes 2–7, F panel) were quantified by with MultiGauge (FugiFilm) on the basis of the phosphorimager analysis to determine the percentage of F expressed at the cell surface. The error bars represent the standard deviation of the mean of 3 separate experiments.

(Fig. 7B). When standardized to two different housekeeping genes (GADPH and AOZ1) and expressed relative to the viral mRNA levels measured in normal conditions, the mean level of viral message expression in TIP47-suppressed cells was increased by a factor of 1.5–2.0 compared to control cells. We note that the levels of the GADPH and AOZ1 messengers were not affected by TIP47 suppression in these experiments (not shown).

These results, taken together with the observation that the levels of ^{35}S-labeled cellular proteins found in cellular extracts after long labeling times were unchanged after TIP47 suppression (see Figs. 2 and 5A), suggested that the increase of virus particle production was SeV-specific. To confirm this, GFP expression driven by SeV, by VSV or by a plasmid that mimics its regular cellular expression was compared. Fig. 7C compares GFP expression in the three conditions with and without TIP47 suppression. Interestingly, only GFP expression from SeV genome appears to respond positively to TIP47 suppression, what is consistent with lack of effect of this suppression on VSV VP production and on cellular protein level.

4. Discussion

Our results show that TIP47 suppression increases the production of SeV and HPIV3 virus particles, an effect that is not observed for MeV, PIV5 and VSV. The increase of viral particle production is paralleled by an increased viral protein synthesis, making this latter a likely explanation for this former. A corresponding increase in the incorporation of proteins into virus particles explains why higher levels of viral proteins are not observed in cellular extracts after TIP47 suppression.

Boosting viral protein synthesis to optimize virus production is a viral strategy. A recently studied example of this scenario involves HPIV3, which has been shown to potentiate the EGF cellular response, resulting in an increase in viral protein synthesis (Caignard et al., 2009) and Fig. S1A). The work presented in this paper provides an example of a cellular counter-reaction that occurs through TIP47, which is described here as a restriction factor. As mentioned in the Introduction, TIP47 has been shown up to now to positively participate in virus production.

The mechanism by which TIP47 suppression leads to increased viral protein synthesis remains unclear. Considering that stimulation of the EGF signaling pathway increases protein synthesis in HPIV3-infected cells (Caignard et al., 2009) activation of the same pathway could occur in SeV-infected cells after TIP47 suppression. However, we have shown that SeV infection only marginally stimulates the EGF signaling pathway. This finding correlates with the inability of SeV-C protein to interact with hGRB2 (Fig. S1-C), an essential adaptor of the EGF signaling pathway with which HPIV3-C, which stimulates the pathway (Fig. S1-B), has been shown to interact. In the end, TIP47 suppression has no effect on the EGF signaling pathway, regardless of whether it can be stimulated by the viral infection (Fig. S1-D). Alternatively, because TIP47 plays a role in the maintenance of mitochondrial membrane integrity (see Introduction), its suppression could weaken the mitochondria-dependent interferon (IFN) induction pathway. This would lower PKR activation and decrease the inhibition of protein synthesis. Unfortunately, our experiments failed to demonstrate a decrease in β-interferon promoter activation upon TIP47 suppression, invalidating this second hypothesis (Fig. S2).

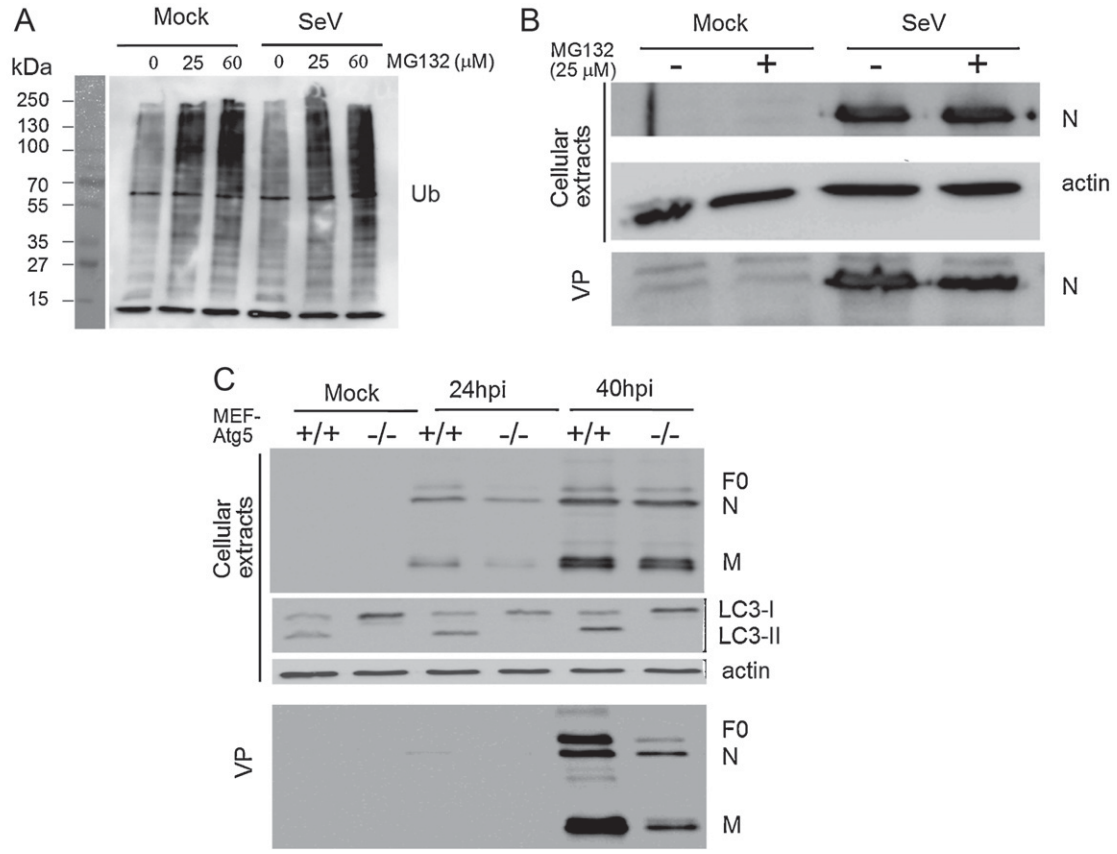

Fig. 6. Blocking proteasome and autophagosome activity. (A) Mock-treated or SeV-infected HeLa cells were incubated with two different concentrations of MG132, and extracts from the cells were probed with an α-ubiquitin (Ub) antibody. (B) As in A except that the cells were incubated for 16 h in the presence of the proteasome inhibitor MG132. Cell extracts and supernatants were analyzed by Western blot. (C) Wild type (+/+) and Atg5 KO (−/−) MEF cells were infected with SeV or not infected. Twenty-four or 40 h post-infection, cellular extracts and supernatants (VP) were collected and analyzed by Western blot. Actin served as a loading control. LC3-II is an autophagosomal marker. Note that in Atg5 KO (−/−) MEF cells, in which autophagy is impaired, LC3-II is not observed; only the LC3-I precursor is present.

Based on the fact that increased viral protein synthesis will increase viral RNA polymerase (vRdRp) production and *vice versa*, it remains likely that the mechanism of TIP47 restriction of SeV production is linked to an activation of viral translation or of viral transcription. Because the two processes are interdependent, the boosting of one of them is, in principle, sufficient to explain the observed effect. In addition, unlike cellular translation, viral translation is coupled to transcription. This coupling could contain an element of specificity that explains how cellular protein synthesis is not affected by TIP47 suppression. With respect to our data, coupled transcription–translation, which is observed as a positive factor for viral gene expression, would be counteracted by TIP47. Similarly, viral transcription could be down regulated by interaction of TIP47 with vRdRp or with the viral nucleocapsid. The results of *in vitro* coupled transcription–translation assays using VSV templates have led investigators to propose an unusual translation process in which viral transcripts are protected by physical interaction of the translation machinery with the viral nucleocapsid (Leyrer et al., 1998; Orenstein et al., 1975). A similar mechanism has been suggested for SeV but has received little further attention (Pelet et al., 2005). Cellular factors have been shown to interact with MeV or VSV RNA polymerase and to increase its activity (Mathur et al., 1996; Bose et al., 2003; Watanabe et al., 2011; Zhang et al., 2005). On the other hand, cellular factors that target the HIV capsid and are likely involved in capsid disruption have been identified (reviewed in Luban, 2012). Although some cellular factor(s) likely interact with SeV RdRp, nothing is known at present about factors that, in contrast to those that positively influence MeV and/or VSV RdRp, restrict respiroviral RdRp, nor about cellular restriction factors that target viral nucleocapsids. If such mechanisms exist, they would represent either a direct restrictive action of TIP47 or its ability to make available, possibly through its role in LE to TGN transport, another cellular restriction factor.

With respect to another known function of TIP47, the respirovirus translation machinery could be localized in a particular cytoplasmic compartment in which translation is maintained at a suboptimal level to minimize detection by the innate immune cytoplasmic sensors. This localization would require perilipin 3 (the other name of TIP47). The involvement of phospholipid-enriched subcellular compartments in plus strand RNA virus replication has been well described; in that case, they act as facilitators (Miller and Krijnse-Locker, 2008). Here we suggest, in contrast, the possible existence of a subcellular compartment that damps down respirovirus transcription and/or translation, an idea that has not been proposed previously. In any case, it seems clear that the known functions of TIP47 do not provide a plausible explanation for the effect observed in the present study.

Obviously, the data reported in this work produce more questions than answers. However, our results are valuable in that they suggest several new lines of research concerning single-stranded RNA viruses of negative polarity, which, although they belong to a single family, have nevertheless evolved peculiarities in their mechanisms of multiplication and of interaction with the infected host.

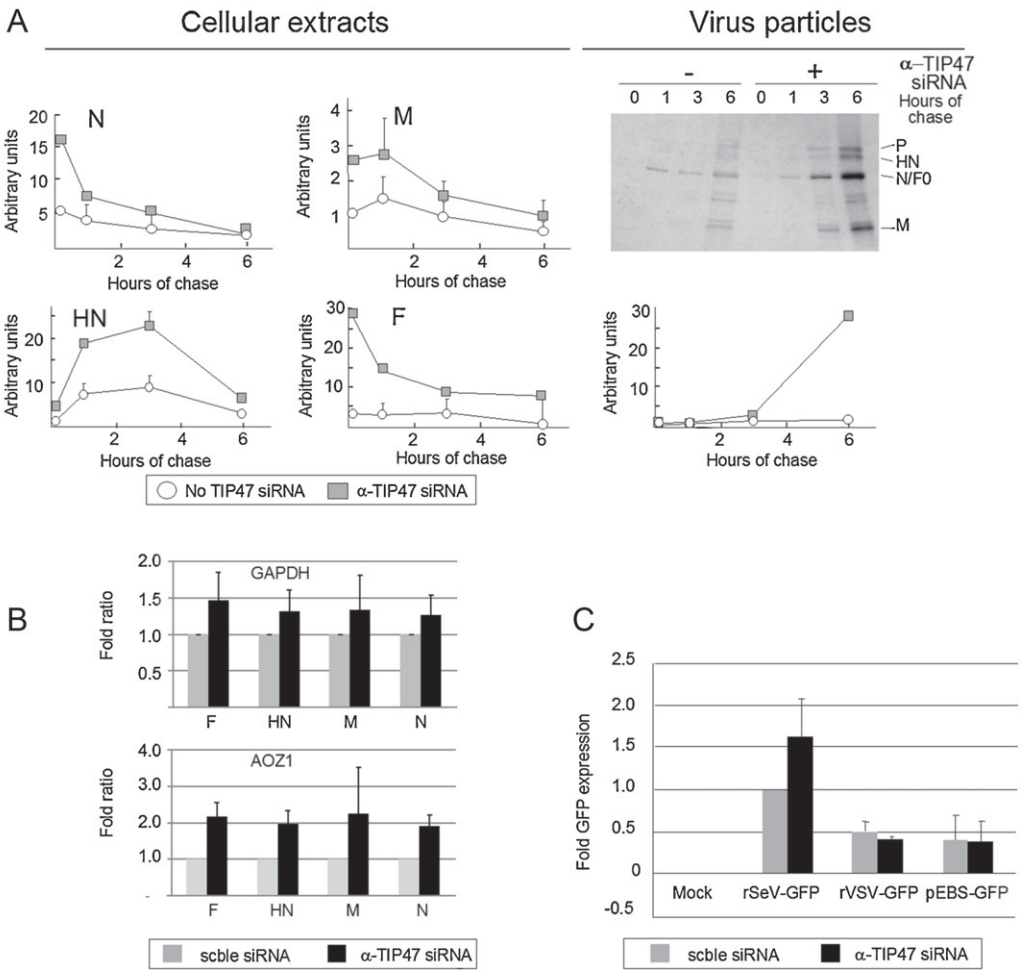

Fig. 7. Synthesis and fate of SeV viral proteins. (A) HeLa cell samples were transfected or not with α-TIP47 siRNA and infected with SeV (moi = 3). At 20 h post-infection, infected cells were pulse-radiolabeled for 15 min and chased for the indicated periods of time. At the end of the chase period, cells were collected and cellular extracts were prepared in RIPA buffer. The indicated proteins were immunoprecipitated (see Section 2), analyzed by PAGE and detected by autoradiography. The autoradiographs were quantified, and the values obtained were plotted as a function of the length of the chase period. Error bars indicate the mean standard deviation of the mean from at least three independent experiments. Upper panel: virus particles. Autoradiography of a representative example of the pulse-radiolabeled viral proteins produced in virus particles as a function of chase periods. Lower panel: The viral N/F0 protein bands shown above were quantified and plotted as a function of the length of the chase period. (B) Total RNA extracts were obtained from rSeV-infected HeLa cells that had been previously transfected with scrambled or α-TIP47 siRNAs. RNA samples were used for reverse transcription using oligo-dT primers. The resulting cDNAs were further quantitatively PCR amplified with primers specific for SeV N, P, M, F, and HN mRNAs or for cellular GADPH and AOZ1 cellular mRNAs, as described in Section 2. (C) HeLa cell samples were transfected with α-TIP47 or scrambled (scble) siRNAs and then infected with recombinant SeV or VSV expressing GFP (rSeV-GFP, rVSV-GFP) or transfected with a plasmid expressing GFP (pEBS-GFP). At appropriate times after infection or transfection (see Section 2), GFP expression was measured by FACS and expressed as fold expression, taking as 1.0 the level of rSeV-Gfp expression measured after transfection with the scrambled siRNA. Errors bars refer to deviation from the mean in three independent experiments.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.virusres.2013.01.006.

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