Sendai virus particle production: A more detailed role of F and HN through, namely, their association with M.

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Abstract

The Paramyxovirus membrane associated proteins are composed of two integral membrane glycoproteins, HN (H, G) and F, and of a matrix protein (M) carpeting the membrane inner layer. For Sendai virus (SeV), F and M have been proposed to form a complex at the endoplasmic reticulum that further migrates to the cell periphery where it represents a nucleation site for viral assembly completion (Essaidi-Laziosi et al., 2013). HN is recruited in the assembly complex once expressed at the cell surface. In contrast to F and M, HN appears dispensable for virus particle production. However, upon F suppression, concomitant HN suppression restricts viral particle production much more severely than F suppression alone, suggesting that HN plays a role as well. In this study, we demonstrate that the transmembrane and cytoplasmic regions of F are sufficient to promote virus particle production and incorporation of a foreign protein in viral particles. We further identify in the F cytoplasmic tail the site of interaction with M. We next confirm HN participation in viral particle production and we provide genetic evidence for a participation [...]
Sendai virus particle production: A more detailed role of F and HN through, namely, their association with M

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A B S T R A C T
The Paramyxovirus membrane associated proteins are composed of two integral membrane glycoproteins, HN (H, G) and F, and of a matrix protein (M) carpeting the membrane inner layer. For Sendai virus (SeV), F and M have been proposed to form a complex at the endoplasmic reticulum that further migrates to the cell periphery where it represents a nucleation site for viral assembly completion (Essaidi-Laziosi et al., 2013). HN is recruited in the assembly complex once expressed at the cell surface. In contrast to F and M, HN appears dispensable for virus particle production. However, upon HN suppression, concomitant HN suppression restricts viral particle production much more severely than F suppression alone, suggesting that HN plays a role as well. In this study, we demonstrate that the transmembrane and cytoplasmic regions of F are sufficient to promote virus particle production and incorporation of a foreign protein in viral particles. We further identify in the F cytoplasmic tail the site of interaction with M. We next confirm HN participation in viral particle production and we provide genetic evidence for a participation of M in the process. We finally derive observations that may provide a mechanism by which the viral C protein participates in viral particle production by mediating HN–M interaction.

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1. Introduction
Sendai virus (SeV) is an envelope virus that belongs to the Paramyxovirus family. Two glycoproteins, HN and F, protrude from the envelope forming spikes composed of HN tetramers and F trimers. The matrix protein (M) carpets the inner side of the envelope forming a two dimensional organized array as seen at the inner surface of the infected cell (Buechi and Bachi, 1982). This array of M is thought to play a central role in virus particle assembly, condensing the two glycoproteins in patches – which serve as attachment sites for the viral nucleocapsids – and completing the ready to bud assembly complex. Moreover, M association with F in the cytoplasm has been recently recognized. Indeed, in the case where F is restricted to the endoplasmic reticulum, following mutation of a TYLTE/SA motif in its cytoplasmic tail, M level is reduced at the cell periphery leading to a blockade of viral particle production (Essaidi-Laziosi et al., 2013). This crucial role of the F cytoplasmic tail has been otherwise recently documented (Stone and Takimoto, 2013). On the other hand, HN was proposed to join the assembly complex at the plasma membrane through a SYWST motif present in its cytoplasmic tail (Takimoto et al., 1998). Indeed, the mutation of SYWST (substituted with AFYKD, the corresponding peptide in measles virus H cytoplasmic tail) induced accumulation of HN at the plasma membrane, without incorporation in viral particles (Fouillot-Coriou and Roux, 2000). The role of the three membrane associated proteins has been recently revisited in experiments where they were suppressed by siRNA technology, individually, or in concert for HN and F (Gosselin-Grenet et al., 2010). In these experiments, F or M individual suppression led to similar moderate but significant decreases of virus particle production. This contrasted with HN suppression effect, confirming HN dispensability for virus particle production (Portner et al., 1974, 1975; Stricker and Roux, 1991; Fouillot-Coriou and Roux, 2000). Despite this well established fact, F and HN concomitant suppressions led to the largest drop in virus particle production, larger than that observed after F suppression alone (Gosselin-Grenet et al., 2010). These results raised questions about the respective role of the two glycoproteins and pointed to yet less well described features, namely their interaction with M.

The present study shows that the F cytoplasmic tail and transmembrane domain are sufficient to drive particle production and to allow incorporation of a foreign protein in virus particle. Secondly, it allows identification of a motif in F cytoplasmic tail prone to interact with M. Thirdly, it consolidates the surprising observation of
HN participation in virus particle production under F level restriction, although HN appears totally dispensable in normal conditions. Finally, it provides genetic evidence of M participation in HN incorporation in virus particles. In this respect, it points to the possibility that HN–M interaction be mediated by a third party, namely the viral C protein, proposed previously to participate, among other functions, in virus particle formation (Kurotani et al., 1998). In the end, the results presented give a more detailed picture of SeV particle formation and open new lines of research that may help reconciling previous data concerning the role of the C proteins.

2. Materials and methods

2.1. Cells and virus infection

MDCK and LLCMK2 cells were maintained in DMEM (Dulbecco’s Modified Eagle Medium, Gibco-Invitrogen) supplemented with 5% fetal calf serum (FCS, Brunshwing) and penicillin and streptomycin antibiotics (Gibco). Cells obtained from K. Conzelmann (Max von Pettenkofer-Institute and Gene Center, Ludwig-Maximilians-University, Munich, Buchholz et al., 1999) were grown in GMEM (BHK-21 Glasgow minimum essential medium, Gibco-Invitrogen) with 5% FCS and a mix of penicillin/streptomycin antibiotics (Gibco) and treated with 0.5 μg/ml of Geneticin (Gibco) once every two passages. MDCK and LLCMK2 cell lines expressing α-gfp siRNA have been described in detail before (Mottet-Osman et al., 2007). All cell lines were maintained at 37 °C under 5% CO₂ atmosphere. For infection, DMEM diluted Sendai virus (SeV) stocks (multiplicity of infection ~ 10) were added to the cells and adsorbed during 1 h at 33 °C. Cells were then washed once with DMEM and 2% FCS–DMEM medium was added. Cells were then incubated for appropriate time period at 33 °C. After 24–48 h of infection (see legends of figures) cells and supernatants were collected and used for further investigation. Virus particles were obtained by pelleting the clarified supernatants through 25% glycerol–Tris–NaCl–EDTA cushion at 13 K rpm at 4 °C for 30 min.

2.2. Construction of full-length cDNA plasmids

SeV full-length cDNA genomes were generated from FL5 through in house standard molecular cloning strategies as previously described (Mottet-Osman et al., 2007). The cDNA constructs recombinant rSeV-Fg, rSeV-HNg, rSeV-Fg-HNg are as described previously (Mottet-Osman et al., 2007; Gosselin-Grenet et al., 2010; Essaidi-Laziosi et al., 2014). For denomination simplification the gfp (standing for green fluorescent protein target) sequence was indicated by “g”. rSeV–HN<sub>APKYD</sub> was generated by site directed mutation of the sequence encoding the SYWST peptide using an insert flanked by two unique cloning sites, ClaI (in the F gene) and Apal (in the L gene). The supplemental genes encoding the F or HN cytoplasmic tail fused to the Biotin polypeptide (derived from pcDNA3/BioEase-DEST, Invitrogen life technologies) are described in Essaidi-Laziosi et al. (2013), with modifications implying additions of the C₁₋₂₃ peptide and HA tag (see text of Section 3 for further explanations). The dTomato protein (TMT) encoding gene was from Shaner et al. (2004). The gene encoding the HA of H1N1 influenza virus was obtained from the laboratory of Laurent Kaiser (Faculty of Medicine, University of Geneva, Switzerland). These supplemental genes were inserted in the unique Mlu restriction site flanked by the gene-start and gene-end sequences of the additional transcription unit inserted between the M and F genes present in FL5. Schematic representations of the rSeV genomes prepared in this paper were shown in Figs. 1, 4A, 5, 7A, S1A, S2A and S3A. All prepared rSeV cDNA constructs were sequenced for at least the modified regions involved (see Table 1 for further information) or in totality when indicated. They all obey the rule of six (Calain and Roux, 1993).

Supplementary Figs. S1–S3 can be found, in the online version, at http://dx.doi.org/10.1016/j.virusres.2015.01.010.

2.3. Recombinant virus rescue and virus stock production

Rescue of recombinant Sendai viruses (rSeV’s) form full-length cDNA genomes was performed as described in Mottet-Osman et al. (2007). Shortly, FL5 cDNA and pTM1 based plasmids expressing the N, P/Cstop and P viral proteins were used to transfect BSRT7 cells. After 48 hours, transfected cells were incubated with 1.5 μg/ml of acetylated trypsin in a medium lacking FCS. Twenty-four hours later, cells were collected and injected into 9 day-old embryonated chicken eggs. After 3 days of incubation at 33 °C, allantoic fluid (AF) was collected. The pellet of 1 ml of AF was analyzed on SDS-PAGE to check for the presence of virus after Coomassie blue staining. Titration of virus stocks was done as described before Sugita et al. (1974).

2.4. Antibodies

For Western blot detection, rabbit polyclonal antibodies α-HN, α-N, α-M and anti F cytoplasmic tail (α-Fc) have been described (α-HNSp, α-Nsp, α-Msp (Tuffereau and Roux, 1988; Mottet-Osman et al., 2007; Mottet et al., 1996). Monoclonal anti-HA tag is from Covance (#16B12). Polyclonal rabbit anti-H1N1 HA protein (A/California/14/2009) is from Sigma–Aldrich (SAB3500059). Anti-dTMT is from Chemicon International (#AB3216). The biotinylated fused proteins were detected with Streptavidin–HRP (Pierce). For immune-fluorescence staining, α-HN (clone M68) and α-F (clone M38) mouse monoclonal antibodies were obtained from Allen Porter (St Jude Children’s Research Hospital, Memphis, Tennessee) and rat monoclonal anti-HA (Clone 3F10, Roche ref 11867423001). TRITC-conjugated donkey anti-rat IgG (Jackson ImmunoResearch) and Alexa488 conjugated goat anti-mouse IgG (Invitrogen) were used as secondary antibodies.

2.5. Western blot analysis

Western blots were performed according to standard protocol. Viral proteins, after migration in 12.5% acrylamide SDS–PAGE gel, were transferred onto polyvinylidene difluoride membrane (PVDF, Millipore) using Trans-Blot SD Transfer Cell (Biorad). Proteins were visualized using the PNR 2106 ECL Western Blotting Detection System (Amersham). Blot imaging was done using a Fujifilm LAS–40.

<table>
<thead>
<tr>
<th>No.</th>
<th>Virus description&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Phenotype&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Motif sequence&lt;sup&gt;c&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>1</td>
<td>wt HN&lt;sub&gt;APKYD&lt;/sub&gt;/NF-C rescued</td>
<td>Mutant</td>
<td>FAPKVD</td>
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<tr>
<td>3</td>
<td>wt HN&lt;sub&gt;APKYD&lt;/sub&gt;/NF-C rescued</td>
<td>Mutant</td>
<td>FAPKVD</td>
</tr>
<tr>
<td>4</td>
<td>wt HN&lt;sub&gt;APKYD&lt;/sub&gt;/NF-C rescued</td>
<td>Mutant</td>
<td>FAPKVD</td>
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<sup>a</sup> Viruses were obtained through standard rescue procedures as described in Section 2 of this paper and of the papers cited here after. NF-C: as in Fouillot-Coriou and Roux (2000), ME-L: as in Manel Essaidi-Laziosi et al., 2014, ASG-G: as in Gosselin-Grenet et al. (2010).

<sup>b</sup> Phenotype is established upon infection of LLCMK2 cells and analysis of the level of incorporation of HN in virus particles: mutant = highly restricted HN incorporation relative to wild type (wt).

<sup>c</sup> Sequence analysis of the HN gene only and outline of amino acid sequence in the motif <sub>10</sub> of the HN cytoplasmic tail. Number of passages not precisely recorded.
2.6. Isotopic radio-labeling of cells

After 16 h of infection, cells were overlaid with FCS free medium containing 1/10th the amount of methionine and cysteine and 30 μCi/ml of 35S-methionine and 35S-cysteine (Pro-mix-[35S]-Amersham, Biosciences). Twenty-four hours later, cells and supernatants were collected and used for SDS-PAGE gel analysis. Radioactive proteins were then detected using Typhoon FLA 7000 Phospho-Imager (GE Healthcare).

2.7. Streptavidin agarose pull-down assays

Pull down with streptavidin agarose resins was performed according to the manufacturer’s instructions (Pierce Streptavidin Agarose Resins, Thermo-Scientific) and as previously described (Essaidi-Laziosi et al., 2013). Briefly, cells were infected with viruses expressing Biotip fused to F or HN cytoplasmic tails (represented in Figs. 6–A, 7-A, 51-A and 52-A) and incubated in DMEM supplemented with biotin 1 mg/ml (Sigma–Aldrich). Infected cells were washed three times with phosphate buffered saline (PBS), collected 30 h post-infection and lysed in NaCl 10 mM, Tris–HCl pH 8.0 50 mM, NP40 0.6%. The cellular extracts were then incubated with 20 μl of streptavidin agarose for 2 h at 4°C. Pulled-down proteins were pelleted and washed five times with NaCl 150 mM, EDTA 5 mM, 50 mM Tris–HCl pH 7.8, NP40 2%. Finally, the bound complexes were eluted with SDS PAGE sample buffer and analyzed by Western blotting.

2.8. Immunofluorescence staining and confocal microscopy

Immunofluorescence analysis was performed according to the protocol previously described in details (Essaidi-Laziosi et al., 2013). Briefly, MDCK cells were grown on sterilized coverslips coated with poly-Lysine (Sigma). After 24 h of incubation infected cells were washed with 20 mM Hapes pH7.5-buffered DMEM and fixed with 4% PFA 15 min at room temperature. In case of surface immunofluorescence (surface IF) staining primary and secondary antibodies were added on the PFA-fixed cells and for total immunofluorescence (total IF) staining, cells were permeabilized using methanol (−20°C) before staining. Washing using PBS followed any incubation. The nucleus was stained with DAPI (Boehringer Mannheim GMBH). Stained cells were mounted in Fluoromount-G (SouthernBiotech). Confocal microscope LSM700 (Carl Zeiss) via 63×/1.4 oil immersion objective was used to take images and Zen software for the acquisition and then the analysis and treatment of confocal images.

2.9. Analysis of revertant viruses

For analysis of spontaneous reversion, viral RNA was extracted from allantoic fluid by Trizol (Life Technology) according to the manufacturer’s instructions. Reverse transcription was carried out with M–MLV reverse transcriptase (Promega). Viral genome cDNA was amplified by PCR using specific primers (primer sequences available upon request). The RT-PCR product was then purified (Qagen DNA gel extraction kit), fully sequenced and analyzed using the Applied Biosystems software. Alternatively, 1 μm of purified viral RNA was used for deep sequencing. Libraries were pooled and sequenced on an Illumina Genome Analyzer GAIIx (Illumina). Obtained results (reads) were visualized by Integrative Genomics Viewer (IGV). Plasmids containing full length cDNA sequences of rSeV-wt and rSeV-HN_AFYKD were equally sequenced (see Table 2).

3. Results

3.1. The F cytoplasmic tail (+ trans-membrane region) is fully competent for virus particle production and glycoprotein incorporation into virion

If the F cytoplasmic tail (Fc) represents a minimal requirement for SeV particle production, then a protein expressing this domain in the right conformation should be sufficient to replace F. Consequently, one would predict that this protein be itself incorporated in particles. At first, a rSeV-Fg/HNg virus, in which the F and HN proteins are open to suppression by siRNA technology (see Section 2) was constructed, carrying the supplemental H1N1 influenza hemagglutinin/neuraminidase (HA0) gene (Fig. 1, line 2). In a second version of this construct, the F transmembrane (Ft) and cytoplasmic (Fc) regions replaced the original HA corresponding regions, creating HA_{Ft-Fc} (Fig. 1, line 3). Fig. 2 shows that the two HA0
Table 2
Summary of the genotypic and phenotypic analysis of wild type, mutated viruses and revertant viruses obtained spontaneously or after serial passages.

| No. | Virus description* | Phenotype** | Motif sequence*** | Sequence in M**** | Sequencing features**†  
|-----|-------------------|-------------|-------------------|-------------------|-------------------------
| 1   | wt/ME-L           | wt          | SYWST             | Leu Arg           | Classic, HN/M           
| 2   | wt/NF-C           | wt          | SYWST             | Leu Arg           | Classic, HN/M           
| 3   | HNAFYKD/ME-L      | Mutant      | AFYKD             | Leu Arg           | Classic, HN/M           
| 4   | HNAFYKD/NF-C      | Mutant      | AFYKD             | Leu Arg           | Classic, HN/M           
| 5   | HNAFYKD/NF-C rescued/ASG-G1 | Mutant  | AFYKD             | Leu Arg           | Classic, HN/M           
| 6   | HNAFYKD/NF-C rescued/ASG-G2 | Mutant  | AFYKD             | Leu Arg           | Classic, HN/M           
| 7   | HNAFYKD/NF-C rescued/ASG-G3 | Mutant  | AFYKD             | Leu Arg           | Classic, HN/M           
| 8   | HNAFYKD/NF-C rescued/ASG-G4 | Mutant  | AFYKD             | Leu Arg           | Classic, HN/M           
| 9   | HNAFYKD/NF-C rescued/AS P5† | Revertant  | AFYKD             | His Arg           | Deep, total             
| 10  | HNAFYKD/NF-C rescued/AS clP6‡ | Revertant  | AFYKD             | His Arg           | Deep, total             
| 11  | AS cDNA rescued* | Mutant      | AFYKD             | Leu Arg           | Deep, total             

* Virus description as in legend to Table 1 with the addition of viral cDNA and viruses prepared by Anastasia Shevtsova (AS).
** Phenotype: as in Table 1.
† Sequence analysis: as in Table 1.
‡ The sequence has been extended to the M gene, and two positions (aa 192 and 239) have been outlined.
§ Sequencing features refer to the methodology used. Classic: regular sequencing covering the M and HN genes (see Section 2). Deep: deep sequencing extending over the entire genome, be it of a rescued virus or of a full length cDNA prepared for virus rescue. “P5” and “clP6” for viruses No. 9 and 10 refer to the number of serial passages in eggs starting from a virus with controlled AFYKD genotype and phenotype (see text). clP6: Plaque purified viruses from the P6 virus.

Fig. 2. Role of F cytoplasmic and trans-membrane domains for formation of and protein incorporation into SeV particles. MDCK cells constitutively expressing or not anti gfpt siRNA (+/− α-gfpt siRNA) were mock-infected or infected with the indicated rSeVs (see fig 1). Cellular extracts (A–C) and virus particles (D–F) were analyzed by Western blots using indicated antibodies. G. Quantification (ImageJ software) of the level of virus particle production is from three independent experiments done in conditions of expression (black bars) or suppression (gray bars) of HN and F glycoproteins. Virus particle levels were taken as the N levels in viral particles normalized to the levels of intracellular N. They are expressed as a percentage of those found in conditions of HN and F expression, taken as 100%. p values are indicated.
proteins (and HA1) are expressed under infection with these two viruses (Fig. 2B, lanes 5–8). When HA harbors the transmembrane and cytoplasmic regions of SeV-F (HA\textsubscript{N-FC}), it can be detected with α-Fc antibodies as well (Fig. 2A, lanes 7 and 8). This expression is equivalent whether endogenous HN and F proteins are expressed or suppressed (−/+ α-g-fpt siRNA). As for the SeV proteins, their expression is not affected by the expression of these supplemental proteins (for F, compare in Fig. 2A, lane 3 with 5 and 7; for HN, N and M see Fig. 2C). Moreover, F and HN levels respond well to suppression (compare lanes −/− in Fig. 2A and C, lanes F\textsubscript{G}/HN\textsubscript{G}), although the corresponding drop in viral particle production does not exceed 70% (Fig. 2G, F\textsubscript{G}/HN\textsubscript{G}), a limitation probably linked to the cell type used in this series of experiments (MDCK as opposed to LLCMK2). Of note, HA and HA\textsubscript{N-FC} are detected in virus particles (Fig. 2D and E). Finally, expression of HA\textsubscript{N-FC} contributes rescuing the highest virus production upon F and HN suppression (Fig. 2F, compare lanes 7–8 with lanes 3–4, or 5–6, and Fig. 2G). Fig. 2G also shows that normal HA, by itself, has already a boosting effect on virus production in condition of HN and F suppression (gray bars).

Because influenza virus HA appears to participate by itself to SeV particle production, likely because it shares some properties with F, we next used a protein that should not share any feature with viral glycoproteins. The gene encoding dTomato protein (dTMT, see Section 2) was then inserted as a supplemental gene, as such (Fig. 1, TMT), or modified so that the protein harbors the F transmembrane and cytoplasmic domains (Fig. 1, TMT\textsubscript{F-FC}). Also, the F N-ter signal peptide (Fs) was added at the dTMT N-ter, to address the protein to ER and to the extracellular pathway. Fig. 3B shows that both versions of dTMT are expressed in infected cells (lanes 5–8), with appropriate PAGE migration features. TMT\textsubscript{F-FC} is also detected by α-Fc antibodies, which do not detect dTMT (Fig. 3A, lanes 5–8). TMT\textsubscript{F-FC} levels, relative to that of F, appear lower than those of the HA\textsubscript{N-FC} (Fig 2A, lanes 7 and 8). SeV proteins, however, are properly expressed or suppressed whenever they should (Fig. 3C). F and HN suppression leads to significant decreases in virus particle production (Fig. 3F, compare lanes 3 and 4). Virus particle production appears not to be affected by TMT expression (Fig. 3F, lanes 5 and 6), but is slightly increased by the expression of TMT\textsubscript{F-FC} (Fig. 3F, compare lanes 6–8). This effect, although not substantial, is confirmed by statistical analysis (Fig. 3G). The modest effect promoted by TMT\textsubscript{F-FC} may result from its reduced presence in the cell, likely due to a faster turn over. This likely explains also the lack of its detection in virus particles (Fig. 3E).

In conclusion, ectopic expression of the F transmembrane and cytoplasmic domains appears sufficient to partly compensate for F and HN suppression in the viral particle production, suggesting that the interactions involved in this process only concern these portions of the protein. In agreement, with this conclusion, these two domains are sufficient to lead to quantitative incorporation of a heterologous protein like influenza HA.

3.2. A region of the F cytoplasmic tail proximal to the transmembrane region is involved in interaction with M

In a previous study by Fouillot-Coriou and Roux (2000), the F cytoplasmic tail (of 42 aa long) could be truncated from its 15 C-ter proximal amino acids without affecting virus particle production and F incorporation in these particles. This result suggested that condensation of the truncated F in the assembly complex – a task likely devoted to M – was not affected. As mutation of the 24TYTLE\textsubscript{29} motif in this cytoplasmic tail, essential for virus particle production, did not disrupt M–F interaction (Essaidi-Laziosi et al., 2013), it became plausible that the point of M–F cytoplasmic tail interaction would lay in the region flanked by TTYTLE and the transmembrane region (see Fig. 4A, aa 25–42). The penta-peptide 30SMLMG\textsubscript{35} was then mutated to 5A (Fig. 4, C-F\textsubscript{mut1}) and interaction with M assessed as previously reported (Essaidi-Laziosi et al., 2013). F cytoplasmic tail in fusion with a peptide prone to biotinylation in situ (Biot) was expressed from a supplemental SeV gene (Fig. 4A, C-Fc constructs). M pull down assays were then tested using streptavidin beads. Previously, this method allowed us to verify the conservation of M-Fc TYTLE/5A interaction (Essaidi-Laziosi et al., 2013). In the present study, we further added a C1–23 peptide and a HA tag at the N-terminal side of the cytoplasmic tail (Fig. 4A, C-F\textsubscript{mut}). C\textsubscript{1–23} originates from the SeV-C protein N-ter and represents an address to the inner side of the plasma membrane (Marq et al., 2007). As expected, C\textsubscript{1–23} brought the cytoplasmic tail to the cell periphery, a condition that apparently improved its interaction with M (Fig. S1, see also Section 4). As controls, regular infections not expressing the biotinylated F cytoplasmic tail (Fig. 4, Fg) or expressing the cytoplasmic tail of the measles virus H protein (Fig. 4, Hc) were used. Analysis of the cytoplasmic tail carrying the mutation TYTLE/SA (Fig. 4A, C-F\textsubscript{c5A}) was also repeated. Fig. 4B and C shows that, as previously published, TYTLE/SA mutation did not cancel interaction with M (C-F\textsubscript{c5A}). In this series of experiments, binding of C-F\textsubscript{c5A} to M was rather boosted. This contrasted with the reduced M pull down by C-F\textsubscript{mut}, suggesting that this portion of the cytoplasmic tail has lost its interaction with M.

3.3. HN does participate to SeV particle formation in conditions of F restriction

The larger drop of virus particle production upon F and HN suppression (exceeding that following F suppression alone) was interpreted as the HN participation in conditions of reduced F level (Gosselin-Grenet et al., 2010). Alternatively, HN co-suppression could produce a higher viral glycoprotein turn over, resulting in a more efficient F depletion. To resolve this uncertainty HN was replaced by HN\textsubscript{AFKRD}, excluded from viral particle (Fouillot-Coriou and Roux, 2000), hence likely not to participate in viral particle formation. A recombinant SeV was then produced encoding HN\textsubscript{AFKRD} in the context of an F gene open to suppression (Fig. 5, rSeV-Fg/HN\textsubscript{AFKRD} and description of all the rSeV’s used in the experiment). Each infection was studied in normal conditions (Fig. 6, −α-g-fpt siRNA) or in conditions of effective protein suppression (Fig. 6, +α-g-fpt siRNA). Viral particle production was recorded by direct 35S-labeled viral proteins analysis purified from the cell supernatants (Fig. 6A). Intracellular viral proteins were monitored by Western blots using appropriate antibodies (Fig. 6B). Total radiolabelled cell extracts were also directly analyzed by SDS-PAGE serving as loading controls (Fig. 6C). Fig. 6A, lanes 3 and 4, show restriction of VP production upon F suppression. As soon previously, this restriction increases upon dual suppression of F and HN so that VP proteins are barely detected (lane 8). Interestingly, similar restrictions are seen upon F suppression alone in the presence of HN\textsubscript{AFKRD} (lane 10). So, for VP production, expression of HN\textsubscript{AFKRD} is equivalent to suppression of HN. Note that, as expected from previous results, HN\textsubscript{AFKRD} expression in conditions of normal F expression, does not perturb VP production (lanes 11 and 12). In the end, in conditions of F suppression alone, but with expression of a non-functional HN\textsubscript{AFKRD}, the negative synergistic effect that suppression of both glycoproteins exerts on virus production is reproduced. This rather confirms the HN participation in virus production when F is reduced; when F is fully expressed, HN remains fully dispensable.

3.4. Evidence for M involvement in HN incorporation into virion

In the model of SeV assembly (Essaidi-Laziosi et al., 2013), HN is recruited at the plasma membrane by interaction with a fraction of M that is found by default at that location. This recruitment does not take place when the HN cytoplasmic tail 16SYWST\textsubscript{14} motif is
changed for AFYKD (Fouillot-Coriou and Roux, 2000). On this basis, it was hypothesized that SYWST is directly involved in M interaction. Similar experiments as those described in Fig. 4 were therefore set up for HN cytoplasmic tail. In a first series of experiments a peptide containing only the HN cytoplasmic tail (HNc) fused to Biotp failed to show any pull down of M (Fig. S2B, HNcwt-C). Addition of the C1-23 peptide to the HNc construct brought the cytoplasmic tail to the plasma membrane (Fig.S2C, HNcwt-C) and improved the efficiency of M pull down (Fig. S2B, HNcwt-C lanes). Because it was previously shown that a deletion of the first N-ter 10 amino acids of HNc had no deleterious effect on HN incorporation into virus particles (Fouillot-Coriou and Roux, 2000), two motifs downwards of SYWST were next mutated (17SGST12 and 24SGWE28) into alanine to create HNcmut1-C and HNcmut2-C respectively (Fig. 7A). None of the three mutants exhibited a decreased ability to pull down M. It is particularly surprising that even mutation HNcAFYKD-C, known to be associated with lack of HN incorporation in virus particles, appears to perform better than HNcwt-C. In the end, not only the data do not support the notion that the SYWST motif participates in HN–M interaction, but also no motif of interaction appears to exist in the HN cytoplasmic tail. Consequently, lack of HN incorporation into virus particle can apparently not be explained by a defect of HN interaction with M (in the limit of the motifs tested here).

Confronted with this conundrum, we developed another approach based on natural selection of HNAFYKD phenotypic revertants. We rationalized that poor HN incorporation into viral particles would certainly lower viral fitness, a fitness that could only improve with virus passages. Besides, as the mutation involves 5 amino acids of which the relevant ones were not known), reversion would now target the relevant site(s). And last but not least, the probability that reversion would involve a site on the putative protein interacting with HN was possible. In fact, sequence data limited to the mutated region had shown increased variability correlating with virus passages (Fig. S3B). It was therefore not surprising that two out of three repeatedly passed SeV–HNAFYKD viruses exhibited revertant phenotypes (Fig. S3C, lanes 3 and 4), in contrast to SeV–HNAFYKD analyzed just after recovery from cDNA (lane 1). Interestingly, sequence analysis showed that the two revertant viruses carried the substitution AFYKD to APFVK (Table 1). This result was fully consistent, but contribution of the putative interacting M protein was not addressed. More SeV–HNAPFYKD viruses, including randomly passaged viruses, were considered with sequence analysis extending to the M gene as well (Table 2). Moreover, a rSeV–HNAPFYKD virus, just obtained from rescue, was repeatedly passed in a controlled way until the phenotypic reversion was observed (Fig. 8A and B). The full length cDNA plasmid used for rescue (not shown), the rescued virus genome (Fig. 8A, wt and Table 2, No. 3) and the phenotypic revertant virus genomes (Fig. 8A and B, Table 2, No. 9, P5 and No. 10, P6) were submitted to deep sequencing. The results of the deep sequencing are shown in Tables 3 and 4. Only the sequence data that differ from those of the rSeV–HNAPFYKD are outlined, i.e. the remaining of the entire genome sequence was found unchanged. Interestingly, the AFYKD motif, which likely provokes a loss of fitness, showed signs of variability with minor changes compared to the original at every single nucleotide position. Only in position 44, the change

![Fig. 3. Role of F cytoplasmic and trans-membrane domains in incorporation of a virally irrelevant protein (dTMT) into SeV particles. As in Fig. 2 except that the analyzed viruses are those described in Fig. 1, lines 1, 4 and 5 with appropriate antibodies. α-TMT: anti-tomato protein antibody. In panel G, p values are indicated.](image-url)
Fig. 4. Interactions of the F cytoplasmic tail with M protein. (A) Schematic representations of recombinant SeV's harboring endogenous F protein open to conditional suppression by α-gfpt siRNA and additional transcription units (line 1) expressing the F cytoplasmic tail (Fc, green) in its wt (Fcwt, line 2) or mutated (Fc5A and Fcmut1, lines 4 and 5) configurations. Fc is further fused to a peptide (Biotp, yellow) open to biotinylation, to a HA tag (HA, gray) and to a 23 amino acid peptide originating from the SeV C protein N-terminus (C1-23, purple). In the Fc sequence (line 3, highlighted in green), motifs αTYTLE[20] and αSMLMGL[20] are substituted by 5 alanines to create C-Fc5A and C-Fcmut1, respectively (lines 4 and 5). Light green: part of Fc that can be removed without affecting glycoprotein incorporation in viral particle and virion production (Fouillot-Coriou and Roux, 2000). Hc (red/green, line 6), cytoplasmic tail of measles virus H protein fused to Biotp, used as control. (B) Anti-gfpt siRNA expressing MDCK cells were infected with the indicated viruses in medium supplemented with biotin. Sixty hours post infection cellular extracts were prepared and incubated with streptavidinagarose resins to pull down Fc fused to Biotp (see Section 2). Left panels and right panels: Western blot analysis of pull down samples and cellular extracts, respectively, probed with α-M antibodies and streptavidin-HRP; Fg: Fg virus, see Fig. 5, line 2. (C) Quantification of the ratio of M protein to Biotp’s using ImageJ software from 2 independent experiments. p values are indicated.

A44G became predominant and led to an amino acid change Δ14G (Table 3). The other change observed concerns the M ORF, where, at nucleotide position 574, a T to A change was evident (93% of the reads), leading to a L192H amino acid change (Table 4). Note that the positions surrounding the mutation show no variability at all, 100% of the reads correspond to those of the rSeV-HNAYKD parent strain. Table 2 presents a summary of all the sequencing data and many features are remarkable. All revertant viruses carry a mutation(s) of

Table 3

Deep sequencing results of the HNAYKD viruses: AFYKD motif highlighted. HNAYKD and clp6 as in Table 2 viruses No. 3 and 10, respectively. For HNAYKD, the % of total number of reads for the positions highlighted is >99% (not shown).

<table>
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<tr>
<th>Sequence</th>
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<th>NT Position in HN ORF</th>
<th>Clp6 % Read in clp6</th>
<th>% Total Read in clp6</th>
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Bold values are stressing the high percentage of the major genome species.
Fig. 5. Schematic representation of rSeV's used to confirm the role of HN in virus particle formation. 1. Outline of the parental SeV genome with its 6 genes flanked by the genomic promoter (GP) and the complement of the antigenomic promoter (AGPc). 2. Outline of rSeV-Fg9 virus. The F transcription unit is flanked by the start/stop transcription signals (−/+). In F open reading frame (ORF), the ecto- (gray), trans-membrane (red) and cytoplasmic (green) domains are shown, with the SYWSTAFYKD motif outlined. In the 5′ non-coding region of the gene, the sequence derived from the green fluorescent protein gene serving as target for siRNA based suppression is shown (gfp, black). 3. Outline of rSeV-HNg virus. As for the F gene in 2. Domains of HN ORF as well as gfp are outlined using the same color code as in 2. The HN cytoplasmic domain carries the wild type motif SYWSTAFYKD. 4. Outline of rSeV-Fg/HNg virus. Both F and HN genes are targeted with gfp sequence. 5. Outline of rSeV-Fg/HNafykd virus. As in 2, but the SYWST motif is replaced with AFYKD. 6. Outline of rSeV-HNafykd virus. As in 3, with the SYWST motif is replaced with AFYKD and without gfp in the F gene. Virus denominations, as in Fig. 1.

A

B

C

Fig. 6. HNafykd behaves as HN suppression in the negative synergy promoted by F suppression. LLCMK2 cells constitutively expressing or not anti gfp siRNA (+/−α-gfp siRNA) were infected with indicated viruses (see Fig. 5). Sixteen hours post-infection, the infected cells were radiolabelled with 35S-methionine and cysteine (see Section 2). (A) Autoradiogram of virus particle samples analyzed by SDS-PAGE. (B) Cellular extracts analyzed by Western blot using α-HN, -F, -N and -M antibodies. (C) Autoradiogram of cellular extracts analyzed by SDS-PAGE.

4. Discussion

In summary, this study provides a few different sets of original information concerning the process of SeV particle assembly and production. In general, it makes ample use of supplemental protein expression and illustrates the possibilities of the integrated suppression complementation system (ISCs) to perform viral protein structure/function analysis in the context of the viral infection. At this point, it is fair to point to the major drawback of this approach, which resides in the impossibility to generally suppress more than 95% of the siRNA targeted protein. On the other hand, this methodology allows expression of supplemental (complementing) proteins to levels corresponding to those of the other viral proteins and in the same “context”. By this, one refers not only to the general context of an infection (in comparison to viral protein expression resulting from plasmid transfection), but more precisely to the same molecular mechanisms and to the same subcellular topography, features that are not fully appreciated yet for Paramyxoviruses, but are better described for positive strand RNA viruses (Van Kuppeveld et al., 2010).

The critical involvement of F was targeted down to its cytoplasmic and transmembrane domains. Ectopic expression of these two domains was enough to restore a significant part of the drop in particle production that follows F and HN suppression. In view of the fact that the cytoplasmic tail alone appears to contain the site of F interaction with M, one would tend to conclude that the transmembrane region may not be critical. Interestingly, influenza virus HA appeared to partially rescue the drop of viral particle...
production caused by F and HN suppression. Whether this reflects partial functional similarity of SeV F and influenza HA, and/or partial lack of specificity in the interactions required for the process remains open; no primary sequence similarity between SeV-F (42 aa) and H1N1 HA (16 aa) cytoplasmic tails could possibly be identified. Regardless, in the same way the HN cytoplasmic and trans-membrane regions allowed measles virus H (type II glycoprotein) uptake into SeV particle (Essaidi-Laziosi et al., 2014), the corresponding domains of F (type I glycoprotein) favored incorporation of a glycoprotein of the same type. Uptake of heterotypic glycoproteins in SeV particles appears then to require the homotypic cytoplasmic and trans-membrane domains, in accordance with the human parainfluenza 3 (HPIV3) requirement to be subtyped by the human parainfluenza 2 (HPIV2) glycoproteins (Tao et al., 2000). This requirement, however, may depend on the source of the heterotypic proteins, since HPIV3 could be readily subtyped with the human parainfluenza 1 (HPIV1) glycoproteins without substitution (Tao et al., 1998).

The resolution of the apparent paradoxical role of HN in virus particle formation received further clarification with the HNAFYKD expression accompanying F suppression. In this situation, a negative synergy for virion production was created between presence of the non-functional HNAPYKD and F suppression, similar to that observed upon simultaneous suppression of both proteins. The coherence of the two sets of data firmly validates the notion of partial and unilateral redundancy of the two proteins in virus particle formation, a finding that was already made using the viral like particle (VLP) system (Sanderson et al., 1993, 1994). When F can fully replace HN, this latter can only partially substitute for F. The higher deficit in viral particle production created by F suppression

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| cIP6 | Nt sequence | G | C | A | G | A | C | C | T | G | C | A | T | T | T | G |
|      | aa sequence | A190 | D191 | H192 | K193 | C194 |

Bold values are stressing the high percentage of the major genome species.
can then be seen only when HN cannot kick in, either because it is suppressed or non-functional. This interpretation establishes the essential role of the two glycoproteins in SeV particle formation: a role that had only been recently recognized for SeV (Gosselin-Grenet et al., 2010), although reported earlier for PIV5 (Schmitt et al., 2002; Waning et al., 2002) and influenza virus (Chen et al., 2007).

It is generally accepted that HN is recruited in the assembly complex by the M protein. As substitution of the SYWST domain into AFYKD abrogates HN recruitment, it appeared coherent to postulate that SYWST was the site of HN–M interaction. This postulate could not be verified, using the pull down assay. Either, the HN cytoplasmic tail would not pull down M (Fig. S2), or if it would, as when sent to the plasma membrane by the C peptide, M would now be pulled down by HN cytoplasmic tail indistinctly in SYWST and AFYKD configurations (Figs. 7 and S2). Even the attempts to mutate two other oligo-peptides in the cytoplasmic tail failed to destroy this interaction, as if, in this case, the HN–M interaction would involve another domain than the cytoplasmic tail (the trans-membrane region?). At this point, questions could be raised concerning the validity of the approach used. The fact that HN–M interaction is seen only when the HN-cytoplasmic tail is addressed to the plasma membrane is reassuring, as it fits with the notion of HN recruitment at the plasma membrane (Essaidi-Laziosi et al., 2013). On the other hand, binding of the HN cytoplasmic tail in fusion to the BioEase and C peptides could create sticky cytoplasmic tails with loss of proper behavior. Alternatively, as HN recruitment must in some way involve SYWST, a third party could serve as an intermediate in HN–M interaction relying on SYWST. HNAFYKD would then have lost interaction with this third partner. It may be pertinent to introduce here the viral C protein claimed to participate, among other functions, in the virus particle formation (Kurotani et al., 1998), C was proposed to recruit Alix, a member of the ESCRT machinery (Sakaguchi et al., 2005; Irie et al., 2007). This conclusion could not be validated in the context of an infection, where the inhibition of the ESCRT machinery by Alix suppression and VPS4A neutralization had no effect on viral particle production (Gosselin-Grenet et al., 2007). This controversy should not necessarily disqualify C for a role in virus assembly. The C protein could bind a fraction of M to address the protein to the cell plasma membrane. The C–M complex would then recruit HN through a C–SYWST motif interaction, further priming a direct HN–M interaction. In this model, the results of the pull down assay could now be explained if the C1–23 peptide contains the site of interaction with M. HN–M interaction could be through the HN transmembrane domain or through the cytoplasmic tail, except that in this latter case, it could only form after a priming role of C. Although quite sophisticated, such a model (presented in Fig. S4) has the merit to reconcile a whole set of data, including the results presented in this study and is moreover open to experimentation. In the case of the C-Fc constructs, where the C1–23 peptide is present at the N-ter of the C-Fc constructs (as opposed to the C-ter of the HN-C constructs, compare Figs. S1 with S2), the configuration of the C1–23 is predicted not to provide efficient binding to M, since this can be abrogated by mutation in Fc (Fig. 4). If this interpretation has some ground, then the pull down assay, as performed by the BioEase-C1–23 peptides fused to the HN cytoplasmic tail becomes inadequate to demonstrate a putative HN–M interaction. However, the genetic analysis of the HN_AFYKD revertant viruses remains. With the exception of one reversion that can be explained by the regaining of the SY dipeptide at proper position in the AFYKD motif (virus No. 5, Table 2), the other reversions all involve substitutions of the last amino acid of the AFYKD motif (AFYKD/V or/G) plus a second site mutation in the M protein (L192R, L192H or R239G). As correction of H192L in the context of AFYKG did not restore the wild type phenotype (Table 2, No. 11), this result strongly suggests that the mutation in M is necessarily involved in phenotypic reversion, and argues for a HN–M interaction.

Supplementary Fig. 4 can be found, in the online version, at http://dx.doi.org/10.1016/j.virusres.2015.01.010.

In the end, this study participates in a further description of the molecular mechanisms involved in the assembly of SeV particles and their production. In its present form it is far to propose a definitive model to account for these processes. One of its merits, however, may reside in the opening of new lines of research based on the ISCS approach toward a better understanding of the sequence of events prevailing in the late steps of Paramyxovirus multiplication cycle.

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References


