Minimal features of efficient incorporation of the hemagglutinin-neuraminidase protein into Sendai virus particles

ESSAIDI, Manel, SHEVTSOVA, Anastasia, ROUX, Laurent

Abstract
Two transmembrane glycoproteins form spikes on the surface of Sendai virus, a member of the Respirovirus genus of the Paramyxovirinae subfamily of the Paramyxoviridae family: the hemagglutinin-neuraminidase (HN) and the fusion (F) proteins. HN, in contrast to F, is dispensable for viral particle production, as normal amounts of particles can be produced with highly reduced levels of HN. This HN reduction can result from mutation of an SYWST motif in its cytoplasmic tail to AFYKD. HNAFYKD accumulates at the infected cell surface but does not get incorporated into particles. In this work, we derived experimental tools to rescue HNAFYKD incorporation. We found that coexpression of a truncated HN harboring the wild-type cytoplasmic tail, the transmembrane domain, and at most 80 amino acids of the ectodomain was sufficient to complement defective HNAFYKD incorporation into particles. This relied on formation of disulfide-bound heterodimers carried out by the two cysteines present in the HN 80-amino-acid (aa) ectodomain. Finally, the replacement of the measles virus H cytoplasmic and transmembrane domains with the corresponding [...]
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Two transmembrane glycoproteins form spikes on the surface of Sendai virus, a member of the Respirovirus genus of the Paramyxovirinae subfamily of the Paramyxoviridae family: the hemagglutinin-neuraminidase (HN) and the fusion (F) proteins. HN, in contrast to F, is dispensable for viral particle production, as normal amounts of particles can be produced with highly reduced levels of HN. This HN reduction can result from mutation of an SYWST motif in its cytoplasmic tail to AFYKD. HNAFYKD accumulates at the infected cell surface but does not get incorporated into particles. In this work, we derived experimental tools to rescue HNAFYKD incorporation. We found that coexpression of a truncated HN harboring the wild-type cytoplasmic tail, the transmembrane domain, and at most 80 amino acids of the ectodomain was sufficient to complement defective HNAFYKD incorporation into particles. This relied on formation of disulfide-bound heterodimers carried out by the two cysteines present in the HN 80-amino-acid (aa) ectodomain. Finally, the replacement of the measles virus H cytoplasmic and transmembrane domains with the corresponding HN domains promoted measles virus H incorporation in Sendai virus particles.

Parameyxoviruses are enveloped viruses containing two integral envelope glycoproteins, the hemagglutinin-neuraminidase protein (HN), which is responsible for cell receptor binding/cleavage, and the fusion protein (F), which is responsible for fusion of the viral envelope with the cellular membrane. The inner side of the viral envelope is carpeted by a layer of the matrix M protein that bridges the envelope to the nucleocapsid, the inner core of the particle. The nucleocapsid is composed of a single-stranded RNA of negative polarity, tightly wrapped by nucleocapsid proteins (N) in a structure of helicoidal symmetry, and is associated with the viral RNA-dependent RNA polymerase made of the two proteins P and L (for recent reviews about Paramyxoviridae, see reference 1). In Sendai virus (SeV), a member of the Paramyxovirinae subfamily, genus Respirovirus, HN is a type II glycoprotein of 576 amino acids (aa) having at its N terminus a cytoplasmic tail of 35 aa followed by a transmembrane domain of 25 aa (2). HN has two functions. On one hand, it binds to the sialic acid-containing sugars on cellular glycoproteins, allowing the specific attachment of the virus particle on the cell surface. In this turn triggers the fusion of the viral envelope with the cell membrane effectuated by the F protein. On the other hand, HN contains a neuraminidase (NA) activity, which, by analogy with the influenza virus NA, is likely to allow detachment of the viral particles at the end of the life cycle. HN protrudes from the viral envelope (and is expressed at the cell surface) as a homotetramer composed of two homodimers in which the monomers are covalently linked by disulfide bonds (1,3,4). SeV HN protein shares these structural features with the other members of the family. The C-terminal portion of HN forms a globular head that is the site of its biological activities (receptor binding and cleavage), and the N-terminal tail contains the signals for HN incorporation into cellular and viral membranes. Between the membrane anchor and the globular head is a stalk region of 80 to 90 aa containing the cysteines that covalently link the dimers (5,6).

The cytoplasmic tail (c) of HN is thought to be responsible for its incorporation into viral particles (7–11). For SeV HN, an SYWST motif situated at aa 10 to 14 from the N terminus was found to be essential for this incorporation. Its deletion or mutation resulted in viral particles depleted of HN (7,12). This surprising result also highlighted the dispensability of HN for viral particle production. This was known for some time due to studies of a thermosensitive mutant (ts271) (13,14) in which HN was degraded at nonpermissive temperatures before reaching the cell membrane (15,16). The finding was then confirmed with the replacement of the SYWST motif by AFYKD, a motif present at the same position of the measles virus (MeV) H protein (7). This finding was revisited more recently using small interfering RNA (siRNA) suppression of HN expression (8). In that study, in an effort to better describe the mechanism of HN incorporation in viral particles, a chimeric protein consisting of the SeV HN cytoplasmic tail and transmembrane domains (hn-ct) fused to the MeV H ectodomain was ectopically expressed. Surprisingly, when SeV HN<sub>AFYKD</sub> is normally excluded from SeV particles, was coexpressed with the chimeric MeV hn<sub>ct</sub>H protein, HN<sub>AFYKD</sub> was recovered in virus particles (8).

The present study was devoted to understanding the mechanism of this transcomplementation. To this effect, hn-ct, plus increasing extensions of the ectodomain (hn-cte), was fused to green fluorescent protein (GFP) and expressed from a supplemental transcription unit of the SeV genome, which in addition expressed HN<sub>AFYKD</sub>. The analysis of the rescued recombinant viruses (rSeV) showed that as soon as the ectodomain portion in the fused hn-cte-GFP protein extended past 80 amino acids, the GFP open reading frame (ORF) was systematically interrupted, leaving expression of an hn-cte capable of bringing the HN<sub>AFYKD</sub> into virus particles. This was achieved by formation of hetero-oligomers (hn-cte-HN<sub>AFYKD</sub>) dependent on cysteine residues at positions...
129 and 138. In this case, then, hn-c-te was found in virus particles as well, along with HN_{AFYKD}.

**MATERIALS AND METHODS**

**Cells and virus infection.** LLC-MK2 cells were grown at 37°C under a 5% CO\(_2\) atmosphere in Dulbecco's modified Eagle medium (DMEM) (Gibco-Invitrogen) supplemented with penicillin, streptomycin, and 5% fetal calf serum (FCS) (Brunswhig). BSRT7 cells were provided by K. K. Conzelmann (17) and grown under the same conditions except for the medium (BHK-21 Glasgow minimum essential medium [GMEM]; Gibco-Invitrogen). They were treated with 0.5 \(\mu\)g/ml of Genetecin (Gibco) once every two passages. During infection, cells were incubated for 1 h at 33°C with virus appropriately diluted in basic salt solution (BSS). After adsorption, virus-containing BSS was removed; cells were rinsed and incubated for 24 to 48 h at 33°C in 2% FCS–DMEM. Cells were lysed in lysing buffer II (150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, and 50 mM Tris-HCl, pH 8.3). Viral particles in the cell supernatants were pelleted through a 25% glycerol–Tris–NaCl–EDTA cushion at 13,000 rpm at 4°C for 60 min and resuspended in appropriate buffers (see the figure legends).

**Constructions of full-length cDNA plasmids.** SeV full-length cDNA genomes were generated by in-house standard molecular cloning strategies using an F15 backbone as previously described (18). Mutations in the HN gene (SYWST motif or cytoeine at positions 69 and 78) were introduced by site-directed mutagenesis of the insert flanked by two unique restriction sites: ClaI (in the F gene) and Apai (in the L gene). Supplemental genes were inserted in the unique MluI restriction site flanking the gene start and end sequences of an additional transcription unit inserted between the M and F genes. Schematic representations of the rSeV genomes prepared in this study are shown in Fig. 1A, 3A, 4A, and 7A. All endogenous HN genes encoded a protein carrying a hemagglutinin (HA) tag at the C terminus, as has been described previously (7) (Fig. 1B). All prepared rSeV cDNAs were sequenced, and they all obey the rule of six (19).

**Rescue and stock preparation of recombinant viruses.** Mutated and chimera rSeVs were rescued as previously described (18). Briefly, BSRT7 cells were transfected simultaneously with full-length genome cDNA constructs and three pTM1-based plasmids expressing the N, P/Gatop, and L viral proteins. At 48 h posttransfection, cells were rinsed, treated with acetylated trypsin (1.5 \(\mu\)g/ml, 15 min, 33°C), and further incubated for 24 h at 33°C. Finally, about 2 million cells were injected into the allantoic cavities of 9-day-old embryonated chicken eggs. After 72 h of incubation at 33°C, allantoic fluids (AF) were collected and served as viral stocks. Viral particles were pelleted from 1 ml AF and analyzed by SDS-PAGE and Coomassie blue staining to monitor the presence of virus particles. After incubation with appropriate antibodies and the corresponding anti-mouse or anti-rabbit horseradish peroxidase (HRP)-coupled secondary antibodies (Bio-Rad), proteins were detected using ECL substrate (PNR 2106 ECL Western blotting detection system [Amersham] or hyper-sensitive ECL [Pierce]) and a Fujifilm LAS-4000 development system.

**Isotopic radiolabeling of cells.** Pulse-chase radiolabeling assays were performed at 24 h postinfection. Cells were deprived of methionine, serine, and FCS for 30 min at 37°C and then pulse-labeled for 10 or 20 min with 300 \(\mu\)Ci/ml of \[^{35}S\] labeled methionine and cysteine (Pro-mix-[\(^{35}\)S]; Amersham Biosciences). Cells were then either collected or chased for 90 min in DMEM supplemented with 10 mM cold methionine or cysteine. \[^{35}\]S-labeled proteins in cellular extracts or viral particles were immunoprecipitated using appropriate antibodies or directly analyzed. Prolonged [\(^{35}\)S]methionine-cysteine radiolabeling assays were performed from 16 to 24 h postinfection. Infected cells were incubated in FCS-free medium containing 1/10 the amount of cold methionine and cysteine and 30 \(\mu\)Ci/ml of [\(^{35}\)S]methionine and [\(^{35}\)S]cysteine at 33°C. Finally, the \[^{35}\]S-labeled samples were resuspended in sample buffer (under reducing or nonreducing conditions) and analyzed by SDS-PAGE. The proteins were detected in the dried gel using a Typhoon FLA 7000 phosphorimager (GE Healthcare).

**Immunofluorescence staining and confocal microscopy.** Infected LLC-MK2 cells were seeded on sterilized coverslips coated with polylysine (Sigma). Twenty-four hours later, cells were buffered with 20 mM HEPES (pH 7.5) in DMEM and fixed for 15 min at room temperature with 4% paraformaldehyde in \(H_2O\), pH 7.3 (PFA). The nuclei were stained with DAPI (4',6'-diamidino-2-phenylindole) (Boehringer Mannheim GmbH). Cells were mounted in Fluoromount-G (Southern Biotech) and analyzed with an LSM510 (Carl Zeiss) confocal microscope via a 63×/1.4 oil immersion objective. Acquisition, analysis, and treatment imaging were performed using the Zeiss LSM Image Browser.

**RESULTS**

The cytoplasmic and transmembrane domains of HN are not sufficient to promote HN_{AFYKD} incorporation into viral particles. As Sendai virus (SeV) HN protein is dispensable for virion production, its incorporation into particles appears to be independent of the steps needed to form these particles. In attempts to clarify the mechanism underlying this incorporation, we have previously shown that ectopic expression of the cytoplasmic and transmembrane domains of HN_{WT} was apparently sufficient for the uptake into viral particles of a HN_{AFYKD}, that was otherwise excluded (see the introduction) (8). In an attempt to clarify the mechanism underlying this transcomplementation, we generated various rSeVs which expressed green fluorescent protein (GFP) fused to HN cytoplasmic and transmembrane domains (hn-ct). In
some cases, this included increasing lengths of amino acids from the HN ectodomain proximal to the transmembrane domain (Fig. 1, hn-cte’s).

Figure 2 presents our first attempts to rescue HN_{AFYKD} incorporation by coexpression in trans of hn-ct or hn-cte fused to GFP. Three ectopic HN domains were tested: (i) the cytoplasmic tail in its wt configuration plus the transmembrane domain (Fig. 1, hn-ct-GFP), the same two domains with the AFYKD mutation (hn-cte_{AFYKD}-GFP), and the same two domains in the wt configuration plus 26 aa of the ectodomain proximal to the membrane (hn-cte_{26}-GFP). No differences were noted in the levels of the viral proteins detected in cells infected with the various viruses indicated (Fig. 2A, IC); that is, HN was uniformly expressed. The analysis of the viral particles produced shows that HN_{AFYKD} levels were highly reduced compared to those of HN_{wt} (Fig. 2A, VP, lane 2 and lanes 3 to 6) in all cases. Figure 2A (IC, middle panel, lanes 4 to 6) shows the level of hn-ct expression detected with antibodies raised against a peptide from the HN cytoplasmic tail (Fig. 1B, /H9251-HNc). It appears that addition of the 26 aa of the ectodomain favors the expression of the chimeric GFP (Fig. 2A, IC, middle panel, lane 5); this is detected as well with /H9251-GFP (Fig. 2A, lower panel, lane 5). When cell surface immunoprecipitations were performed (Fig. 2B, S lanes), no sign of cell surface expression of the chimeric GFP proteins was obtained (/H9251-GFP lanes). This contrasts with the efficient cell surface expression of
HNwt and HNAFVKD (α-HA lanes), confirming previous results (7, 8). GFP expressed alone (Fig. 2B, GFP/HNAFVKD and α-GFP lanes) is found exclusively in total cell immunoprecipitates, and this validates the technique of total cell versus cell surface immunoprecipitation.

Confocal microscopy images of the hn-ct-GFP constructs failed as well to show the characteristic ring of cell surface-expressed proteins (Fig. 2D); the GFP staining appears to be more consistent with a perinuclear endoplasmic reticulum (ER) localization. Pulse-chase35S labeling (Fig. 2C) shows that the three chimeric GFP proteins are unstable compared to unmodified GFP and confirms the more efficient synthesis of hn-cte26-GFP. In conclusion, ectopic expression of hn-ct's fused to a virally irrelevant protein (GFP) could not restore the HNAFVKD uptake in viral particles.

Approximately 80 amino acids of the HN ectodomain are needed for HNAFVKD incorporation into virus particles. Since the addition of a portion of the ectodomain (e26) was beneficial for the level of expression of the chimeric protein, as well as for a possible endoplasmic reticulum (ER) localization (Fig. 2D), new constructs were prepared in which the ectodomain was extended to 80 and 130 aa (Fig. 3A, hn-cte80-GFP and hn-cte130-GFP). Analysis of the cellular extracts and viral particles produced after infection with these new rSeVs showed that, in contrast to the finding for hn-cte26-GFP (repeated here), coexpression of hn-

### TABLE 1

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* Schematic descriptions of these viruses can be found in Fig. 1A, 3A, 5B, 6A, 7A, and 8A.

Successful rescues/total independent rescue attempts. * virus obtained with no GFP expression due to the insertion of stop codons in ORF.

Number of serial egg-to-egg passages to obtain a high-titer virus stock.

PFU/ml of the allantoic fluid stock.

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**FIG 2** First attempt to rescue incorporation of HNAFVKD in SeV particles. LLC-MK2 cells were infected with the indicated viruses described in Fig. 1A. (A) Western blot analysis of the cellular extracts (IC) and viral particles in the cell supernatants (VP) probed with α-HN(1-255), M(1-250), N(1-155), and F(1-250) (upper panel), α-HNc (middle panel), and α-GFP (lower panel) antibodies. GFP (black arrowhead) and hn-ct-, hn-cte-, and hn-cte26-GFP proteins (*) are indicated. (B) Infected cells were radiolabeled with [35S]methionine and cysteine from 16 to 24 h postinfection. Surface (S) and total (T) immunoprecipitations (IP) were performed using α-GFP or α-HA as indicated. Lane V, 35S-SeV labeled viral proteins loaded as protein markers. Immunoprecipitated GFP (green arrow) and hn-ct- or hn-cte-GFP proteins (*) are indicated. (C) Eighteen hours postinfection, LLC-MK2 cells infected with the indicated viruses were radio-pulse-labeled for 10 or 20 min to control synthesis. After the 20-min pulse, the cells were further chased for 90 min. The GFP-containing proteins were then immunoprecipitated using α-GFP and directly analyzed by PAGE and detected by autoradiography. (D) LLC-MK2 cells grown on coverslips were infected with the rSeVs expressing the hn-ct- and hn-cte26 fused to GFP as described for panel A. At 24 hours postinfection, the cells were fixed, stained with DAPI, and observed by confocal microscopy. GFP proteins, green; nuclei, blue.
ct80-GFP and hn-cte130-GFP was associated with increased levels of HN\textsubscript{AFYKD} in virus particles (Fig. 3B, VP, upper panel, lane 4 and lanes 5 and 6). These levels could reach about 60% of normal (Fig. 3C). Surprisingly, the detailed analysis of the construct expression failed to reveal the presence of the new chimeric proteins. For instance, in Fig. 3B (upper left panel), hn-cte26-GFP is clearly visible above the M protein at its expected molecular mass (lane 4, *), but in lanes 5 and 6, no visible bands are detected at positions where hn-cte80- and hn-cte130-GFP should migrate (above the band in lane 4). In the middle panel, however, two bands were found at about 15 to 20 kDa, a much smaller molecular mass than expected (lanes 5 and 6, ●). When probed with α-GFP (Fig. 3B, lower panel), these two bands were not detected, compared to free GFP or hn-cte26-GFP (lanes 2, 3, and 4 [*] compared to lanes 5 and 6). Finally, we note that two smaller bands are detected in virus particles when probed with α-HNc (Fig. 3B, VP, middle panel, lanes 5 and 6, ●). In conclusion, uptake of HN\textsubscript{AFYKD} in viral particles can be partially restored by coexpression of chimeric GFP containing an HN ectodomain longer than 26 aa.

Normally, HN is found at the cell surface in the form of a homotetramer composed of two dimers in which the monomers are linked by disulfide bonds, which are visible when analyzed under nonreducing (NR) conditions. Figure 4A shows the results of such an analysis using immunoprecipitates of the samples presented in Fig. 3B. The expected forms (HN monomer, HN\textsubscript{2}, and HN\textsubscript{2-HN\textsubscript{2}}) were readily detected, either in total cell or in cell surface immunoprecipitates, for HN\textsubscript{wt} (lanes GFP/HN\textsubscript{wt}), for HN\textsubscript{AFYKD} (lanes-GFP/HN\textsubscript{AFYKD}), or for hn-cte26-GFP. However, the profile obtained from cells infected with hn-cte80-GFP or hn-cte130-GFP clearly differed by exhibiting a more complex pattern, namely, by a distinct band between the HN monomer (HN) and dimer (HN\textsubscript{2}) and by multiple bands between the dimer and the tetramer (HN\textsubscript{2}-HN\textsubscript{2}) (Fig. 4A, ?). Similar results were obtained when a direct analysis of HN (detected by α-HNc) present in the viral particle was made (Fig. 4B, NR). HN dimers and tetramers were readily detected for HN\textsubscript{wt} (in this case, HN monomer is not present), and other forms were detected in lanes hn-cte80- and hn-cte130-GFP (marked with a question mark). This pattern was better resolved with the use of 17.5% PAGE and a more sensitive ECL (Fig. 4C, NR). In particular, we note two forms migrating faster than the N protein (●), which mirror the two small bands detected under reducing conditions (Fig. 4C, R, ●) and in Fig. 3B.

**FIG 3** Second attempt to rescue incorporation of HN\textsubscript{AFYKD} in SeV particles. (A) Schematic representation of rSeV viruses encoding hn-cte26-GFP, hn-cte80-GFP, and hn-cte130-GFP. Note that the hn-cte-GFPs are all in the wt (SYWST) configuration and that the endogenous HN is in its mutated (AFYKD) one. The same color code as for Fig. 1A is used. (B) LLC-MK2 cells were infected with the indicated viruses, and Western blot analysis of the cellular extracts (IC) and the viral particles in the cell supernatant, probed with the indicated antibody preparations, are presented. *, hn-cte26-GFP protein detected with α-HNc, α-HNc, and α-GFP antibodies at the expected molecular mass (all panels). α-HNc recognizes both full HN and GFP HN tails, ?, potential hn-cte80-GFP and hn-cte130-GFP proteins detected using α-HNc antibody but with an unexpected molecular mass (middle panel). (C) HN\textsubscript{AFYKD} incorporation in VP after infections with the indicated viruses, quantified (using ImageJ software) in relation to N and normalized to incorporation of wild-type HN (lane 2 of panel B) taken as 100%. Data were obtained from three separate experiments.
The complexity of the HN oligomer pattern observed upon infection by rSeV-hn-cte80-GFP and hn-cte130-GFP prompted us to verify the sequences of these viral transgenes (Fig. 5A). Remarkably, in both cases a stop codon that prevented translation of the GFP protein had been introduced. For the hn-cte80-GFP gene, this stop codon created an hn-cte protein carrying an ectodomain of 80 aa with an additional 4 aa of GFP. In the case of the hn-cte130-GFP, the stop codon arrested the ectodomain translation at aa 98 (Fig. 5B). These findings are consistent with the sizes of the proteins detected in Fig. 3 and 4 and with the lack of reactivity with /H9251-GFP antibody. In the end, rescue of HNAFYKD incorporation into viral particles was achieved with coexpression in trans of wt-hn-cte domains where the ectodomain was at least 80 aa long. These hn-cte’s were likely incorporated in viral particles (Fig. 3B and 4B) and were likely responsible for the complex pattern of HN isoforms found under nonreducing conditions, either at the cell surface (Fig. 4A) or in viral particles (Fig. 4B), as they form heterodimers with HNAFYKD. This would explain the complexity of the HN pattern observed in Fig. 4. For example, the band above HN in Fig. 4A could represent an oligomer (HN-hn-cte). Similarly, the two bands below N in Fig. 4C (NR, ●) could represent homodimers of hn-cte’s as seen in Fig. 4C (R, ●), suggesting that these hn-cte’s are indeed incorporated in viral particles.

Importance of Cys129 and Cys138 for rescue of HNAFYKD incorporation into virus particles. Remarkably, the counterselection of the GFP protein expression in rSeV-hn-cte80-GFP and -hn-cte130-GFP resulted in retaining an HN ectodomain longer than 80 amino acids in both cases. At positions 129 and 138 of the ectodomain (Fig. 1B), two cysteines are found. We therefore examined the possibility that formation of disulfide-bound heterodimers constituted the driving force for retaining this ectodomain length and that the ability to form HN oligomers had

**FIG 5** Sequence comparison of hn-cte-GFPs in plasmids and rescued viruses. (A) Viral RNA was recovered from recombinant virus particles, and the genes encoding hn-cte80-GFP and hn-cte130-GFP were reverse transcribed (RT) using a primer of positive polarity positioned in the 5’ extremity of the M gene. The RT products were then amplified by PCR using primers flanking the additional transcription units carrying the hn-cte-GFP genes situated between the M and F genes. RT-PCR products were sequenced and compared to the sequence of the original cDNA used for virus rescue. Highlighted nucleotides in gray indicate the G-to-T and the G-to-A substitutions leading to the insertion of stop codons in the hn-cte80-GFP and hn-cte130-GFP ORFs, respectively. (B) Schematic representation of hn-cte80-GFP and hn-cte130-GFP proteins theoretically expressed by the full-length cDNA plasmid used for virus rescue (plasmid) and the proteins actually expressed by the recovered viruses (virus).
selected for the closing of the chimeric GFP ORFs. New rSeVs were therefore generated (Fig. 6A), which simply expressed hn-cte's with an 80-aa-long ectodomain, both in the wt (hn-cte80) and AFYKD (hn-cmte80) configurations, as transgenes. Moreover, cysteines 129 and 138 were alternatively replaced by serines. The viruses were tested with their appropriate controls expressing HNwt or HNAFYKD. In the cellular extracts, the viral proteins, namely, HN, were equally expressed (Fig. 6B, IC). For virus particles (VP), lanes 1 and 2 serve as controls, showing normal and restricted HN incorporation (Fig. 6D). We found that HN was recovered to a significant level only when hn-cte was in its wt configuration (Fig. 6B, lane 3, and D). Cysteine-to-serine substitutions had a negative effect on this rescue (Fig. 6B, lane 4). As for hn-cmte, where AFYKD replaces SYWST, HN uptake in viral particles was minimal if not undetectable (Fig. 6B, lanes 5 and 6, and D). We note the presence in Fig. 6B (VP, lanes 3 and 4) of a small band in VP samples corresponding to hn-cte60. Analysis performed under nonreducing conditions showed evidence of hn-cte involvement in heterodimer (HN-hn-cte) formation only when the 2 cysteines were present (Fig. 6C, IC, lanes 3 and 5, and NR, *). In addition, hn-cte was present in immunoprecipitations obtained with α-HA (Fig. 6C, IC, lanes 3 and 5, R, ●), and evidence of mixed oligomer (HN-hn-cte) correlated with HNAPYKD incorporation in viral particles only when hn-cte was wild type (Fig. 6C, VP, lane 3, *). hn-cte dimer (hn-cte2) was formed only with the cysteine-containing hn-cte (Fig. 6C, VP, compare lanes 3 and 4, lower panel, *).

In summary, these data show that one or both of the two cysteines are engaged in disulfide-bound oligomer formation (likely hn-cte-HNAPYKD) resulting in incorporation of HNAPYKD in viral particles. Replacement of cysteines with serines reduced HNAPYKD uptake, possibly by weakening the hn-cte-HNAPYKD interaction (Fig. 6B and C, VP, lane 4).

Cysteines 129 and 138 are not essential for HNwt incorporation in SeV particles. We next examined the role of the two cysteines, namely, the ability to form covalently linked dimers, in the normal situation where integral HNwt are incorporated into virus particles. A new rSeV was produced where the endogenous HNwt carried the two serine substitutions in place of the two cysteines (S2) (Fig. 7A). Clearly, S2 substitutions had no detrimental effect on HN incorporation in virus particles (Fig. 7B, VP, lanes 1 and 3, and C). Remarkably, this normal incorporation takes place in the apparent absence of homodimers and homotetramers at the infected cell surface and in virus particles, detectable by PAGE under nonreducing conditions (Fig. 7B, NR, IC, and VP). The two cysteines (or one of the two) are thus required for formation of detectable HN dimers and tetramers. However, they are not essential for HNwt incorporation in virus particles.

Incorporation of MeV H in SeV particles. We previously reported that MeV H expressed in the context of rSeV-HA-HNAPYKD infection was not incorporated into virus particles, even
when MeV H carried hn-ct in place of its own domains (8). In those experiments, MeV H was expressed from minigenomes amplified in mixed virus stocks, a procedure that cannot ensure the same level of expression as that from the nondefective genome. Based on the present results, however, hnct-H would be expected to be incorporated, especially since formation of disulfide-linked dimers appears not to be essential. We therefore prepared rSeVs carrying as supplemental genes MeV H or MeV hnct-H (Fig. 8A) so that MeV H expression now corresponded to that of the other SeV proteins. Infected cells were 35S labeled, and MeV H was recovered by immunoprecipitations from cellular extracts and from purified viral particles (Fig. 8B, lower panel). Figure 8B also shows expression of intracellular HN wt by Western blotting (IC, middle panel, lane 1) or HNAFYKD (IC, middle panel, lanes 2 to 4). In the right part of Fig. 8B (VP), at the position of the missing HN A FYKD, a faint protein band is visible upon HN wt expression, and a strong protein band is detected in the case of hnct-H expression (upper panel, lanes 3 and 4, respectively). Probing the viral particle proteins with an α-HA antibody showed that these proteins were not HNAFYKD (middle panel, lanes 2 to 4), and specific α-H immunoprecipitations confirmed that these proteins were MeV H (lower panel, lanes 3 and 4). Thus, quantitative incorporation of MeV H into SeV particles takes place when the MeV Hct domains are replaced by those of SeV-HN. Remarkably, enough residual incorporation of HN occurred so that virus expressing HNAFYKD could be rescued (albeit after 4 serial passages in embryonated eggs). rSeV-HNAFYKD cDNA was constructed in three different full-length cDNA clone backbones by two different researchers, and this led to the recovery of three virus preparations exhibiting the same phenotype. Despite the four serial passages consistently required for virus rescue, deep sequencing of the virus obtained and used in this study showed no sequence differences relative to the wt reference sequence other than those introduced (unpublished data). The availability of rSeV-HNAFYKD then made possible the study of the mechanism of HN incorporation in viral particles by defining conditions that complement the HNAFYKD incorporation deficiency. These were, in a way, “naturally selected” when chimeric hn-cte (80/130)-GFP proteins were coexpressed with HNAFYKD and led to the selection of hn-cte domains devoid of the GFP sequence. Moreover, the required length of the ectodomain (e) was found in the same experiments to be at least 80 amino acids. This selection presumably required 5 or 6 serial passages to recover hn-cte’s lacking the GFP ORF (Fig. 5; Table 1), since the recovery of virus expressing hn-cte60 alone was achieved after a single egg passage (i.e., similar to the case for wt virus). Thus, hn-cte60 provided an SYWST-containing cytoplasmatic tail and two cysteines at the distal end of the ectodomain (position 129 and 138), allowing disulfide linkage between hn-cte’s and HNAFYKD. In the end, one SYWST-containing cytoplasmatic tail appears to be sufficient to recruit an otherwise deficient HN for

**DISCUSSION**

The 10SYWST14 amino acid motif in the SeV HN cytoplasmic tail was recognized as essential for HN incorporation in SeV particles. Replacement of SYWST with the corresponding sequence A FYKD present in MeV H made HN highly defective for incorporation into virus particle (20- to 50-fold reduction). Remarkably, enough residual incorporation of HN occurred so that virus expressing HNAFYKD could be rescued (albeit after 4 serial passages in embryonated eggs). rSeV-HNAFYKD cDNA was constructed in three different full-length cDNA clone backbones by two different researchers, and this led to the recovery of three virus preparations exhibiting the same phenotype. Despite the four serial passages consistently required for virus rescue, deep sequencing of the virus obtained and used in this study showed no sequence differences relative to the wt reference sequence other than those introduced (unpublished data). The availability of rSeV-HNAFYKD then made possible the study of the mechanism of HN incorporation in viral particles by defining conditions that complement the HNAFYKD incorporation deficiency. These were, in a way, “naturally selected” when chimeric hn-cte (80/130)-GFP proteins were coexpressed with HNAFYKD and led to the selection of hn-cte domains devoid of the GFP sequence. Moreover, the required length of the ectodomain (e) was found in the same experiments to be at least 80 amino acids. This selection presumably required 5 or 6 serial passages to recover hn-cte’s lacking the GFP ORF (Fig. 5; Table 1), since the recovery of virus expressing hn-cte60 alone was achieved after a single egg passage (i.e., similar to the case for wt virus). Thus, hn-cte60 provided an SYWST-containing cytoplasmatic tail and two cysteines at the distal end of the ectodomain (position 129 and 138), allowing disulfide linkage between hn-cte’s and HNAFYKD. In the end, one SYWST-containing cytoplasmatic tail appears to be sufficient to recruit an otherwise deficient HN for
incorporation, providing that a covalently linked heterodimer can form. In some way, GFP must interfere with the proper function of the dimer formed, or its transit through the exocytosis pathway is counterselected. This counterselection does not take place in hn-cte26-GFP, presumably, because this protein does not have the possibility to form a heterodimer with HNAFYKD.

In the end, the present study shows transcomplementation by hn-cte of HNAFYKD uptake in viral particles. This complementation takes place by hn-cte dragging HNAFYKD so that both partners end up in the particles. This result differs from that reported before, where transcomplementation was proposed to take place without detection of the hn-cte-carrying protein (in that case, MeV hnctH) in virus particles (8). In view of the selection of proper hn-cte from hn-cte-GFP described in this study, we propose that a similar hn-ct emerged from hnctH and went undetected. Although this did not occur in the present study (see Fig. 8), the experimental approaches are sufficiently different to support the two different issues. In the 2010 study, the MeV hn_H was encoded by an SeV minigenome, and this makes possible the production of the complementing hn-ct by only a fraction of minigenomes, while the remaining still produced MeV hn_H, albeit in a reduced amount. This could explain the transcomplementation and lack of detection of hn_H in viral particles. Alternatively, the data obtained previously could have resulted from selection of AFYKD motif revertants that have later been obtained (unpublished data). These putative explanations have been excluded in the present study by systematic sequencing of the obtained viruses. In addition, hn_H was well represented in viral particles and showed no ability to restore HNAFYKD incorporation (see Fig. 8).

The requirement for the covalently linked dimers appears not to apply when each monomer harbors the SYWST recruiting signal, as HNwt carrying the S2 substitutions (Fig. 7A) was equally incorporated in viral particles. It is interesting that the disulfide bonds are not required for HN insertion in the plasma membrane, suggesting that the traffic of HN through the exocytic pathway does not require formation of HN oligomers. We find it more surprising that the absence of covalently linked HN dimers does not have a larger overall effect on the virus life cycle but is restricted to the appearance of a small-plaque phenotype, accompanied by only a minimal loss of titer (Fig. 9 and Table 1). In this case, it is not clear whether oligomerization is totally dispensable or whether an oligomer forms that is not stable enough to be detected.

This work also provides information about the need for HN during the virus life cycle. We confirmed in this study that rSeV stocks reaching titers reduced only to a third of those of wt viruses (Table 1) (7) can have 20- to 50-fold less HN on the particles. We also show that the price to pay is a decreased infectivity, as revealed by the small-plaque phenotype of HNAFYKD viruses in titration assays (Fig. 9 and Table 1). We have evidence that the different multiplication steps develop at the same rate (including production of virus particles). It would therefore appear that virus depleted of HN is penalized during the attachment step, where receptor binding is less efficient. Alternatively, HN depletion on the
viral envelope may limit F/HN interactions required to regulate fusion activation (23).

This work has added to our understanding of HN uptake in virus particles. Since HN_{APYKK}, is transported normally to the cell surface, where it accumulates in amounts generally higher than that of HN_{AP}, it is likely that it is at the cell surface that HN is recruited to the assembly complex that participates in virus budding. This raises the question (not addressed in this work) of the mechanism of this recruitment. The simplest explanation would involve an interaction of the HN cytoplasmic tail (via the SYWST motif) with another viral protein. The protein of choice should be M, as numerous studies have shown indirect evidence of M-HN association (reviewed in references 24, 25, and 11). This mechanism has not yet been examined and will be part of another study.

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