

Morbillivirus and henipavirus attachment protein cytoplasmic domains differently affect protein expression, fusion support and particle assembly

Bevan Sawatsky,^{1,2,3,4} Dennis A. Bente,^{2,3} Markus Czub⁵ and Veronika von Messling^{1,4}

Correspondence

Veronika von Messling
veronika.vonmessling@pei.de

¹INRS-Institut Armand-Frappier, University of Quebec, Laval, Quebec, Canada

²Department of Microbiology & Immunology, University of Texas Medical Branch, Galveston, Texas, USA

³Galveston National Laboratory, University of Texas Medical Branch, Galveston, Texas, USA

⁴Veterinary Medicine Division, Paul-Ehrlich-Institute, Langen, Germany

⁵Faculty of Veterinary Medicine, University of Calgary, Calgary, Alberta, Canada

The amino-terminal cytoplasmic domains of paramyxovirus attachment glycoproteins include trafficking signals that influence protein processing and cell surface expression. To characterize the role of the cytoplasmic domain in protein expression, fusion support and particle assembly in more detail, we constructed chimeric Nipah virus (NiV) glycoprotein (G) and canine distemper virus (CDV) haemagglutinin (H) proteins carrying the respective heterologous cytoplasmic domain, as well as a series of mutants with progressive deletions in this domain. CDV H retained fusion function and was normally expressed on the cell surface with a heterologous cytoplasmic domain, while the expression and fusion support of NiV G was dramatically decreased when its cytoplasmic domain was replaced with that of CDV H. The cell surface expression and fusion support functions of CDV H were relatively insensitive to cytoplasmic domain deletions, while short deletions in the corresponding region of NiV G dramatically decreased both. In addition, the first 10 residues of the CDV H cytoplasmic domain strongly influence its incorporation into virus-like particles formed by the CDV matrix (M) protein, while the co-expression of NiV M with NiV G had no significant effect on incorporation of G into particles. The cytoplasmic domains of both the CDV H and NiV G proteins thus contribute differently to the virus life cycle.

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INTRODUCTION

The paramyxovirus attachment protein plays a critical role in viral receptor interaction and the activation or triggering of its homologous fusion (F) protein. The attachment proteins of different paramyxoviruses have adapted to a wide spectrum of target receptors, including terminal sialic acids on cell surface glycoproteins for mumps and parainfluenza viruses (Villar & Barroso, 2006), as well as proteinaceous receptors such as signalling lymphocyte activation molecule (SLAM; Tatsuo *et al.*, 2000) and nectin-4 (Mühlebach *et al.*, 2011; Noyce *et al.*, 2011) for morbilliviruses, and ephrin-B2 (Bonaparte *et al.*, 2005; Negrete *et al.*, 2005) and ephrin-B3 (Negrete *et al.*, 2006) for henipaviruses.

Detailed structures have been solved for several paramyxovirus attachment proteins, including the haemagglutinin-neuraminidase (HN) proteins of Newcastle disease virus (Crennell *et al.*, 2000), parainfluenza virus type 5 (Yuan *et al.*, 2005) and human parainfluenza virus type 3

(Lawrence *et al.*, 2004), the haemagglutinin (H) protein of measles virus (MeV; Colf *et al.*, 2007; Hashiguchi *et al.*, 2007) and the glycoproteins (G) of Nipah virus (NiV; Xu *et al.*, 2008) and Hendra virus (HeV; Bowden *et al.*, 2010). These structures reveal a common architecture where the β -sheets of the globular head are arranged symmetrically around a central axis as a six-bladed propeller. The attachment protein-mediated initiation of the F protein rearrangement and subsequent membrane fusion seem to be highly conserved, despite widely diverse receptors (Chang & Dutch, 2012). Interaction with the respective cellular receptors induces conformational changes in the attachment protein globular head (Bose *et al.*, 2012; Liu *et al.*, 2013; Navaratnarajah *et al.*, 2011), which are in turn transferred to the stalk region (Bose *et al.*, 2011; Liu *et al.*, 2013, 2015; Navaratnarajah *et al.*, 2012; Yuan *et al.*, 2011). This F-triggering function of the attachment protein can be dissociated from its other functions, such as intracellular transport, association with F and receptor binding, which indicates

that it resides in a distinct region. Indeed, the attachment protein stalk domain is still able to trigger the F protein even when the globular head is removed (Bose *et al.*, 2012, 2014; Brindley *et al.*, 2013; Liu *et al.*, 2013).

Paramyxovirus glycoprotein functions are also regulated by intracellular processes, where the amino-terminal cytoplasmic domain serves as a signal peptide for the translocation of the nascent protein chain into the endoplasmic reticulum (ER) (Spriggs & Collins, 1990), thereby modulating protein maturation and surface transport. While the morbillivirus F and H proteins associate early during biosynthesis in the ER and are transported to the cell surface as a complex (Plempner *et al.*, 2001), the henipavirus F and G proteins are transported independently (Whitman *et al.*, 2009), indicating that differences in interactions between glycoproteins may influence the assembly and cell–cell fusion process. Paramyxovirus HN/H/G and F protein cytoplasmic domains also contain tyrosine motifs, which result in transport of proteins to the basolateral surface of polarized cells (Runkler *et al.*, 2009; Weise *et al.*, 2010). These signals, however, are overridden by the homologous matrix (M) protein, resulting in direction of

the glycoproteins to the appropriate cellular compartment for virus assembly (Dietzel *et al.*, 2011; Naim *et al.*, 2000).

Despite the critical role of the cytoplasmic domain, several paramyxovirus attachment proteins tolerate substantial deletions in this domain without their receptor-binding and fusion support properties being adversely affected (Moll *et al.*, 2002; Porotto *et al.*, 2006). To investigate the contribution of the cytoplasmic domain to the different functional aspects of the attachment protein, we characterized cell surface expression and particle assembly support in a series of NiV G and canine distemper virus (CDV) H glycoprotein mutants with heterologous cytoplasmic domain substitutions or progressive deletions in this region.

RESULTS

The NiV G cytoplasmic domain is required for protein expression

The cytoplasmic domains from diverse paramyxoviruses vary widely in length and sequence (Fig. 1), with no

	Cytoplasmic domain	
<i>Henipavirus</i>		
NiV G	MPAENKKVRFENTTSDKGKIPSKVIKSYGTMDIKKINEGLDLSK	TM boundary
HeV G	MMADSKLVSLNNSLGGKIKDQKGVIKNYGTMDIKKINDGLDLSK	
<i>Morbillivirus</i>		
CDV H	MLSYQDKVGFYKDNARANSKLSLVTEEQGRR	
MeV H	MSPQRDRINAFYKDNPHPKGSRIVINREHLMIDR	
RPV H	MSPPRDRVDAYYKDNFQFKNTRVVLNKEQLLIER	
PPRV H	MSAQRERINAFYKDNPHNKNHRVILDRERLVIER	
<i>Respirovirus</i>		
SeV HN	MDGDRSKRDSYWSTSPGGSTTKLVSDSERSGK	
HPIV1 HN	MAEKGKTNSSYWSTTRNDNSTVNTHTINTPAGR	
HPIV3 HN	MEYWKHTNHGKDAGNELETSMATHNNKLTNK	
<i>Rubulavirus</i>		
MuV HN	MEPSKLFITISDNATFAPGPVNNAADKKTFRCTCFR	
SV5 HN	MVAEDAPVRATCRVLFRR	
HPIV2 HN	MEDYSNLSLKSIPKRTCRIIFRR	
HPIV4 HN	MQDShGNTQILNQANSVMKRTWRLLFR	
<i>Avulavirus</i>		
NDV HN	MDRAVSQVALENDEREAKNTWRLIFRR	

Fig. 1. Variability of paramyxovirus attachment glycoprotein cytoplasmic domains. Cytoplasmic domains of selected viruses from officially classified genera in the subfamily *Paramyxovirinae*. Sequences are shown from the amino-terminus up to the carboxy-terminal transmembrane boundary, indicated on the right. Accession numbers: Nipah virus (NiV) G Malaysia strain, AF212302; Hendra virus (HeV) G, AF017149; canine distemper virus (CDV) H 5804P strain, AY386316; measles virus (MeV) H IC-B strain, NC_001498; rinderpest virus (RPV) H Kabete O strain, NC_006296; peste-des-petits ruminants virus (PPRV) H Tu/00 strain, NC_006383; Sendai virus (SeV) HN Ohita strain, NC_001552; human parainfluenza virus type 1 (HPIV1) HN Washington/1964 strain, AF457102; human parainfluenza virus type 3 (HPIV3) HN, NC_001796; mumps virus (MuV) HN Jeryl Lynn strain, AF201473; parainfluenza virus 5 (PIV5) HN W3A strain, NC_006430; human parainfluenza virus type 2 (HPIV2) HN Toshiba strain, NC_003443; human parainfluenza virus type 4 (HPIV4) Toshiba strain, M34033; and Newcastle disease virus (NDV) HN LaSota strain, AF077761.

obvious common structural elements. To evaluate the extent of functional complementarity, we exchanged the cytoplasmic domains between the CDV H and NiV G glycoproteins (Fig. 2a). The parental FLAG-tagged CDV H and NiV G proteins were readily detected by Western blot, as was the chimera CDV H/Gcyt, whereas the NiV G/Hcyt chimera was very poorly expressed (Fig. 2b). To quantitatively compare the overall and cell surface expression levels of the chimeric glycoproteins, transfected cells were permeabilized or left untreated, stained with the anti-FLAG antibody and analysed by flow cytometry. The expression of the mutant CDV H protein bearing the NiV G cytoplasmic domain (CDV H/Gcyt) was comparable to that of the parental CDV H protein in both cases (Fig. 2c, d). However, the chimera NiV G/Hcyt was again very poorly expressed and was not detected on the cell surface (Fig. 2c, d), which confirms the Western blot results (Fig. 2b), indicating that the NiV G cytoplasmic domain plays a much more important role in protein expression or stability than the corresponding region of CDV H.

NiV G cannot tolerate exchange of its cytoplasmic domain

Since the fusion-triggering function of paramyxovirus attachment glycoproteins resides in the extracellular domain, fusion support is dependent on correct expression of functional protein on the cell surface (Bose *et al.*, 2012; Liu *et al.*, 2013; Navaratnarajah *et al.*, 2011). We therefore assessed the fusion support function of the parental and cytoplasmic domain exchange mutants in a quantitative fusion assay with the homologous or heterologous F proteins. While CDV H/Gcyt induced levels of fusion similar to those of the parental CDV H protein (Fig. 3a, b), the mutant NiV G/Hcyt protein induced virtually no fusion when it was co-expressed with the homologous NiV F protein (Fig. 3a, c), which is consistent with its low level of surface expression (Fig. 2d). The CDV glycoproteins induced much higher levels of fusion in VerodogSLAMtag cells than the NiV glycoproteins when the absolute levels of luminescence produced in the assay were compared (Fig. 3b, c). VerodogSLAMtag cells stably express high levels of the CDV receptor canine SLAM, but only

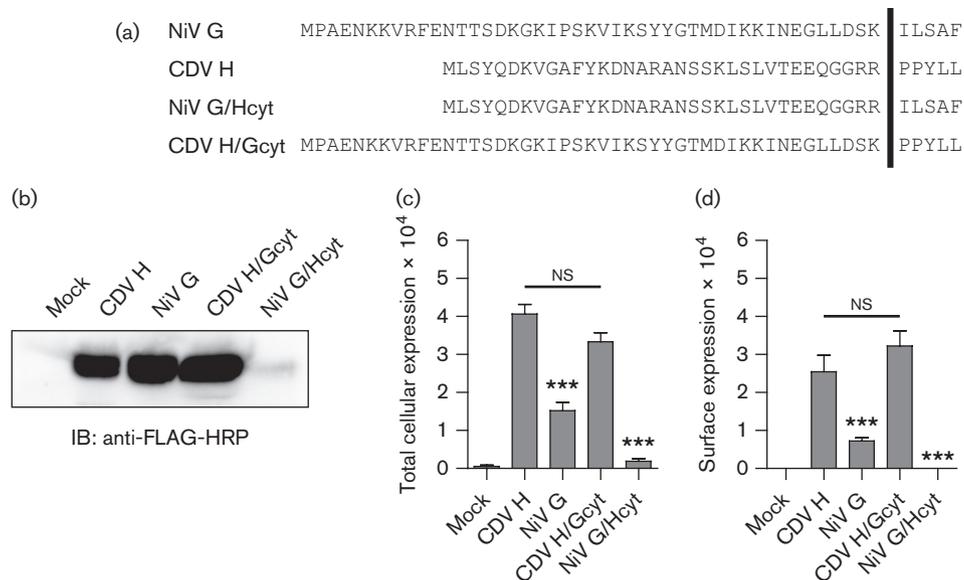


Fig. 2. Expression of chimeric glycoproteins with heterologous cytoplasmic domains. (a) Chimeric glycoproteins constructed for this study. NiV G and CDV H are shown, as are NiV G with the CDV H cytoplasmic domain (NiV G/Hcyt), and CDV H with the NiV G cytoplasmic domain (CDV H/Gcyt). The vertical bar represents the boundary between the cytoplasmic and transmembrane domains. (b) Detection of CDV H, NiV G and the chimeric glycoproteins CDV H/Gcyt and NiV G/Hcyt by Western blot. 293T cells were transfected with control expression plasmids or the respective mutants, and were lysed 48 h after transfection. Proteins were detected with an anti-FLAG M2 antibody directly coupled to HRP. (c, d) Cell surface and total cellular expression of CDV H, NiV G and the chimeric glycoproteins CDV H/Gcyt and NiV G/Hcyt. 293T cells transfected with control expression plasmids and the respective mutants were either permeabilized for total protein (b) or fixed for cell surface (c), then stained with anti-FLAG M2 antibody followed by anti-mouse Alexa Fluor 488 secondary antibody. The mean fluorescence intensities for positively stained cell populations are shown. Bars on graphs represent the mean of a sample group and error bars represent SEM. Groups were analysed by one-way ANOVA with Tukey post-test. *** $P < 0.001$; NS, not significant. Statistical significance is indicated on the graphs compared to CDV H cellular expression in (b) and surface expression in (c).

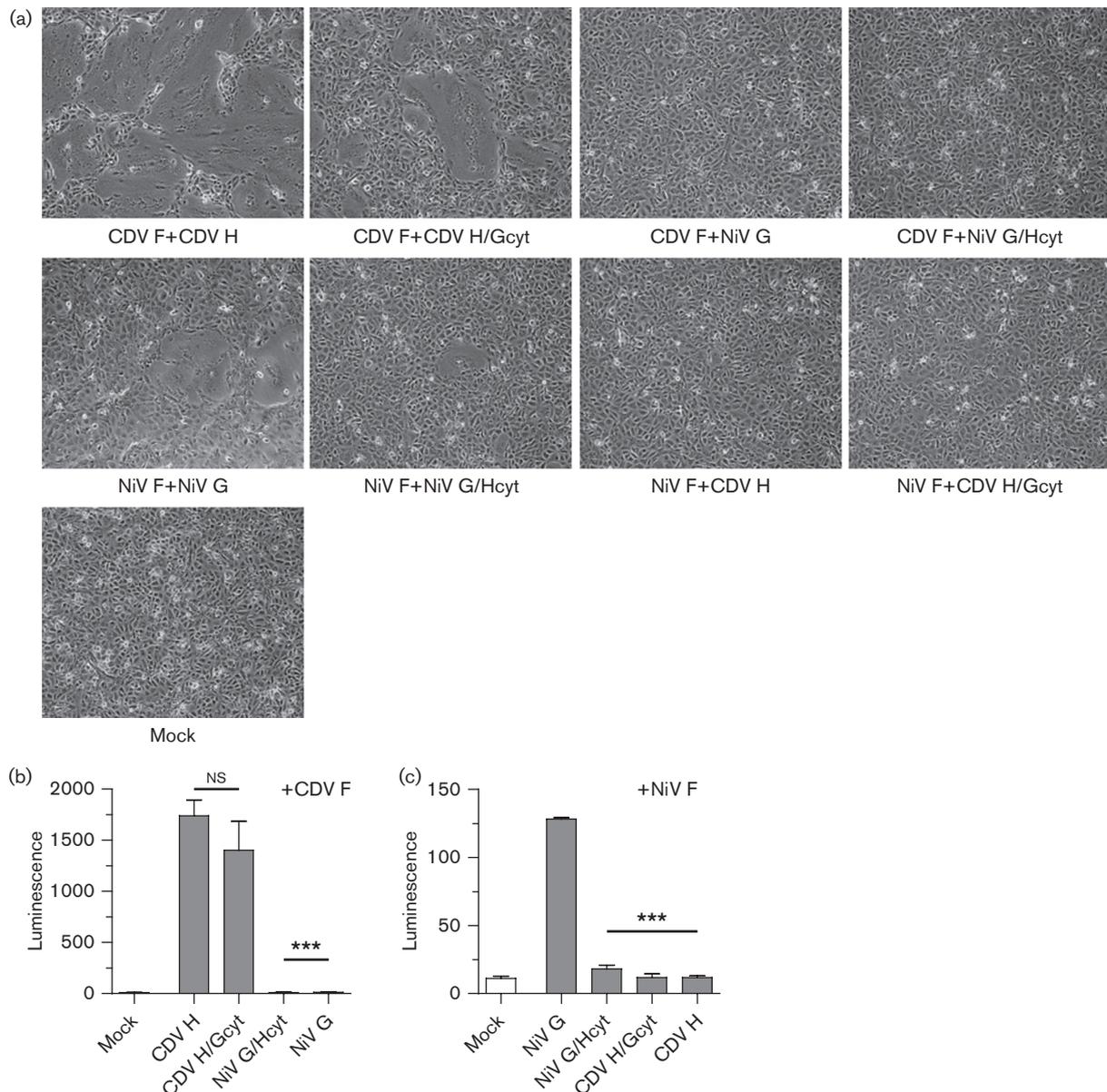


Fig. 3. Fusion support activity of CDV H, NiV G and the chimeric glycoproteins CDV H/Gcyt and NiV G/Hcyt. (a) Phase-contrast microscopy of cells transfected with either CDV or NiV F in combination with CDV H, NiV G or chimeric glycoprotein expression plasmid. Pictures were taken at 24 h post-transfection at a magnification of $\times 100$. (b, c) Quantitative fusion assay. Semi-confluent monolayers of VerodogSLAMtag cells were independently transfected in triplicate with firefly luciferase plasmid and either CDV or NiV F in combination with CDV H, NiV G or chimeric glycoprotein expression plasmid as described in Methods. After 6 h, the transfected cells were overlaid with 10^5 cells per well VerodogSLAMtag cells transfected with T7 RNA polymerase expression plasmid and incubated at 37°C for 24 h. Cells were lysed and the luciferase signal was read in duplicate for each sample. Graphs show luminescence signals from fusion mediated by CDV F (b) or NiV F (c). Bars on graphs represent the mean of a sample group and error bars represent SEM. Groups were analysed by one-way ANOVA with Tukey post-test. $***P < 0.001$; NS, not significant. Statistical significance is indicated on the graphs in (b) compared to CDV H, and in (c) compared to NiV G.

endogenous levels of the NiV receptor ephrin-B2. Furthermore, the NiV glycoproteins are known to be less fusogenic in most cell lines than the glycoproteins from other paramyxoviruses (Bossart *et al.*, 2002). None of the heterologous glycoprotein combinations CDV F+NiV G and NiV F+CDV H induced fusion, which confirms the

specificity of our assay. The cytoplasmic domain of the NiV G protein is thus essential for protein expression, which directly affects fusion support activity, whereas the fusion support function of CDV H is relatively insensitive to substitutions in this region providing protein expression is maintained. This illustrates that the cytoplasmic domain

(a)

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CDV H      M L S Y Q D K V G A F K D N A R A N S S K L S L V T E E Q G G R R
CDV HcytΔ10 M ----- K D N A R A N S S K L S L V T E E Q G G R R
CDV HcytΔ20 M ----- K L S L V T E E Q G G R R
CDV HcytΔ30 M ----- G R R
CDV HcytΔ32 M ----- R

NIV G      M P A E N K K V R F E N T T S D K G K I P S K V I K S Y Y G T M D I K K I N E G L L D S K
Niv GcytΔ10 M ----- N T T S D K G K I P S K V I K S Y Y G T M D I K K I N E G L L D S K
Niv GcytΔ20 M ----- S K V I K S Y Y G T M D I K K I N E G L L D S K
Niv GcytΔ30 M ----- M D I K K I N E G L L D S K
Niv GcytΔ40 M ----- L D S K
Niv GcytΔ43 M ----- K
    
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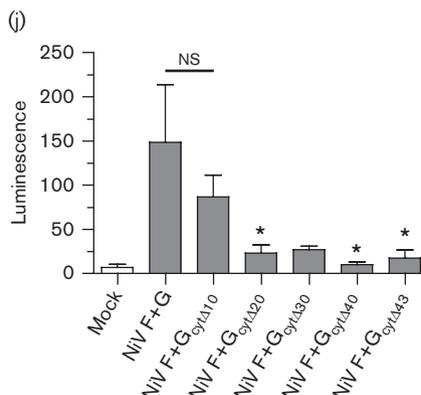
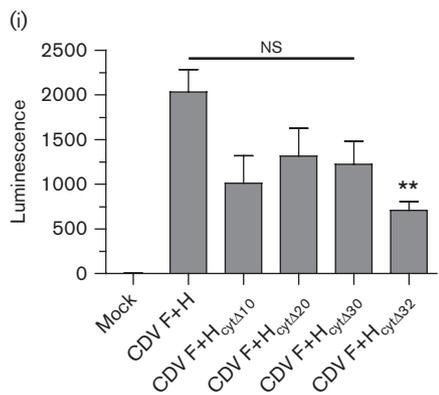
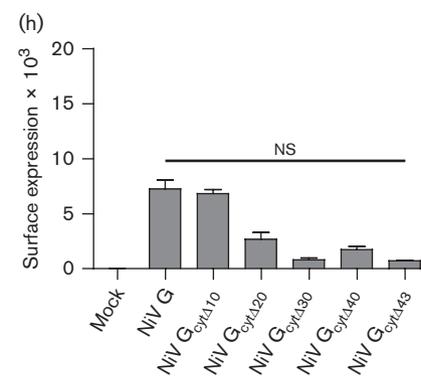
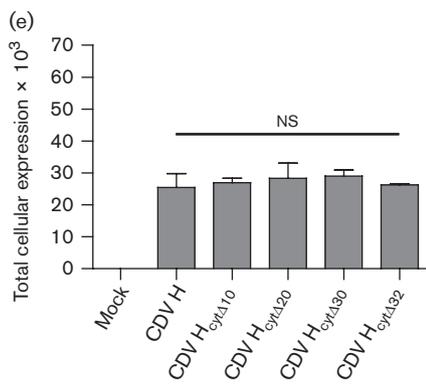
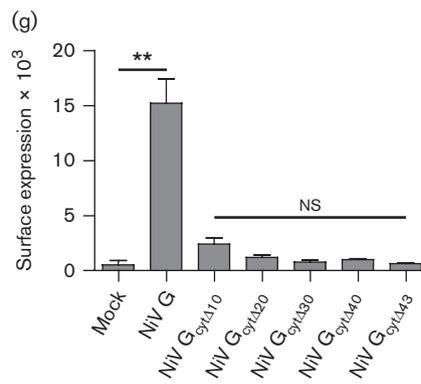
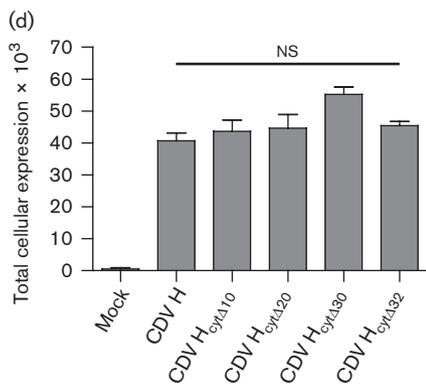
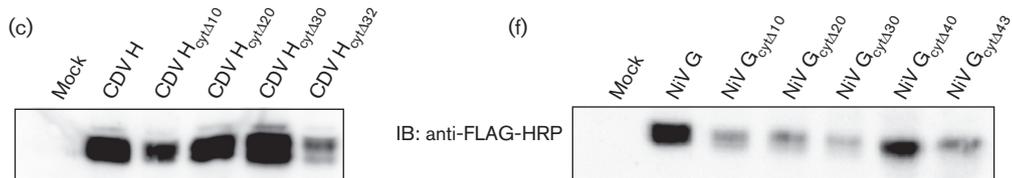


Fig. 4. Expression of cytoplasmic domain deletion mutants. (a, b) Schematic diagrams of (a) CDV H and (b) NiV G cytoplasmic domain deletion mutants constructed for this study. Sequences are shown from the amino-terminus up to the carboxy-terminal transmembrane boundary on the right. Tyrosine residues that act or may potentially act as targeting or signalling motifs are shown in black boxes to highlight which residues have been deleted from constructs containing shorter domains. (c–h) Total cellular and cell surface expression of CDV H, NiV G and the CDV H (c–e) or NiV G (f–h) cytoplasmic deletion mutants. (c, f) Detection of CDV H (c), NiV G (f) and the respective deletion mutants by Western blot. 293T cells were transfected with control expression plasmids or the respective mutants, and were lysed 48 h after transfection. Proteins were detected with an anti-FLAG M2 antibody directly coupled to HRP. Blots are representative of four replicates. FACS analysis was performed with 293T cells transfected with control expression plasmids and the respective mutants, which were either permeabilized for total protein (d, g) or fixed for cell surface (e, h), then stained with anti-FLAG M2 antibody followed by anti-mouse Alexa Fluor 488 secondary antibody. The mean fluorescence intensities for positively stained cell populations are shown. (i, j) Cell–cell fusion of WT and cytoplasmic domain deletion mutant glycoproteins. Semi-confluent monolayers of VerodogSLAMtag cells were independently transfected in triplicate with firefly luciferase plasmid and either CDV in combination with CDV H and the respective deletion mutants (i), or NiV F in combination with NiV G and the respective deletion mutants (j). After 6 h, the transfected cells were overlaid with 10^5 cells per well VerodogSLAMtag cells transfected with T7 RNA polymerase (T7RNAP) expression plasmid and incubated at 37 °C for 24 h. Cells were lysed and the luciferase signal was read in duplicate for each sample. Graphs show luminescence signals from fusion mediated by CDV F (i) or NiV F (j). Bars on graphs represent the mean of a sample group and error bars represent SEM. Groups were analysed by one-way ANOVA with Tukey post-test. * $P < 0.05$; ** $P < 0.01$; NS, not significant. Statistical significance is indicated on the graphs compared with CDV H in (d) and (e), compared to NiV G in (g) and (h), compared to CDV F + H in (i), and compared to NiV F + G in (j).

has no direct role in this function of paramyxoviral attachment proteins.

The cytoplasmic domain is not required for CDV H surface expression and fusion support function

To investigate these different tolerances for structural modifications of CDV H and NiV G cytoplasmic domains in more detail, we next generated a series of FLAG-tagged mutants with 10-residue deletions of the respective proteins (Fig. 4a, b). The total amount of protein detected by Western blot and FACS staining for the CDV H cytoplasmic domain deletion mutants was comparable to the parental CDV H (Fig. 4c, d), and yielded similar levels of cell surface expression (Fig. 4e). Overall NiV G expression was generally lower than that of CDV H, and deletion of the first 10 residues of NiV G was sufficient to result in a dramatic reduction of protein levels (Fig. 4f, g). However, NiV G_{CytΔ10} surface expression was equivalent to that of parental NiV G, suggesting a regulatory function of this region in cell surface transport or protein turnover (Fig. 4h). All further deletions abolished protein expression, demonstrating an essential role for the cytoplasmic domain of NiV G, but not CDV H, in biosynthesis.

We then assessed the CDV H and NiV G cytoplasmic deletion mutants for their ability to promote fusion. All CDV H deletion mutants showed a modest reduction in fusion promotion that was not statistically significant, with the exception of CDV H_{CytΔ32} (Fig. 4i). Deletion of the first 10 residues of NiV G resulted in a similarly modest reduction in fusion (Fig. 4j), but further deletions in the NiV G cytoplasmic domain significantly reduced fusion to background levels (Fig. 4j). Since the level of fusion promotion corresponds to the amount of protein detected on

the cell surface, this indicates that the level of fusion activity is regulated through modulation of the amount of protein on the cell surface.

Efficient CDV particle formation involves interactions of the H protein cytoplasmic domain with the M protein

Interaction between the M protein and the cytoplasmic domain is involved in the incorporation of attachment proteins into viral particles (Cathomen *et al.*, 1998a; Tahara *et al.*, 2007), and many paramyxovirus glycoproteins carry motifs in their cytoplasmic domains that mediate these interactions (Schmitt *et al.*, 1999, 2002; Takimoto *et al.*, 1998; Waning *et al.*, 2002). However, for some paramyxoviruses, expression of the attachment protein alone is sufficient to result in the release of virus-like proteins (VLPs) into the culture supernatant (Diederich *et al.*, 2008; Patch *et al.*, 2007), indicating an intrinsic particle formation capacity of the protein. To investigate the role of the cytoplasmic tails, we analysed the VLP formation upon expression of either the CDV H or NiV G glycoproteins, or the respective deletion mutants, in the absence or presence of the homologous M proteins. We then measured relative incorporation of the attachment proteins into VLPs containing the M protein, and compared these levels to the amount of attachment protein that buds into the culture supernatant in the absence of M. Indeed, co-expression of the CDV M and H proteins resulted in a four- to fivefold enhancement of H incorporation compared with H protein expression alone (Fig. 5a), but this enhancement was completely lost when the first 10 residues of CDV H were deleted (Fig. 5a). There was a slight, albeit non-significant, enhancement of NiV G incorporation into particles when it was co-expressed with NiV

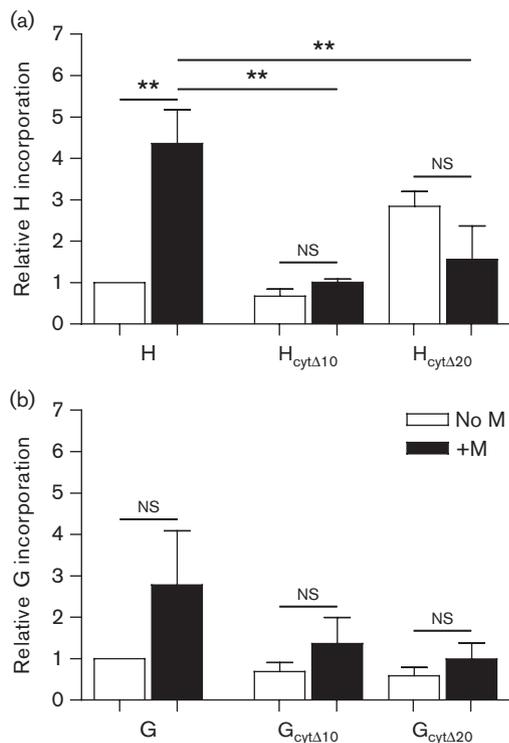


Fig. 5. Quantification of glycoprotein incorporation into VLPs. Comparison of glycoprotein-only and glycoprotein+matrix VLPs and lysates from CHO-K1 cells transfected with either CDV H and M, and the respective mutants (a) or NiV G and M, and the respective mutants (b). Western blots were performed as described in Methods, and the signals from the lysate and VLP fractions from each replicate were normalized to either CDV H or NiV G expressed alone and are presented as the ratio between the signals in the VLPs and the lysate, and represent three independent replicates. White bars represent glycoprotein incorporation into VLPs in the absence of homologous M protein, and black bars represent glycoprotein incorporation into VLPs when the homologous M protein is expressed. Groups were analysed by one-way ANOVA with Tukey post-test. $**P < 0.01$; NS, not significant. Statistical significance is indicated on the graphs compared with CDV M+H in (a), while no statistically significant differences were found in (b).

M (Fig. 5b). However, there was no difference in NiV G deletion mutant particle incorporation regardless of the presence of NiV M (Fig. 5b). The CDV H protein thus contains a motif in its extreme amino-terminus that enhances its incorporation into particles containing the M protein, while the incorporation of NiV G into VLPs seems to be less dependent on the M protein.

DISCUSSION

In addition to their role in signal transduction, the cytoplasmic domains of surface glycoproteins frequently influence protein processing and transport (Moll *et al.*, 2002; Parks & Lamb, 1990; Spriggs & Collins, 1990; Wilson

et al., 1990). Generally, a minimal domain of 10–15 amino acids is required for type II glycoproteins to ensure correct translocation of the nascent protein chain into the ER and subsequent post-translational modifications (Parks & Lamb, 1990; Spriggs & Collins, 1990; Wilson *et al.*, 1990). Here we show that the CDV H cytoplasmic domain is dispensable for protein expression and fusion promotion activity, but has an important role for its incorporation into VLPs. In contrast, the same domain of NiV G is critically important for protein expression and consequently fusion promotion, but has no obvious role in incorporation of the protein into VLPs. The assembly of morbillivirus thus appears to be a highly coordinated process in which the glycoproteins are directed into particles by the M protein, whereas G–M protein interactions do not seem to be important for henipavirus assembly.

Disruption of NiV G cytoplasmic signalling motifs decreases protein expression

The MeV and Sendai virus attachment protein cytoplasmic domains can be reduced to 14 amino acids without loss of function (Moll *et al.*, 2002; Waning *et al.*, 2002), but further deletions result in a considerable decrease of cell surface expression and lack of incorporation into virus particles (Cathomen *et al.*, 1998b). Likewise, using an alternative in-frame start codon, the HeV G protein cytoplasmic domain can be reduced to only 13 residues while still yielding a functional protein that induces fusion more efficiently than the WT protein (Palomares *et al.*, 2013; Porotto *et al.*, 2006). We found that the NiV G protein clearly cannot tolerate deletions of this length, which is largely due to greatly decreased expression of the truncated proteins. Some early studies of henipavirus fusion demonstrated that the homologous HeV glycoproteins fuse more efficiently than the NiV glycoproteins (Bossart *et al.*, 2002), but it has not been clear why this is the case. Since CDV and NiV attachment protein mutants with cytoplasmic domains of 15 residues or fewer lack tyrosine motifs important for post-translational sorting to the basolateral surface of polarized cells (Moll *et al.*, 2001; Runkler *et al.*, 2009; Weise *et al.*, 2010), the resulting loss of appropriate intracellular transport likely disrupts protein homeostasis and decreases protein expression levels.

Independence of CDV H protein expression and fusion support function from the cytoplasmic domain

While the amount of cell–cell fusion observed when paramyxovirus glycoproteins are co-expressed generally corresponds to the relative amounts present on the cell surface, the amount of attachment protein is usually believed to be the more important determinant since it triggers the F protein after reaching the appropriate threshold of binding to the cellular receptor (Hasegawa *et al.*, 2007; Whitman & Dutch, 2007). This held true for both the CDV H and NiV G cytoplasmic domain deletion mutants, where the levels of surface expression corresponded to the overall expression levels of

each protein. NiV G was more sensitive to alterations in this domain than CDV H; the expression of CDV H was slightly reduced when deletions were made from the cytoplasmic domain, regardless of the length, while exchange or even deletions of modest length dramatically reduced NiV G expression. CDV H may thus represent an outlier among paramyxoviruses in this respect, since the MeV H protein is affected by cytoplasmic domain deletions in a manner similar to that which we observed for NiV G (Moll *et al.*, 2002).

Incorporation of NiV G into VLPs is non-specific

Consistent with previous reports (Ciancanelli & Basler, 2006; Li *et al.*, 2009; Patch *et al.*, 2007), expression of the CDV and NiV M proteins alone was sufficient for VLP formation. The incorporation of many paramyxovirus glycoproteins into nascent viral particles is a coordinated process and requires interaction with the homologous viral M protein. While both attachment proteins were enriched to varying degrees in VLPs containing either CDV or NiV M, deletion of 10 residues at the amino-terminus of CDV H abolished this enrichment, and the incorporation pattern for CDV H mutants with further deletions was similar to those for VLPs that did not contain the CDV M protein. The morbillivirus H protein therefore has a functional motif at its amino-terminus that directs preferential incorporation into viral particles. While morbillivirus F and H proteins associate very early during biosynthesis (Plempner *et al.*, 2001), F also forms VLPs when co-expressed with M (Cathomen *et al.*, 1998a; Pohl *et al.*, 2007), and recombinant MeVs with partially deleted cytoplasmic tails were viable (Cathomen *et al.*, 1998b), it remains unclear which glycoprotein interacts more strongly with the homologous M protein to drive morbillivirus particle formation. In contrast, henipavirus glycoproteins are transported independently and only associate transiently on the cell surface (Lamp *et al.*, 2013; Whitman & Dutch, 2007; Whitman *et al.*, 2009). Recent studies on VLP formation by the NiV M protein have demonstrated that henipavirus glycoprotein incorporation is a haphazard process, where at least half of the VLPs produced have no surface glycoproteins (Landowski *et al.*, 2014). The M-protein-mediated incorporation of NiV G is also much less efficient than that of the F protein (Landowski *et al.*, 2014), further supporting our findings. In addition, infectious NiV particles are released into the culture supernatant even when the entire M ORF is deleted (Dietzel *et al.*, 2016), demonstrating that the role of the henipavirus M protein in virus assembly is less prominent than for other paramyxoviruses. This implies a greater contribution of the henipavirus glycoproteins to the budding process, suggesting a fundamentally different process of henipavirus assembly compared with other members of this virus family.

METHODS

Cells and transfections. Vero E6 (ATCC CRL-1586), CHO-K1 (ATCC CCL-61) and 293T (ATCC CRL-3216) cells and Vero cells

stably expressing the canine SLAM protein (VerodogSLAMtag) (von Messling *et al.*, 2003) were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 5 % heat-inactivated FBS (Gibco). For the biochemical analysis of recombinant proteins, 6- or 12-well plates of VerodogSLAMtag or Vero E6 cells were transfected at approximately 90 % confluence as described previously (Sawatsky & von Messling, 2010; Sawatsky *et al.*, 2012). Briefly, each well was transfected with 2 µg of the parental CDV H or NiV G protein expression plasmid, or the respective mutants, using Turbofect (Thermo Fisher). For fusion assays, CDV or NiV F protein expression plasmid containing a C-terminal haemagglutinin tag (HA, YPYDVPDYA) was included in the transfection and fusion activity was evaluated after 24 h. Cells were lysed in 200 µl RIPA buffer [50 mM Tris/HCl pH 8.0, 150 mM NaCl, 1 % (v/v) NP-40 and 0.5 % (w/v) sodium deoxycholate] containing protease inhibitor cocktail (Complete; Roche) 48 h after transfection, and the clarified lysate was stored at -20 °C. For evaluation of protein expression by FACS staining, 293T cells were seeded into six-well dishes and transfected with the respective CDV H or NiV G expression plasmid using Lipofectamine 3000 (Thermo Fisher) according to the manufacturer's instructions.

Production of chimeric CDV and NiV glycoproteins. CDV H genes or gene fragments were amplified from the expression plasmid pCG-H5804PZeo (von Messling *et al.*, 2005), while NiV G genes or gene fragments were amplified from the plasmid pczCFG5-NiV G (Sawatsky *et al.*, 2007) and cloned into the *Bam*HI and *Sph*I sites of the pCG1-IRESzeomut expression plasmid (von Messling *et al.*, 2003). FLAG epitope tags (DYKDDDDK) were introduced into the 3' primer immediately upstream of the stop codon of each gene to facilitate detection (Plempner *et al.*, 2001). FLAG tags were also introduced into the carboxy-termini of all subsequent CDV H and NiV G mutants made for this study. The cytoplasmic domain exchange genes for the mutants CDV H/Gcyt and NiV G/Hcyt were produced by overlapping PCR and assembled before cloning into the *Bam*HI and *Sph*I sites of pCG1-IRESzeomut. CDV H and NiV G cytoplasmic domain truncation mutants were produced by PCR amplification using primers in which blocks of 10 residues in the N-terminal region immediately downstream of the start codon were progressively deleted. The CDV M (5804P strain) gene was amplified from the genomic plasmid p5804PeGFP (von Messling *et al.*, 2004), while the NiV M (Malaysia strain) gene was amplified from purified viral RNA. The 3' primers for the NiV M gene contained the coding sequence for the c-myc epitope tag (EQKLISEEDL) immediately upstream of the stop codon (Salditt *et al.*, 2010), while the corresponding primer for CDV M contained no tag. The CDV F gene was amplified from the expression plasmid pCG-F5804PZeo (von Messling *et al.*, 2005), while the NiV F genes or gene fragments were amplified from the plasmid pczCFG5-NiV F (Sawatsky *et al.*, 2007). The 3' primers for the CDV and NiV F expression plasmids both contained the coding sequence for the haemagglutinin epitope tag (HA, YPYDVPDYA).

FACS staining. Six-well dishes of 293T cells were transfected with the respective NiV G and CDV H mutant expression plasmids as detailed above. Cells were treated with 0.05 % trypsin-EDTA 48 h after transfection, resuspended in DMEM containing 10 % FBS and then transferred to centrifuge tubes and washed with PBS. For total protein staining, cell pellets were treated with Cytofix/Cytoperm (GE Healthcare) for 30 min at 4 °C, and were then washed with PBS. Mouse anti-FLAG M2 primary (Sigma) and goat anti-mouse Alexa Fluor 488 secondary antibodies (Thermo Fisher) were used. For cell surface staining, cell pellets were first stained with the mouse anti-FLAG M2 antibody, and then fixed with 4 % paraformaldehyde before incubation with the secondary antibody.

Quantitative fusion assays. Confluent monolayers of VerodogSLAMtag cells in a six-well dish were transfected with 5 µg per

well pCAGGS-T7RNAP, which expresses the bacteriophage T7 DNA-dependent RNA polymerase (T7RNAP) under the control of the chicken β -actin promoter. The transfected cells were trypsinized 18 h later and pooled for use as target cells in the assay. In parallel, VerodogSLAMtag cells were seeded into 24-well dishes at 40–50% confluence. These cells were transfected with 1 μ g per well of plasmid that expresses the firefly luciferase under the control of the T7RNAP promoter (pTM1-ffLuc) along with 0.5 μ g per well of expression plasmid for either the CDV or NiV F_{HA} proteins, and 0.5 μ g per well of expression plasmid for either CDV H, NiV G or the respective cytoplasmic domain exchange and truncation mutants. After incubation for 6 h at 37 °C, 10⁵ T7RNAP-expressing target cells per well were added to the 24-well dishes and were incubated at 37 °C for 24 h. The cells were lysed with 100 μ l per well Cell Culture Lysis Reagent (Promega) at room temperature, transferred to fresh Eppendorf tubes, and briefly centrifuged at 20000 g to pellet cell debris. The clarified supernatant was used for the reporter assay. Each sample (20 μ l) was read in duplicate in black clear-bottom 96-well plates using the Luciferase Assay System (Promega) according to the manufacturer's recommendations. Luminescence was read on an Infinite M200Pro automated plate reader (Tecan).

VLPs. VLPs were produced based on a protocol previously used to purify NiV VLPs (Ciancanelli & Basler, 2006). Confluent monolayers of CHO-K1 cells in six-well plates were transfected with 3 μ g CDV M or NiV M_{myc} protein expression plasmid and 3 μ g of the respective FLAG-tagged CDV or NiV glycoprotein expression plasmid. All VLP experiments were incubated at 32 °C. Control wells were transfected with either M or H/G expression plasmid and an equal amount of empty pCG1-IRESzeomut vector. For glycoprotein-only VLPs, CHO-K1 cells in six-well plates were transfected with 3 μ g of the respective FLAG-tagged glycoprotein expression plasmids. The transfection medium was removed after 16 h and replaced with fresh DMEM supplemented with 5% FBS. At 48 h post-transfection, cells and medium were resuspended and centrifuged at 250 g for 20 min to pellet cellular material. CDV M+H VLPs were incubated for 72 h. The pellet was washed once with PBS and then lysed in 250 μ l RIPA buffer. The clarified culture supernatant was overlaid onto 20% sucrose cushions in TNE buffer (10 mM Tris pH 7.5, 100 mM NaCl, 1 mM EDTA) and centrifuged for 2 h at 4 °C in a Beckman SW-41Ti rotor at 30 500 r.p.m. (approximately 160 000 g). The supernatant was aspirated and the VLPs were resuspended in 50 μ l 1 \times SDS-gel loading buffer (GLB) containing 10% β -mercaptoethanol per tube. An equal volume of 2 \times SDS-GLB containing 20% β -mercaptoethanol was mixed with the cellular lysate before separation in parallel on polyacrylamide gels. The NiV M_{myc} and CDV M proteins were detected using monoclonal anti-myc clone 9E10 (Millipore) or anti-measles M protein antibodies (MAB8910; Millipore), respectively, and a monoclonal goat anti-mouse secondary antibody conjugated to HRP (Southern Biotech). The FLAG-tagged glycoproteins were detected with an anti-FLAG antibody directly conjugated to HRP. β -Actin was detected in cell lysates using an anti-actin antibody directly conjugated to HRP (mAbcam 8226; Abcam).

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